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OX513A Technical Dossier

Part A – Characterisation of OX513A *Aedes aegypti*

Submission to the GMO Office of the National Institute of Public Health and the Environment of the Netherlands (RIVM) for the technical evaluation of the release of *Aedes aegypti* OX513A in Saba.

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Introduction

Effective vector control measures are recognised as critical to achieving and sustaining disease reduction for mosquito borne arboviruses[1]. *Aedes aegypti*, the principal mosquito species that transmits the Zika, dengue, and chikungunya viruses, has a number of characteristics that make it extremely difficult to control by existing conventional methods[2].

Integrated Vector Management (IVM)[3] is significantly reliant on chemical controls, and principally includes source reduction such as insecticidal treatment or elimination of larval habitats, the application of insecticides that target the adult mosquito population, as well as the use of trapping methods. On a smaller scale, biological control methods such as the use of predatory fish, and non-chemical treatments in larval breeding sites are also used. Even the most progressive IVM programs cannot generally achieve control targets sufficient to bring *Aedes aegypti* populations below disease transmission thresholds, as it is not possible to access all of the mosquito breeding sites with the current control measures. In very few cases IVM strategies have achieved reduction to a low enough level to prevent disease transmission (Egger et al., 2008; Heintze et al., 2007; Halstead, 2000). Additionally, certain aspects of the biology of *Aedes aegypti* make it less susceptible to interventions that are routinely used against other mosquito species. Bed-nets for example, which are designed to protect people from mosquito bites during night time and are regularly used in malaria prevention programmes, are ineffective against *Aedes aegypti* because this species actively searches for blood during the day time. Control efforts worldwide for *Aedes aegypti*, have thus had limited success to date as evidenced by the continued global threat of arboviruses.

Oxitec Ltd. has developed a non-chemical vector control program for *Aedes aegypti* which involves the repeated controlled release of genetically modified (GM) male *Aedes aegypti* strain OX513A which pass on a self-limiting gene preventing the next generation from surviving to functional adulthood. OX513A is homozygous for a recombinant DNA (rDNA) construct, stably integrated at a specific site in the OX513A genome, that confers both late-acting cell death in the developing larvae in the absence of tetracycline (the self-limiting trait), and contains a gene that encodes a fluorescent marker (DsRed2) for use in field monitoring.

OX513A was first constructed in 2002, published in 2007 (Phuc et al., 2007) and has been characterized for over 12 years in both contained experiments, and in regulated environmental releases, the details of which are presented in this Technical Dossier. Male OX513A mate principally with the wild females of their own species, leading to a reduction in the local population of *Aedes aegypti*, thus limiting the direct impact on the environment to the target organism. This intended species-specific feature also ensures that OX513A cannot become established in the environment. Furthermore, male mosquitoes do not bite and therefore are unable to transmit or vector viruses or other saliva constituents. An OX513A program can be used in two ways:

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[1] http://apps.who.int/iris/bitstream/10665/75303/1/9789241504034_eng.pdf?ua=1 (accessed 16/05/2016)
[2] http://www.who.int/emergencies/zika-virus/articles/mosquito-control/en/ (accessed 16/05/2016)
[3] http://www.who.int/neglected_diseases/vector_ecology/ivm_concept/en/ (accessed 16/05/2016)

- To reduce and potentially eliminate the *Aedes aegypti* population in an area,
- To prevent resurgence of the *Aedes aegypti* population once control in the area has been achieved.

Aedes aegypti eggs are robust and can be shipped without undue losses and can also be stored for several months. OX513A eggs are produced in the UK for shipment to a locally based mobile rearing unit (MRU) located at or near the geographic area where the target *Aedes aegypti* populations are located, where they are hatched and reared to pupae. The male pupae are then sorted mechanically from the females with over 99.9% accuracy (Carvalho et al., 2015; Gorman et al., 2015; Harris et al., 2012) using the difference in size between male and female pupae (sexual dimorphism). Males, which do not bite or transmit disease, are used for the release. Continuity in quality assurance processes from the UK based egg production facility to the MRU and release phase allow Oxitec to maintain oversight on the efficacy and stability of OX513A.

The World Health Organization (WHO) Vector Control Advisory Group on new paradigms (VCAG) is tasked to support the control and elimination of vector-borne diseases by providing a pathway forward for novel forms of vector control. In March 2016 the VCAG issued a recommendation in support for the carefully planned pilot deployment under operational conditions, accompanied by rigorous independent monitoring and evaluation of OX513A, programs[4].

The objective of the OX513A Technical Dossier – Part A and B is to present data which supports the Environmental Risk Assessment (ERA) of OX513A (Part C) and is not in itself intended to derive conclusions on environmental risk, although observations in some cases may be correlated to risk conclusions. Technical and scientific details relevant to the ERA are ordered in the following subject areas:

[4] http://www.who.int/neglected_diseases/news/mosquito_vector_control_response/en/ (accessed 16/05/2016)

Part A:

1. Recipient organism: *The biological characteristics of Aedes aegypti, including information on taxonomic status, common name, origin, centres of origin, and a description of the habitat where Aedes aegypti may persist or proliferate.*

2. Donor organisms: *Taxonomic status and common name, source, and the relevant biological characteristics of the donor organism sequences in the rDNA construct.*

3. Vector used in the transformation of OX513A: *Characteristics of the transformation vector, including its identity, if any, and its source or origin, and its host range.*

4. rDNA Insert and characteristics of modification: *Genetic characteristics of the inserted nucleic acid and the function it specifies, and/or characteristics of the modification introduced;*

5. Characterisation of OX513A: *Identity of OX513A, and the differences between the biological characteristics of OX513A and those of wild or wild type Aedes aegypti;*

6. Detection and identification of OX513A: *Suggested detection and identification methods and their specificity, sensitivity and reliability;*

7. Regulated environmental Releases of OX513A: *Previous Aedes aegypti vector control projects using OX513A*

Part B:

1. Information relating to the intended use of OX513A in Saba: *Details of the proposed release programme in Saba.*

2. Receiving environment: *Information on the location, geographical, climatic and ecological characteristics, including relevant information on biological diversity and centres of origin of the likely potential receiving environment.*

In each of the above subject areas, a summary of the technical study is presented and may represent an internal Oxitec study, weight of evidence from peer reviewed scientific literature, or a study contracted by an independent laboratory or technical service. In some cases, existing external reports by third parties or national, regional, or international organisations are referred to and supplied as appendices to the OX513A Technical Dossier(s).

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1 Recipient organism- *Aedes aegypti*

The recipient organism is the *Aedes (Stegomyia) aegypti* (L.) mosquito which belongs to the Order Diptera, Family Culicidae, Genus Aedes. *Aedes aegypti* is a tropical species of mosquito found between 15°N and 15°S, typically in Africa and parts of South America but has been reported to have a cosmopolitan habitat extending from 40°N to 40°S latitude (Mousson et al., 2005) (Walter Reed Biosystematics Unit[5]). It is considered as an invasive species in several jurisdictions (ISSG, 2016)

Aedes aegypti originated in Africa, which has been confirmed by a number of studies using genetic analysis to create a detailed phylogeny of the species (Brown et al., 2011; Failloux et al., 2002). These studies describe a clear genetic structure of the *Aedes aegypti* population which supports the idea that the *Aedes aegypti* species harbours two genetically distinct forms or subspecies *Aedes aegypti aegypti* and *Aedes aegypti formosus. Aedes aegypti aegypti* now has a worldwide distribution in all tropical and subtropical habitats whereas the *Aedes aegypti formosus* population remains in Africa (Brown et al., 2011; Urdaneta-Marquez and Failloux, 2011). It is thought from genomic studies that the mosquito radiated from its original distribution as a result of passive transport initially on slave trade ships (Failloux et al., 2002) however with the expansion of the shipping industry in the 18th and 19th centuries the range of *Aedes aegypti* increased as it was transported across the world; first infesting ports then moving inland along transportation routes. During World War II transmission of dengue intensified as troops spread both dengue fever and the vector, *Aedes aegypti,* throughout Asian and Pacific regions (Gubler, 2011).

Aedes aegypti is a peri-domestic species closely associated with human habitations. Breeding is tied to artificial water containers, such as potted plant holders, water tanks, tires, discarded plastic and metal containers such as soda cans, drains and roof guttering as well as ephemeral containers, such as puddles (e.g. Powell and Tabachnick, 2013) . Once eclosed the adult *Aedes aegypti* mosquitoes live in and around houses where females have easy access to the blood meal necessary for egg development. The mosquito eggs are laid individually by females in the damp walls of both natural and artificial containers that can hold water. Eggs are the long-term survival structures of these mosquitoes, surviving up to 6 months. The larvae and pupae prefer relatively clean water typically found in containers such as; water storage containers, flowerpots and waste materials such as tyres, cans, bottles etc. The waste material containers are usually only sources of mosquitoes during the rainy season in other countries but in tropical countries this tends to be year round. The duration of the larval stages is approximately 7-9 days and pupae 2-3 days but this is temperature dependant. The preferred sites for adults are domestic urban environments in sheltered dark spaces within houses/ apartments. *Aedes aegypti* is a day biting mosquito with two peaks, one mid-morning and one mid-afternoon. The average adult lifespan is 8-15 days for female mosquitoes and 3-6 days for male mosquitoes. (see Christophers, 1960)

[5] http://www.wrbu.org/mqID/mq_medspc/AD/AEaeg_hab.html (accessed 15/08/2016)

Spontaneous flight of adults is limited to around 200m depending on availability of breeding sites and hosts from which to take a blood meal (see **Section 5.3**) however the species is dispersed over longer distances by passive transport on boats, trains and modes of long distance transport. International Sanitary Regulations require ports and airports to be clear of *Aedes aegypti* for 400m (WHO, 2005). Climate and the availability of breeding sites are the two main factors that regulate the populations of *Aedes aegypti* in urban environments (Powell and Tabachnick, 2013)

The effect of temperature on larval development of *Aedes aegypti* has been well studied, and has an ecological temperature range of 14-30^{o}C, at which the larval development is a function of temperature. Temperature also affects adult size, dry weight and ovariole number all of which fall as the temperature rises. High temperatures alone (>40^{o}C) are unlikely to limit the species but global historical collections and laboratory experiments on this well-studied vector have suggested its distribution is limited by the 10°C winter isotherm (Christophers, 1960), while a complex stochastic population dynamics model analysis suggests the temperature's limiting value to be more towards the 15°C yearly isotherm (Otero et al., 2006). Low temperatures below the 10^{o}C isotherm are likely to severely limit the geographical range, although the protection of human habitations may afford some protection from lower temperatures. Scholte et al. (2010) indicated that *Aedes aegypti* could not survive winter temperatures in Northern Europe. However, survival at temperatures below freezing is extremely unlikely; Thomas et al. (2012) found that a tropical strain of *Aedes aegypti* eggs could only survive at a threshold of-2^{o}C for 24 hours before hatching broke down completely.

Altitude is thought to affect distribution, with an elevation of 1800-2400 m (6000-8000 ft) likely to be limiting to the species and lower levels in temperate latitudes. Navarro et al. (2010) in an extensive survey of mosquito species in the Andes, did not record the presence of *Aedes aegypti* over 2000 m (6560 ft). The slope of the elevation could also be an influencing factor, with plateaus being more preferable than steep slopes.

OX513A was generated through the transformation of the Rockefeller strain of *Aedes aegypti* (Kuno, 2010) through microinjection of individual embryos collected from females, and subsequently out-crossed into a Latin genetic background provided by Instituto Nacional de Salud Pública (Mexico). See ***Appendix 1*** **-** *Molecular characterisation and lineage of Aedes aegypti OX513A*

2 Donor organisms

The genetic elements in the OX513A rDNA construct and their function, along with the origin of the DNA sequence are detailed in **Section 4 - Table 1.** DNA fragments used in the development of the OX513A rDNA are principally synthetic, and the "donor organism" indicates were the sequence originated. A complete list of all DNA elements used in the OX513A rDNA construct (in order 3'-5') including short non-coding linker DNA sequences, is given in ***Appendix 1 -*** *Table 2 Molecular characterisation and lineage of Aedes aegypti OX513A*

Further information on each of the organisms where DNA sequences originated is given below:

2.1 *Trichoplusia ni* (Cabbage looper moth)

The cabbage looper moth has an extensive global range throughout Central and North America as well as being detected in Northern Europe, most of Russia, the Middle East, Asia, parts of Africa, and most of the Indo-Australian region. It is a pest of particular notoriety in the USA and Canada, where it is a significant pest of brassica crops. The moth is seasonal with an erratic yearly occurrence. Adult moths are present year-round in the warmer climates of the southern USA, although in colder climates such as Canada the adults will only be present in the summer months after migrating long distances from warmer areas (Capinera, 1999). Cabbage looper is a pest that feeds on the leaves of cruciferous plants but does not contain any known toxic or pathogenic properties.

Thibault (1999) describes the transposable element, *piggyBac*[6] which was isolated from a cell culture of cabbage looper (*Trichoplusia ni)*, this non-autonomous transposon has been well-studied and used to transform insects from a range of taxa: Diptera, Lepidotera Coleoptera and Hymenoptera (Labbe et al., 2010; Koukidou et al., 2006; Kuwayama et al., 2006; Handler, 2002; Sumitani et al., 2003; Jasinskiene et al., 1998; Tamura et al., 2000)

2.2 Drosophila melanogaster (Vinegar fly)

Drosophila melanogaster has a widespread global distribution which has radiated from its original range in Africa and parts of Asia. *Drosophila melanogaster* are known to be present throughout North, Central and South America and most of Europe, Asia and the outer regions of Australia. The distribution of the fly is limited to areas with a permissible temperature range, (development is impossible below 12^{o}C and above 32.5^{o}C) (Economos and Lints, 1986) and access to water. *Drosophila melanogaster* are associated with rotting fruits, females lay eggs in fruit that will rot in a sufficient timescale for their larvae to feed on the yeast found in the rotting fruit. The short generation time of the *Drosophila* (7 days from egg to adult) has enabled them to radiate rapidly as a species and there are a number of *Drosophila* species found worldwide. Due to their short generation time they also make excellent model organisms for developmental biology and other disciplines and have been well studied in

[6] https://www.systembio.com/piggybac-transposon/overview [accessed 17/02/2016]

laboratories for over a century (Arias, 2008). These studies have enabled a thorough understanding of genetic function and isolation of a number of genetic sequences, some of which can be used as promoters in different orders of organisms.

2.3 Discosoma spp.

Discosoma genes have been used extensively as markers of gene expression where DsRed[7] or a close variant have been used as a marker gene. *Discosoma* species are also known by their common name of mushroom corals and are found throughout many marine environments. *Discosoma spp* have particular fluorescence proteins which are similar to the green fluorescent protein (GFP) family of proteins. A mutation of DsRed enabled the generation of a close variant, DsRed2, which has improved expression and solubility, assisting its use as a marker gene. The fluorescent marker *DsRed2* has been used extensively as a marker in a wide variety of organisms from viruses (Weber et al., 2006) to fungal species (Nahalkova and Fatehi, 2003) and mammals (Arao et al., 2009).

The fluorescence genes confer no competitive advantage or disadvantage to the recipient and there are no reported adverse consequences to the environment or human health. The following review articles are useful in this regard:

- (Millwood et al., 2010) Fluorescent Proteins in Transgenic Plants. Reviews in Fluorescence 2008:387-403.
- (Stewart, 2006) Go with the Glow: Fluorescent Proteins to light transgenic organisms. Trends in Biotechnology 24(4):155-162.

2.4 Escherichia coli*

Escherichia coli is an intensively studied bacterium which serves as a model organism across a range of disciplines. Its geographical range is worldwide as *E. coli* forms part of the microbiota of the lower gastrointestinal tract of mammals, including humans. There are many strains of *E. coli*, most of which exist as harmless commensals, however a number of pathogenic strains of *E. coli* have been recorded. The genomes of several *E. coli* strains have been completely sequenced and information from the sequencing has been used in designing constructs for genetic engineering. Sequencing genomes of *Salmonella* strains, which last shared a common ancestor with *E. coli* more than a hundred million years ago, has increased our understanding of the evolution of bacterial species (Elena et al., 2005). The *E. coli* strains used in the generation of the tetracycline-repressible system are all laboratory strains listed in Altschmied et al. (1988). These strains are typically benign and non-pathogenic.

[7] http://www.clontech.com/US/Products/Fluorescent_Proteins_and_Reporters/Fluorescent_Proteins_by_Name/DsRed2_Fluorescent_Protein (Accessed 17/05/2016)

2.5 Herpes simplex virus type 1*

There are more than 80 herpes viruses circulating in human populations however only 8 of these are known human pathogens. Different human populations have increased susceptibility to infection with a Herpes virus but there is a worldwide distribution of Herpes within the human population (Fatahzadeh and Schwartz, 2007). Herpes simplex virus type 1 (HSV-1) is a human virus usually associated with infections of the lips, mouth, and face. It is the most common herpes simplex virus and many people develop it in childhood. It is transmitted by contact with infected saliva. By adulthood, 30 - 90% of people will have antibodies to HSV-1

VP16 is a virion phosphoprotein of HSV and a transcriptional activator of viral immediate-early (IE) genes and requires an acidic transcriptional domain, which if absent then the VP16 is impaired in its capacity to support the infectious cycle. VP16 also requires transport to the nuclear membrane and binding to various co-factors in the nucleus for activation. For activation to occur the co-factors must be present.

* In OX513A VP16 is used in a fusion protein with domains from *E. coli* and known as tTAV. Activating regions derived from the HSV-1 have been coupled to control elements derived from *E. coli* in order to develop the conditional lethal tetracycline-repressible transactivator element, tTA, widely used as the tet- repressible control system (Gossen and Bujard, 1992). Although the self-limiting trait tTAV is based on a fusion of E. coli and VP-16 from HSV-1 the DNA used in the rDNA construct is synthetic in nature.

2.6 Small synthetic linking sequences.

Synthetic linking sequences are used to connect genetic elements within the construct.

3 Vector used in the transformation of OX513A

A summary of the vector characteristics and transformation technique is provided below, Comprehensive details on the transformation can be found in ***Appendix 1*** **-** *Section 1 Molecular characterisation and lineage of Aedes aegypti OX513A; Preparation of the vector plasmid and sources of the genetic elements.*

The vector used is *PiggyBac*, DNA (deoxyribonucleic acid) transposons isolated from the Cabbage looper moth, *Trichoplusia ni*. *PiggyBac* is only capable of integrating into DNA flanked by an open reading frame (ORF) within the element when its inverted terminal repeats (ITRs) are intact. (Handler, 2002; Handler and James, 1998). In the construct used for transformation of the mosquitoes the transposase gene of the *PiggyBac* element was irreversibly destroyed by deletion of a section of that gene. Transformation is effected by introducing with the transforming construct, a helper plasmid that supplies transposase activity but is itself unable to transpose into other DNA. One of the ITR's that flank the wild type *piggyBac* transposase has been removed in the helper plasmid so that the helper plasmid cannot itself integrate, even though it encodes for the active transposase. The helper plasmid is not present in the modified mosquitoes.

The transformation of the *Aedes aegypti* OX513A was achieved through micro-injection of individual eggs into germ line cells according to the methods described in Jasinskiene et al. (1998). The micro-injection consisted of the vector plasmid, pOX513 (shown in **Figure 1**) co-injected with a *PiggyBac* 'helper plasmid' as the source of *PiggyBac* transposase (**Figure 2**) (Handler and James, 1998). Once a stable transformed line of laboratory reared *Aedes aegypti* of the Rockefeller strain was identified, it was made homozygous through mating heterozygotes and selecting homozygous offspring over several generations (approximately 8-9). The strain has been continuously maintained since 2002.

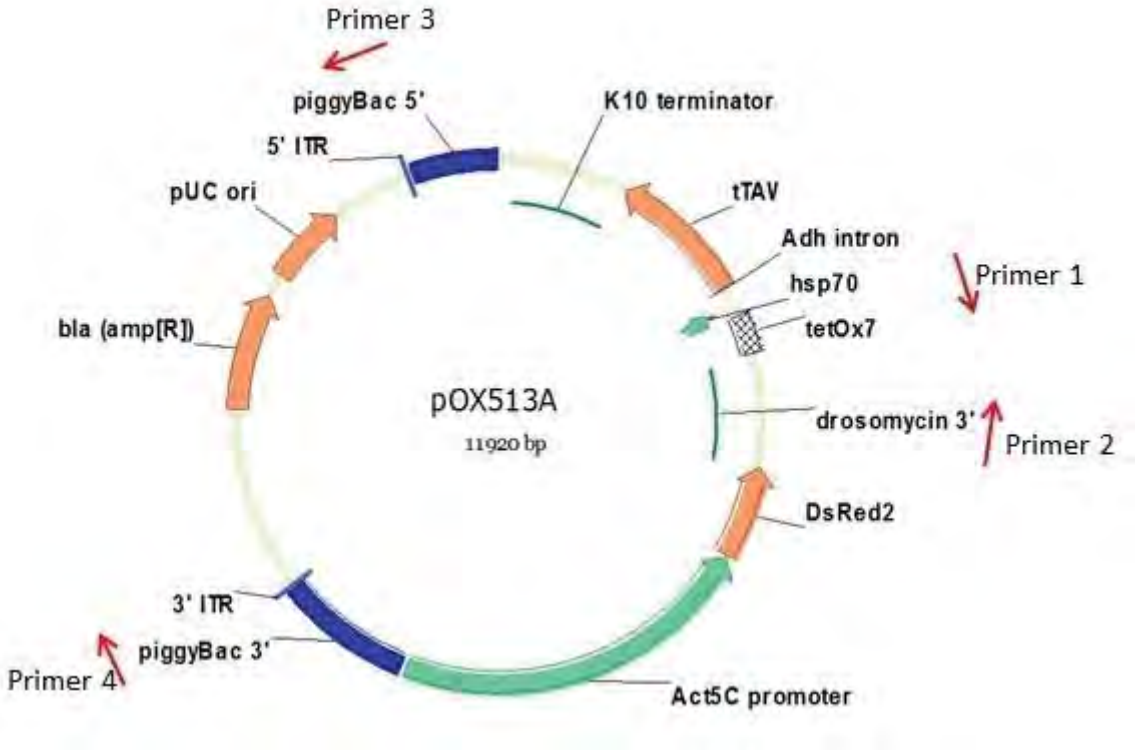

Figure 1. Map of the plasmid used in the transformation of OX513A. Primer locations are a schematic representation intended to represent the general regions of the plasmid amplified as described in Section 4.1 *Detecting the absence of plasmid backbone in transgenic lines.*

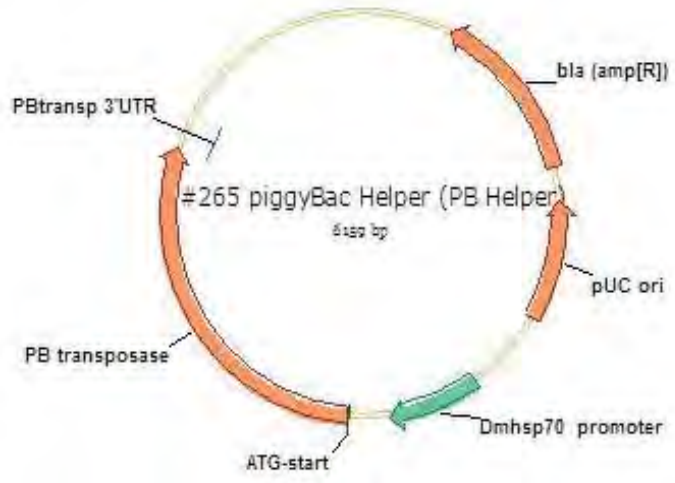

Figure 2. *PiggyBac* helper plasmid vector used in the transformation

4 rDNA Insert and characteristics of modification

An overview of the key characterisation data of the rDNA insertion event, and expressed genes is provided in this section. More details on the molecular characterization can be found in ***Appendix 1.*** *Molecular characterisation and lineage of Aedes aegypti OX513A;* ***Section 3****. Molecular characterization of Aedes aegypti OX513A line.* The inserted genetic elements and their functions are summarised in **Table 1** below. Between the coding regions of DNA short sections of DNAs are present these small fragments are non-coding and are likely to be short incomplete sections of genes. Positional information can be found in ***Appendix 1 - Table 2***

Table 1. Genetic elements contained in the rDNA construct in OX513A *Aedes aegypti.*

Genetic Element	Donor Organism and Common name	Reference	Genotypic and phenotypic effect
piggyBac 3'	*Trichoplusia ni* (Cabbage looper moth)	(Cary et al., 1989);(Thibault, 1999)	DNA transposable element with sequence deletions to prevent mobility. See description under section 1 c). Characteristics of the vector, including its identity, if any, and its source or origin, and its host range.
Act5C	Drosophila melanogaster (Vinegar fly)		Promoter element driving the expression of the marker gene
DsRed2	Discosoma (Coral)	(Lukyanov et al., 2000); (Matz et al., 1999)	Red fluorescent protein marker gene. The fluorescent marker has been used in a wide range of vertebrate and invertebrate species, as marker genes confer no competitive advantage or disadvantage to the recipient, and there are no observed adverse consequences resulting from their incorporation into the mosquito.
Drosomycin 3' UTR	Drosophila melanogaster (Vinegar fly)		Polyadenylation signal
$TetO_7$	Escherichia coli (bacteria)	(Gossen and Bujard, 1992)	Non-coding binding site for tTAV
Hsp70	*Drosophila sp.* (Vinegar fly)		Promoter element driving tTAV
Adh intron	*Drosophila sp.* (Vinegar fly)		Enhances gene expression
tTAV	Synthetic DNA based on a fusion of sequences from *Escherichia coli* and Herpes simplex virus (VP16 transcriptional activator)	(Gong et al., 2005); (Gossen and Bujard, 1992)	Tetracycline repressible transcriptional activator: tTAV protein binds to and activates expression from the tetracycline response element (tRE) which includes the specific DNA sequence to which tTAV binds ($TetO_7$). Note: tRE is included within the tTAV component of the plasmid map (Figure 1) and not separately labelled. tTAV also binds tetracycline with a high affinity, preventing it from binding DNA. The putative mode of action is that high level expression of tTAV is deleterious to cells as it represses normal transcriptional function. tTAV thus acts as a tetracycline regulated switch which confers the self-limiting trait and thus enables the mass rearing of the mosquito in the laboratory when a dietary supplement of the tetracycline family is administered. tTAV has been used in fungi, mice, plants and mammalian cultures with no known adverse effects on the environment or human health
K10 Poly-A	*Drosophila sp*. (Vinegar fly)		Polyadenylation signal
piggyBac 5'	*Trichoplusia ni* (Cabbage looper moth)	(Cary et al., 1989)	DNA transposable element with sequence deletions to prevent mobility.

4.1 Detecting the absence of plasmid backbone in transgenic lines

Also see ***Appendix 1*** *Section 3.5 Detecting the absence of plasmid backbone in transgenic lines*

The plasmid is derived from a cloning vector (Gal4/UAS - pKC26-FB2) which includes an ampicillin resistance gene (*bla (amp[R]*)) and a bacterial origin of replication (*pUC ori*) **Figure 1**). To determine whether these backbone sequences had been integrated into OX513A, PCR was used to amplify a target 4045bp fragment encompassing the entire plasmid backbone using primers 3 and 4 in noted in **Figure 1**. As PCR is more efficient from plasmid templates than from genomic DNA, due to the differences in complexity and size. In order to estimate what dilution of plasmid would be comparable to genomic DNA for analytical controls, PCR on a dilution series of plasmid DNA was carried out using Primers 1 and 2 in **Figure 1**. In order to check that the genomic DNA (gDNA) was of sufficient quality for PCR analysis, an additional PCR was carried out to amplify a 747bp fragment of the endogenous mosquito gene Actin 4.

The results confirm that the gDNA was of sufficient quality to support PCR amplification (**Figure 3**) and the absence of plasmid backbone DNA in OX513A was confirmed (**Figure 4**).

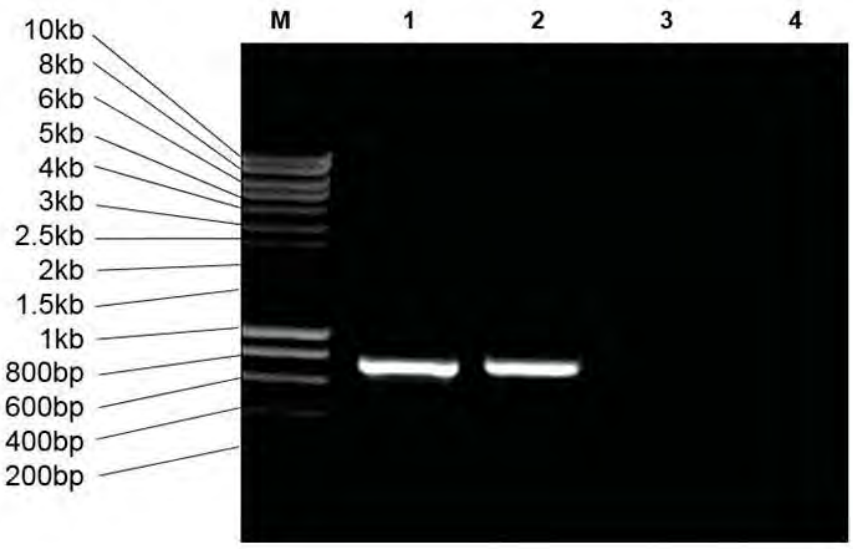

Figure 3: Results of PCR analysis to check gDNA quality using primer set for Actin 4 gene. Lane 1: OX513A genomic DNA, lane 2: Wild-type genomic DNA, lane 3: 1/5000 dilution of pOX513 plasmid and Lane 4: H2O. M: Smart ladder (Eurogentec).

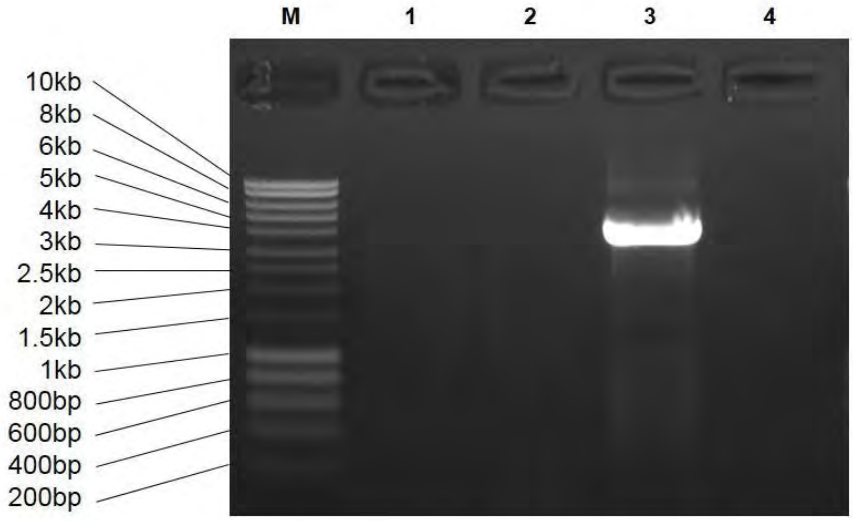

Figure 4: Results of PCR analysis for vector backbone using primer sets 3 and 4. Lane 1: OX513A genomic DNA, lane 2: Wild-type genomic DNA, lane 3: 1/5000 dilution of pOX513 plasmid and Lane 4: H2O. M: Smart ladder 200bp-10Kb (Eurogentec).

4.2 Number of copies inserted

Also see ***Appendix 1*** *Section 3.3 Confirmation of single insertion site.*

The OX513A strain has been continuously maintained in the laboratory for over 115 generational equivalents[8] with no indication of genetic instability or derivation from expected Mendelian inheritance ratios that could be associated with a multiple insertion or segregating loci. Southern blot analysis was conducted to confirm the presence of a single insertion in strain OX513A.

Three restriction enzymes (*Age*1, *Bgl*II, and *Sal*1) were used and their recognition sites in the inserted plasmid are shown in **Figure 5**. The three enzymes used were chosen because each cut only once in the area of the transgene recognised by the probes which allowed minimum restriction fragment sizes to be determined. Exact size of band is unknown as the next

[8] As of 16/08/2016- Generational equivalents for OX513A *Aedes aegypti* are defined as: non-discrete generations with a generation time of approximately 4 weeks under the conditions used, leading to 12-15 generations per year. This number may potentially vary with temperature, and also with the length of time that eggs are stored prior to hatching. For GE insects, the first generation or two from the founder individual (transgenic G_1 individual, where G_0 represents the individuals micro-injected with DNA) can be identified as discrete generations. Thereafter – and sometimes from the outset, especially where the number of initial transformants is high, pooled rearing is more common. Furthermore, this may not involve discrete-generation rearing. For example, in large-scale rearing all life-cycle stages are present, and eggs collected at a particular point in time cannot be assigned to a particular generation by lineage or pedigree tracing, rather a time-based estimate can be made of the rate of progress through generations.

restriction site in the gDNA is unknown. The expected resulting restriction fragments are labelled A, B and C and Southern Blot probes are indicated by green boxes.

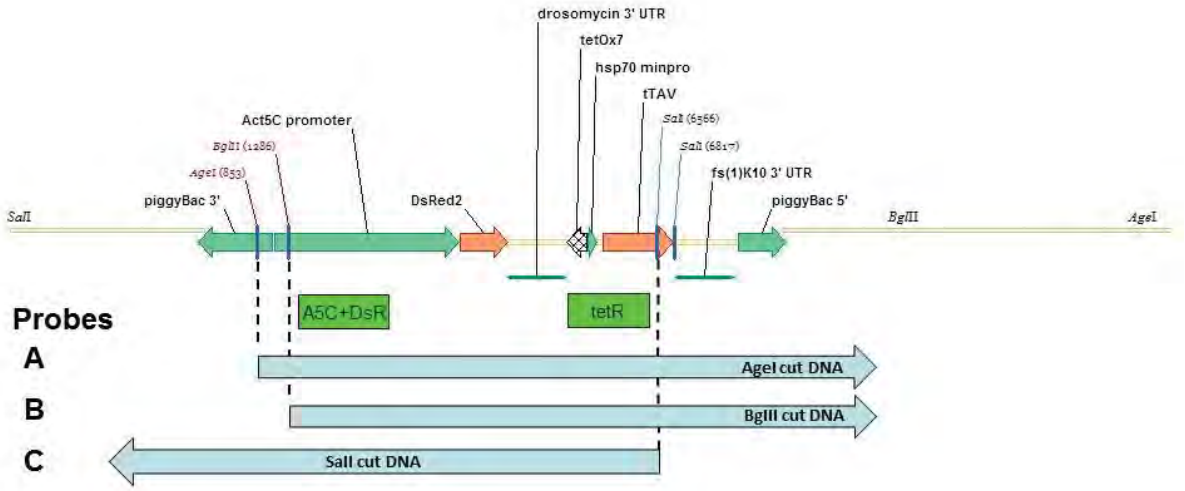

Figure 5. Schematic diagram showing restriction digests strategy and probes to determine copy number by Southern blot. *Age*I cuts within the piggyBac 3' of the transposon at 853 bp and further downstream in the genomic DNA to produce a band expected to be more than 7565 bp. *Bgl*II cuts within the Act5C promoter sequence at 1286 bp so is expected to produce a fragment of more than 7131 bp. *Sal*I cuts within the tTAV sequence at 6566Kb and 6817KB to produce a band expected to be more than 6566 bp on the Southern Blot.

As shown in **Figure 6**, there is only one detectable band larger than the minimum expected size for each restriction digest, supporting evidence for a single insertion of the transgene.

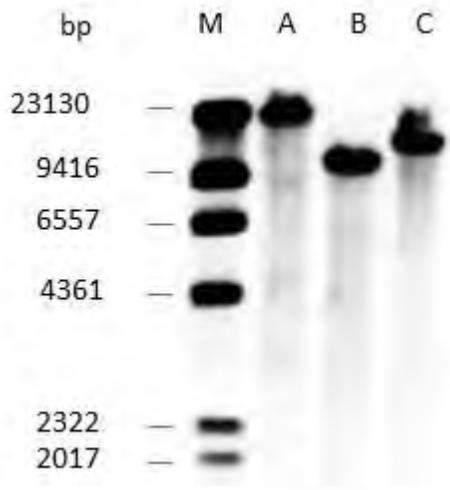

Figure 6. Southern Blot of OX513A. Digoxiginin labelled DNA marker (M), *Aedes aegypti* (Latin) OX513A gDNA (10 µg) digested with *Age*I (A), *Bgl*II (B) and *Sal*I (C) restriction enzymes. Further controls were not deemed appropriate for this test as the hybridizing bands on the Southern blot confirm the data obtained from amplification and DNA sequencing of the integrated OX513A insert within the *Aedes aegypti* genome.

4.3 Verifying the insertion site and sequencing the regions flanking the gene

See also ***Appendix 1*** *Section 3.1 Genome insertion site*

An inverse polymerase chain reaction (PCR) methodology was used to identify the genomic sequence adjacent to the insertion site of OX513A as described in Handler and James (1998). Genomic DNA from OX513A was digested with the restriction enzymes *Hae*III, *Msp*I and *Taq*I (chosen as they cut roughly every 500bp-5kb in the *Aedes aegypti* genome). Restriction fragments were circularised by ligation with T4 DNA ligase, and a PCR reaction using primer sequences in opposite orientation within the region between the *PiggyBac* restriction site and terminus for each junction, was used to amplify the sequences flanking the insertion site. Subcloned PCR products were sequenced and compared to *PiggyBac* terminal sequences by DNA alignment and BLAST analysis (Altschul et al., 1990) to identify the genomic insertion site sequences and distinguish them from those in the OX513 plasmid.

Sequencing of the flanking regions showed that the insertion was mediated by *PiggyBac* into a TTAA target site in the genome as expected for this integration method. The TTAA duplicated target site is characteristic of all *PiggyBac* integrations (Elick et al., 1996) and typically indicates a vector-mediated transposition. DNA flanking sequences of 307bp and 315bp on either side of the insertion site were obtained.

The *Aedes aegypti* genome has been fully sequenced, assembled, and annotated with respect to known genes, expressed sequence tags (ESTs) and transcripts. This information is publicly available via Vectorbase[9]. The combined flanking sequence of 622bp was compared with the genome sequence, transcript and EST databases using the BLAST tool on the Vectorbase website. Both Blastn and Blastx functions were used to compare the sequence in both orientations at the nucleotide level (Blastn) and translated sequence in all 6 reading frames, to deposited amino-acid sequences (Blastx). The sequences were also analysed using the NCBI Blast database[10], which compares the nucleotide (Blastn) and translated sequences (Blastx), again in both orientations, to all sequences deposited in Genbank version 2.2.27. The flanking sequence shows 94.6% identity across its entire length with a single genome sequence contig (1.859), showing an unambiguous match.

No homology to known open reading frames was identified, thus no genes appear to be disrupted by the insertion. In addition, the genome browser view on Vectorbase of this BLAST match shows that the nearest gene/EST hit is 30.5kb away, so is not expected to be affected by this transgene insertion.

4.4 Nature of the inserted traits *DsRed2* and *tTAV*

Aedes aegypti is biologically similar with respect to its life-history characteristics to the wild populations of mosquito except for the introduction of two traits (Phuc et al., 2007)

[9] http://aaegypti.vectorbase.org (accessed 31/08/2016)
[10] https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed 31/08/2016)

4.4.1 Fluorescent marker *DsRed2*

A heritable fluorescent marker protein (DsRed2) has been stably integrated at a specific site in the OX513A genome. The marker gene enables the detection of OX513A in the field, and allows the evaluation of the dissemination of OX513A genes resulting from the release of OX513A males. DsRed is a naturally occurring fluorescent protein which was originally found in various Discosoma spp. The DsRed2 is from Clontech Laboratories[11] and was artificially developed from DsRed to enhance the fluorescence and improve the solubility, which in turn increases the sensitivity of detection (Shagin et al., 2004); (Bevis and Glick, 2002); (Matz et al., 1999); (Lukyanov et al., 2000). In OX513A, there are three additional amino acids (MAR) at the N-terminus, which are from a cloning linker sequence. The DsRed2 protein is expressed constitutively in the developmental stages of the OX513A mosquito and results in a fluorescent phenotype when viewed with diagnostic equipment (excitation wavelength 558nm, emission 583nm).

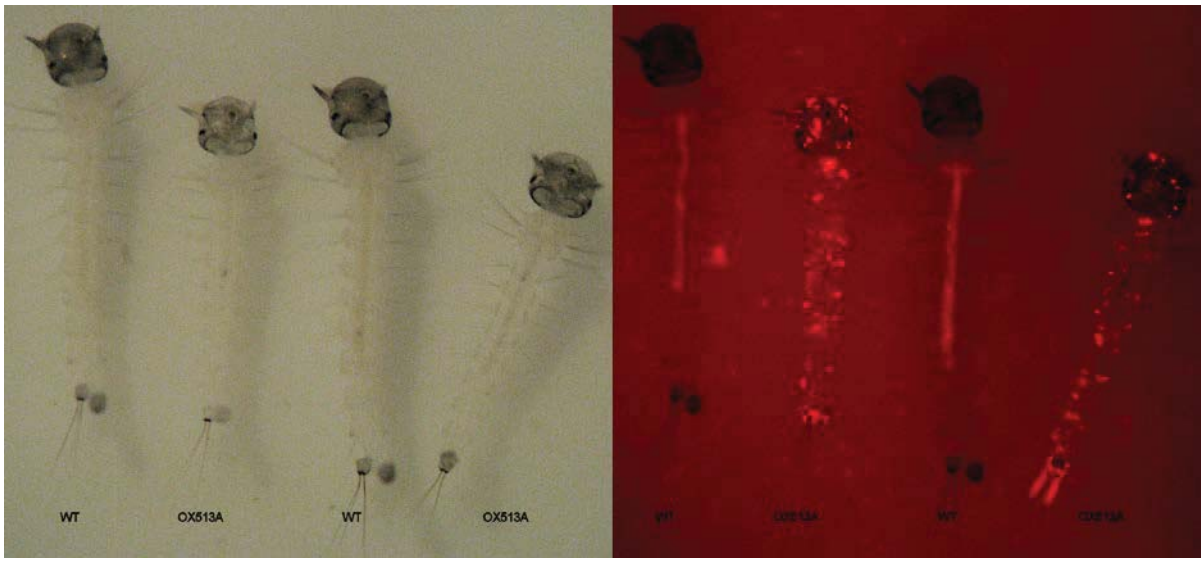

Figure 7. Expression of fluorescent marker (DsRed2) in OX513A *Aedes aegypti* under diagnostic fluorescence microscope. The fluorescent marker is strongly expressed in a characteristic punctate manner,, allowing the easy identification of OX513A individuals.

4.4.2 Self-limiting trait *tTAV*

An insect-optimized tetracycline repressible transactivator protein (tTAV) has been stably integrated into OX513A. This is intended to produce a phenotype whereby the progeny of matings have increased mortality. The tTAV protein binds to and activates expression from the tetracycline response element (tRE) which includes the specific DNA sequence to which tTAV binds (tetO), but in the presence of the antibiotic tetracycline or its analogues, it binds preferentially with high affinity to the tetracycline preventing it from binding DNA in the cell

11 http://www.clontech.com/GB/Products/Fluorescent_Proteins_and_Reporters/Fluorescent_Proteins_by_Name/DsRed2_Fluorescent_Protein?sitex=10030:22372:US (accessed 16/08/2016)

(Gossen and Bujard, 1992), thus preventing the transcription of the gene regulated by that promoter (see **Figure 8**). tTAV thus acts as a tetracycline regulated switch which confers conditional cell death and thus enables the mass rearing of the mosquito in the laboratory when a dietary supplement of the tetracycline family is administered.

The first Tet system was described in Escherichia coli by (Gossen and Bujard, 1992) and is known as the TET-OFF system. In this system, the presence of tetracycline blocked transcription of a "transactivator protein" (tTA) switching off the positive feedback loop driving expression of the tTA protein. The self-limiting trait in OX513A works via a tTAV system (a variant of tTA) which elicits cell death is a result of a build-up of proteins within the cells of larvae, this is purported to be through transcriptional squelching (Lin et al., 2007). High-level expression of tTA is deleterious to cells as it can repress normal transcription.

tTAV is a tTA variant sequence optimized for expression in *D. melanogaster* and other insects. tTA and its variants, such as tTAV, have been used in fungi, rodents, plants, and mammalian cultures with no observed non-target adverse effects on the environment or human health. Its use in animal systems ranges from small mammals such as rats and mice reviewed in (Schönig et al., 2013), to dogs (Kim et al., 2011) as well as fish (Li et al., 2012). There are over 10 000 publications on its use[12] in a wide range of systems. Its wide use is due to the observation that it is well tolerated in eukaryotic systems (Schönig et al., 2013; Naidoo and Young, 2012; Stieger et al., 2009; Muñoz et al., 2005; Zhu et al., 2002).

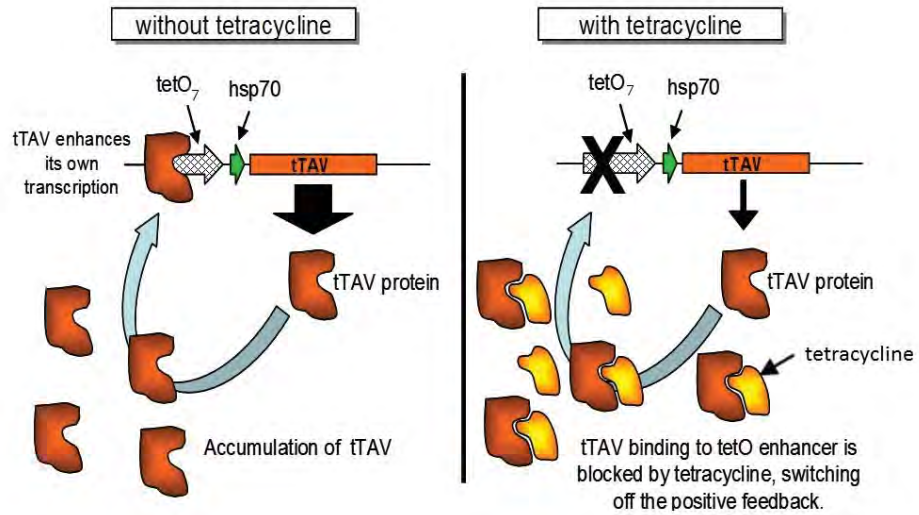

Figure 8. Schematic representation of the tTAV system. In the absence of tetracycline (left panel), small amounts of tTAV protein generated by the effect of the hsp70 promoter (hsp70) can bind to the tetO binding sites ($tetO_7$), creating a positive feedback loop that enhances expression of tTAV. When the tTAV protein accumulates in sufficient quantities it affects cellular function, resulting in cell death in the developing larvae. In the presence of tetracycline (right panel), tTAV is prevented from binding to the tetO sites and can therefore not enhance the expression from the hsp70 promoter. This prevents the accumulation of tTAV.

[12] http://www.tetsystems.com/science-technology/ (accessed 17/05/2016)

4.5 Potential for toxicity and allergenicity of the introduced proteins

To assess whether the tTAV or DsRed2 proteins inserted into OX513A contain sequences that are likely to represent potential hazards to animal or animal health as a result of toxic or allergenic properties, a comprehensive independent bioinformatics analysis was conducted by the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska U.S.A.[13] The program develops and provides expert services relating to allergenic and novel foods and food ingredients including GM products. A summary of the bioinformatics assessment is provided below, for the full report see ***Appendix 2*** *Bioinformatics analysis for risks of allergenicity and toxicity of proteins encoded by the two genes introduced into genetically engineered mosquitos (Aedes aegypti), strain OX513A for production of sterile males to reduce vector transmission of important human diseases.*

The analysis examined the potential toxicity and allergenicity of the inserted proteins tTAV and DsRed2 using a weight of evidence approach based on reviews of the scientific literature and specific studies based on sequence homology. Additionally, a literature search was performed based on the same methodology on additional elements of the OX513A rDNA construct.

4.5.1 Bioinformatics assessment of the allergenic and toxicity potential of DsRed2 and tTAV

A bioinformatics analysis (***Appendix 2***) was conducted by the FARRP in accordance with published guidelines of the International Codex Alimentarius, Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (FAO/WHO, 2001). The guidelines establish highly conservative criteria to identify proteins which are allergens, or are sufficiently similar to an allergen to cause cross-reactions. A procedure is established on how to assess sequence homology between a given protein and known allergens. The Food Allergy Research and Resource Program compared tTAV and DsRed2 protein sequences coded in the OX513A rDNA construct to known allergens, as well as known toxins, to specifically identify:

- sequence matches with > 50% identity over the full-length;
- sequence matches of >35% identity over 80 or more amino acid segments; and,
- identical matches of 8 or more contiguous amino acid segments.

The Amino Acid Sequence of the tetracycline-controlled transactivator (tTAV) protein. The sequence is 100% identical as described in Gong et al. (2005) and Phuc et al. (2007). The Amino Acid sequence of the DsRed2 protein in OX513A is identical to the published sequence of pX-DR (Chen et al., 2009) + 3 amino acids at N- terminus added in constructing the mosquito insertion transposon.

The analysis was conducted using bioinformatics search tools to compare tTAV and DsRed2 protein sequences to two different databases containing over 31.5 million sequences in order

[13] http://farrp.unl.edu/ (accessed 17/05/2016)

to identify regions meeting the established criteria. The databases consulted were: AllergenOnline, a sequence searchable database intended for the identification of proteins that may present a potential risk of allergenic cross-reactivity; and, NCBI Entrez Protein, containing entries from multiple sources maintained by the National Center for Biotechnology Information (NCBI) of the National Institutes of Health (U.S.A.). The search tools used in the analysis, BLAST (Basic Local Alignment Search Tool) and FASTA3, represent two unique computer algorithms designed to compare DNA and amino acid sequences. The tools differ slightly in their functionality and application.

Searches revealed no matches meeting established criteria, suggesting the proteins are not allergens nor are sufficiently similar to an allergen to cause cross-reactions. They did not identify matches to toxins to suggest they may be toxic.

Although not directly or indirectly toxic, it is the specific and intended effect of the genetic modification that the expression of tTAV confers conditional cell death in the developing larvae of the progeny of matings of OX513A males with wild *Aedes aegypti* females, in the absence of a dietary supplement of the tetracycline family.

4.5.2 Literature review on the history of use of the elements in the OX513A rDNA construct.

Literature searches (**Appendix 2**) were conducted through the PubMed (NCBI) database maintained by the US National Library of Medicine (http://www.ncbi.nlm.nih.gov/pubmed). PubMed is a search engine accessing a comprehensive database of references and abstracts on life sciences and biomedical topics. PubMed provides quality control in scientific publishing and only journals that meet PubMed's scientific standards are indexed. The primary question used to build the search parameters was whether the sources of the gene or sequences used in the construct are causes of allergy or toxicity. Searches where conducted broadly using the introduced gene source such as the source organism and/or specific element, and terminology such as “allergen”, “allergenicity”, “toxin”, toxicity”. The data (authors, publication, date and abstracts) from searches were saved to files for review. All publication abstracts were manually reviewed and any likely relevant publications suggesting adverse health risks were investigated further.

Additionally, as the above noted search only represented tTAV and DsRed2, a supplemental literature search using the same methodology, explored the potential toxicity, allergenicity and pathogenicity of the other genetic elements in the pOX513 construct. A description of the methodology can be found in ***Appendix 3*** *Supplement to DsRed2 and tTAV Bioinformatics report.*

The literature search analysis did not uncover any concerns of potential allergenicity, allergenic cross-reactivity or potential toxicity that would demonstrate a need for further testing regarding safety.

Additionally, ***Appendix 3.1*** *Expert opinion 2015 Transgenic protein tTAV - assessment of allergenic risk* presents an analysis by Professor Ian Kimber, currently Professor of Toxicology and Associate Dean for Business Development in the Faculty of Life Sciences at the University

of Manchester[14] who did not identify any risk to human health associated with the tTAV or DsRed2 in OX513A.

4.5.3 Additional Toxicity and Allergenicity Assessment

A New Protein Consultation (NPC0004) has been carried out by the U.S. Food and Drug Administration (FDA) -Center for Food Safety and Applied Nutrition (CFSAN)[15] on DsRed2 expressed in plants as part of an application for deregulation in the USA for DP 32138-1 Maize. A bioinformatic analysis was conducted in accordance with the international Codex Alimentarius; Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (FAO, 2001) and revealed no identities between known or putative protein allergens or toxins and the DsRed2 protein sequence. Additionally, the lability of the protein in simulated gastric fluid (SGF) and an examination of the gene source and history of exposure, was assessed (Pavey, 2006). The Maize line expressing DsRed2 has been further evaluated in an Environmental Assessment (EA) by the United States Department of Agriculture (USDA)[16] which concluded that the corn transformation event that contained the DsRed2 gene was unlikely to become a plant pest risk.

An Environmental Assessment (EA) and Finding of No Significant Impact (FONSI) has been issued by the United States Department of Agriculture- Animal and Plant Health Inspection Service (USDA-APHIS) for the field Release of an Oxitec Diamondback moth expressing the tTAV and DsRed2 genes[17]. Additionally, a Pink Bollworm expressing fluorescent genes similar to DsRed2 (Enhanced Green Fluorescent Protein) was assessed by the USDA-APHIS and it was concluded that it was unlikely to present an environmental risk [18].

The United States Food and Drug Administration- Center for Veterinary Medicine (US FDA-CVM) have issued (August 5, 2016) an EA and FONSI In support of a proposed field trial of genetically engineered (GE) male Aedes aegypti mosquitoes of the line OX513A in Key Haven, Monroe County, Florida under an investigational new animal drug exemption[19] which states "*FDA found that the probability that the release of OX513A male mosquitoes would result in toxic effects in humans or non-target animals or allergenic effects in humans is extremely low and the risk is negligible.*" A link to the full EA and FONSI is provided in ***Section 7 - Table 8***

[14] https://www.liverpool.ac.uk/drug-safety/staff/professoriankimber/ (Accessed 27/06/2016)
[15] http://www.fda.gov/AboutFDA/CentersOffices/OfficeofFoods/CFSAN/ (Accessed 17/05/2016)
[16] http://www.aphis.usda.gov/brs/aphisdocs/08_33801p_dpra.pdf (Accessed 17/05/2016)
[17] https://www.aphis.usda.gov/brs/aphisdocs/13_297102r_fonsi.pdf and https://www.aphis.usda.gov/brs/aphisdocs/13_297102r_dea.pdf (Accessed 31/08/2016)
[18] https://www.aphis.usda.gov/brs/aphisdocs/05_09801r_ea.pdf (Accessed 17/05/2016)
[19] http://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/GeneticEngineering/GeneticallyEngineeredAnimals/ucm446529.htm (Accessed 23/08/2016)

4.6 Conclusions regarding the characterisation of the insert in OX513A

Conclusions drawn from the observations in this section below are also presented in along with additional conclusions in ***OX513A Environmental Risk Assessment Part C- Section 1.2.1 Molecular Characterisation***

- The sequence of the construct in OX513A is as intended without re-arrangements.
- Based on flanking sequence analysis, the insertion is not known to disrupt endogenous gene function and no proteins other than those intended are likely to be produced.
- OX513A does not contain vector backbone sequences from the plasmid used for transformation, including antibiotic resistance genes or origins of replication, verified by as verified by molecular analysis.
- No contaminating materials such as viruses, cells or chemicals were introduced during the transformation process only the relevant parts of the rDNA construct intended to express the desired genotype and phenotype.
- The insert has been shown to be stable and a complete single copy insertion.
- No sequences have been inserted that encode for pathogens, toxins, or allergens as evidenced by both literature searches and bioinformatics studies.
- The expression pattern of the inserted trait is as expected for a single insertion event.
- No sequences have been introduced that encode for pathogens, toxicants, allergens or are likely to have other potential adverse effects on the animal with the exception of the intended effect.
- Evidence has been provided from the literature and bioinformatics studies on the lack of allergenicity and toxicity of the gene sequences in the rDNA construct from the donor organisms.
- Information from searches of the scientific literature on pathogenicity has been provided for the sequences in the rDNA construct, which indicates there is unlikely to be any adverse effect on human health, animal health or the environment.

5 Characterisation of OX513A

5.1 Life table parameters

Several life table parameters have been examined for the OX513A strain in comparison with the unmodified wild-type comparator mosquito and are described below.

Bargielowski et al., (2011b) compared the life history characteristics of OX513A and a wild-type strain of *Aedes aegypti* to increasing larval rearing density using a constant amount of food per larva. Parameters examined were larval mortality, developmental rate (i.e., time to pupation), adult size, and longevity. Only two statistically significant differences were found between the strains: the OX513A *Aedes aegypti* larval survival was 5% lower than the wild-type and there was a reduced adult longevity (20 days OX513A vs 24 days WT mean lifespan). The OX513A line pupated approximately one day sooner than the WT *Aedes aegypti* resulting in smaller adults than the unmodified line. This effect was more pronounced in females than in males. Whilst these differences between OX513A and the WT lines could lessen the performance of the released males in a population suppression program, the likelihood that it results in adverse impacts on the environment is considered negligible compared to the use of current controls for *Aedes aegypti*.

For the complete study report see Bargielowski et al. (2011b)

5.1.1 Reproductive capacity

Several parameters regarding reproductive capacity have been measured for both wild type and OX513A strain in two independent studies.

In a study by Patil et al. (2015) mating and life table assessments were used to compare OX513A with reared *Aedes aegypti* strains collected from New Delhi (DEL) and Aurangabad (AWD) regions in India. The laboratory study demonstrates that only minor life table variations of limited biological relevance exist between OX513A and Indian *Aedes aegypti* populations. Developmental time from first instar to adult emergence was significantly longer for OX513A (10.7±0.04 days) than for New Delhi (9.4±0.04 days) and Aurangabad strains (9.1±0.04 days). This difference is presumed to be from the OX513A strain having been adapted to laboratory rearing since 2002, whereas the DEL and AWD strains were wild collected in 2011 and subsequently laboratory reared. Differences in mean longevities, female reproductive parameters and population growth parameters between the strains were non-significant (**Table 2**). Additionally, the study examined the mean dorsal cephalothorax widths (mm) of OX513A and wild type Delhi *Aedes aegypti* male pupae, as well as mating competitiveness (discussed in **Section 5.1.3**) and found no significant differences.

Table 2. Reproductive parameters of OX513A and wild type strains of *Aedes aegypti*.

Reproductive parameters	OX513A	Wild Type		F value (df)	p value
	Mean ±SE	DEL Strain	AWD strain		
		Mean ±SE	Mean ±SE		
Blood meals per female	9.5±1.1[a]	7.6±0.5[a]	7.5±0.5[a]	1.9 (2)	0.148
Oviposition events per female	7.9±0.9[a]	7.0±0.5[a]	6.7±0.5[a]	0.8 (2)	0.466
Eggs laid per female	546.5±66.9[b]	499.8±41.8[ab]	378.8±32.1[a]	3.1 (2)	0.050
Hatch rate (%)	91.8±1.24[a]	91.7±0.95[a]	94.9±1.09[a]	2.5 (2)	0.088
Pupation rate (%)	81.0±3.06[ab]	75.2±3.16[a]	88.0±1.70[b]	5.1 (2)	0.010
Adult emergence (%)	79.0±3.13[ab]	73.7±3.20[a]	86.9±1.62[b]	5.3 (2)	0.009

Differences in the mean values indicated by the same letters within the rows are non-significant at 0.05 level by ONE-WAY ANOVA using Tukey's-b test.

For the complete study report see (Patil et al., 2015)

Life table parameters were also previously examined (Lee et al., 2009) by comparing OX513A to the parental wild type strain a strain, and results are consistent with the 2015 Indian study. The data did not reveal significant differences between OX513A and the parental wild-type strain when examining: number of eggs laid per female, number of larvae hatched per egg batch, number of sterile eggs per egg batch, days spent in L4, days spent at pupal stage, and the number of days from hatching to adult.

5.1.2 Insemination capacity and cost of mating

The insemination capacity of males (i.e., the number of females a male is capable of inseminating over the course of his lifetime), and the cost of investing in courtship and mating on longevity for a wild-type strain of Malaysian origin ('WT') and the OX513A line of mosquitoes were evaluated. Experimental details and the results of this study have been published (Bargielowski et al., 2011a) and are summarized in **Figure 9**.

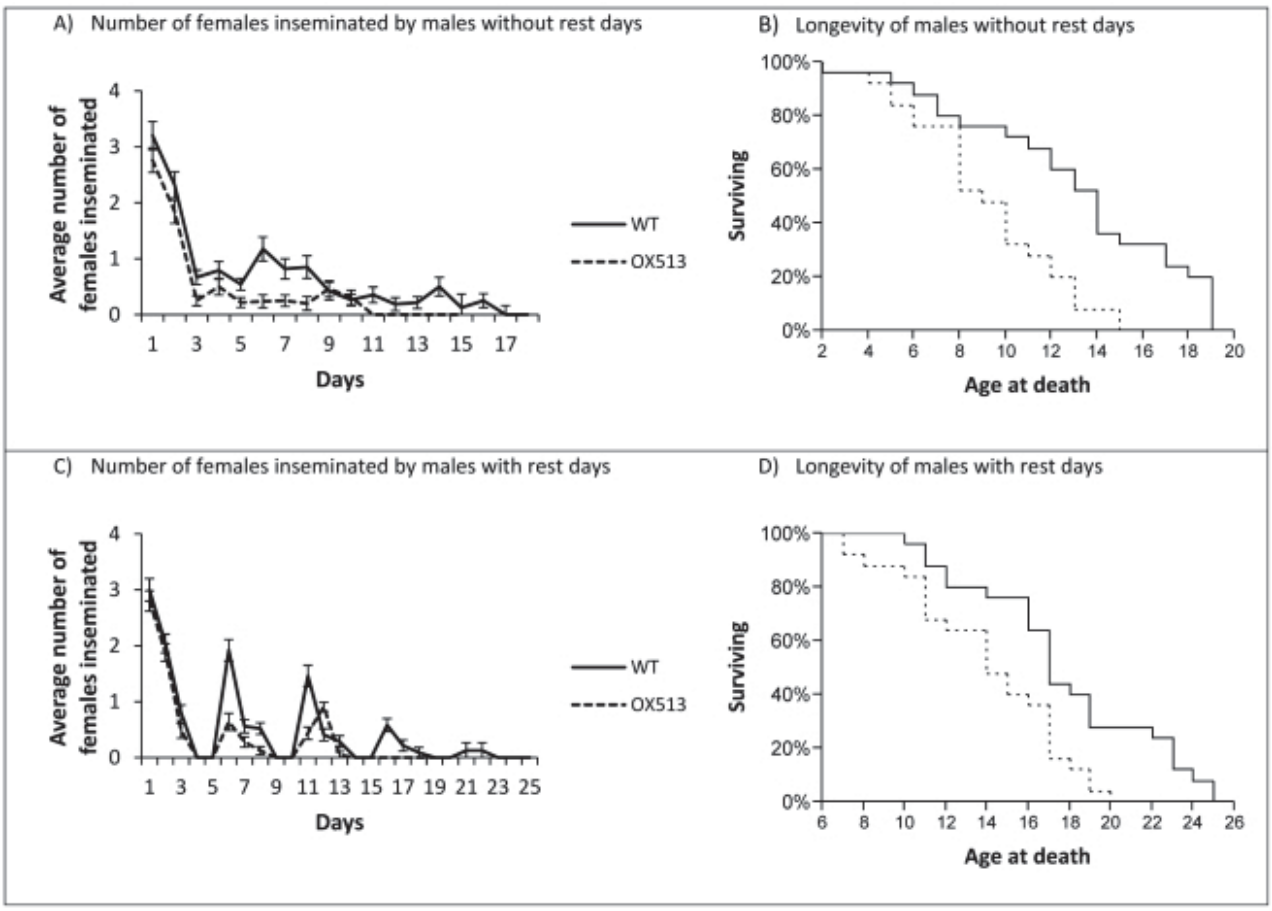

Figure 9. Insemination Capacity of OX513A males (from Bargielowski *et al*, 2011)

Results show distinct differences in the insemination capacity and the cost of mating in males of the OX513A and the WT lines. OX513A males inseminated just over half as many females (on average 6.6) as the WT males (on average 11.5) during their lifetime. Providing days of rest from mating had no significant effect on the total number of females inseminated by males of each line, yet it did increase their longevity. The reduced insemination capacity observed in this study may be evidence of a slight fitness penalty in the OX513A compared to the wild-type.

For the complete study report see (Bargielowski et al., 2011a)

5.1.3 Mating competitiveness

Male mating competitiveness is a key requirement for success in the self-limiting release program. The competitiveness of an organism is defined as its ability to compete with conspecific organisms for a limited environmental resource. In the case of OX513A mating competitiveness, it is the ability of OX513A males to compete with wild males in successfully mating females, and is essential to effect *Aedes aegypti* population suppression. Therefore, extensive testing of the OX513A strain mating competitiveness in a range of environments has been carried out. This includes studies in laboratory cages and in regulated environmental releases in the Cayman Islands (Harris et al., 2012; Harris et al., 2011) and Brazil (Carvalho et al., 2015).

Calculations for evaluating mating competitiveness in the laboratory and in regulated environmental releases are by necessity approached differently due to the nature of the data that is available, and values are not comparable across the two different methodologies. Values generated from laboratory data (**5.1.3.1**) and release data (**5.1.3.3**) differ in terms of thresholds for successful use in vector control programs due to additional considerations that must be given in releases conducted in vector control projects. Further details on considerations for regulated environmental releases are further discussed in ***Section 7*** *Regulated environmental releases of OX513A*.

5.1.3.1 Mating competitiveness in the laboratory

Mating competitiveness studies for OX513A against wild-type strains from around the world have been carried out in a wide variety of laboratory settings with collaborative partners internationally. Confined studies are generally performed with equal numbers of OX513A and wild-type (WT) males competing for WT females (in a ratio of 1:1:1, respectively) in a confined arena. After mating, WT females are individually isolated and allowed to lay eggs, and those which mated with OX513A males can be identified through expression of the fluorescent marker DsRed2 passed to the progeny. The proportion of WT females mated to OX513A males is an indication the competitiveness of OX513A males as compared to WT. If the OX513A males were equally attractive to the WT female as a WT male, equal numbers of each would have mated WT females and mating competitiveness would be equal to 0.5; a lower number is less competitive, and a value of 1 is the maximum upper limit.

The OX513A strain performed successfully against all the WT strains tested regardless of the genetic background as none of the mating competitiveness estimates differ significantly from 0.5 (**Figure 10**). For comparison, based on information from International Atomic Energy Agency (IAEA) with irradiated SIT programs, for the medfly *(Ceratitis capitata*) program, a mating competiveness of 0.2 assessed in the laboratory is acceptable for a successful SIT program[20].

[20] http://www-naweb.iaea.org/nafa/ipc/public/ipc-mass-reared-tephritid.html (accessed 31/08/2016)

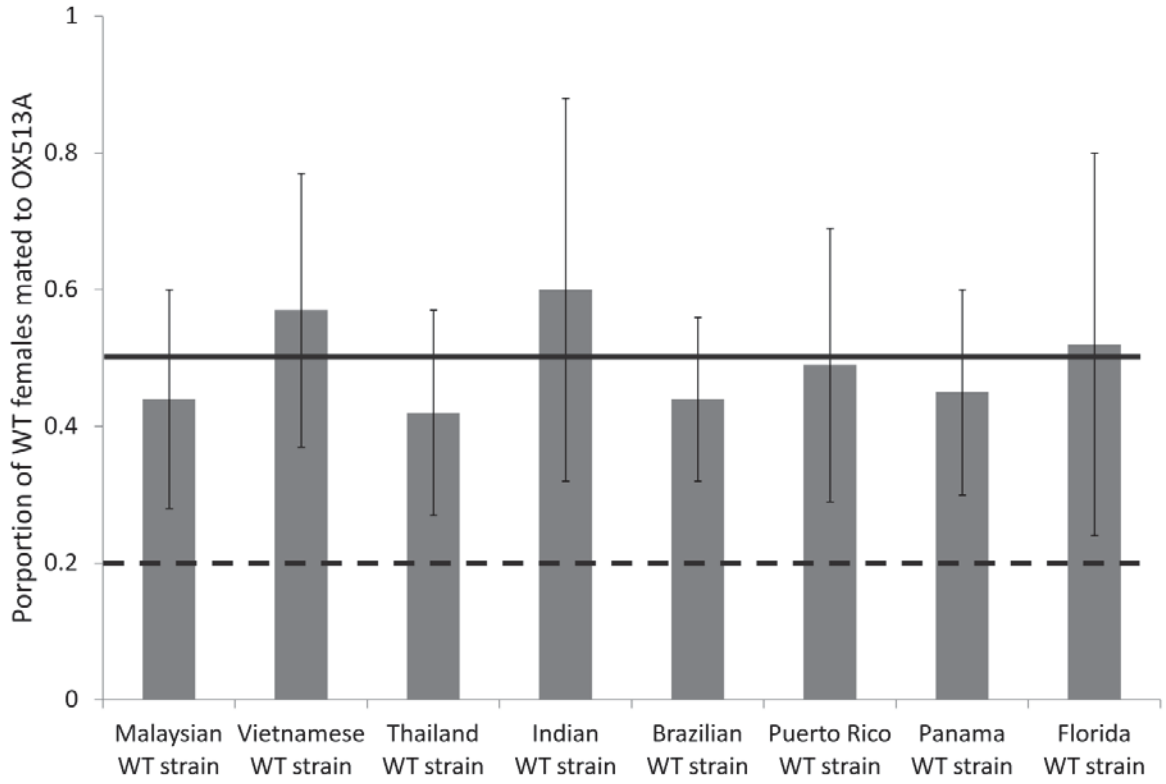

Figure 10. Summary of OX513A mating competitiveness results against wild-type *Aedes aegypti* strains worldwide in the laboratory. The dotted line represents 0.2 mating competitiveness for irradiated SIT and the solid line represents equal mating competitiveness of 0.5.

5.1.3.2 Mating competitiveness in semi field conditions

Lee et al. (2012) evaluated mating competitiveness in a purpose-built fieldhouse in Kuala Lumpur Malaysia using, per each trial, ten mosquitoes of each type in a simulated three-room apartment of 86m^3, which represents a more realistic density. Two variants of the OX513A strain were used, one in an Asian-derived genetic background, the other in a Latin American genetic background. The results showed that approximately 50% of the OX513A males from either background found mates. This is equivalent of a fully competitive strain. Furthermore, these results also showed that the Malaysian females did not discriminate between males of an Asian-derived or a Latin-American-derived genetic background, which suggests that a single strain could be used over a very large geographic area. This confined-field study represents a realistic assessment of OX513A male mating competitiveness, minimizing the typical urban environment for *Aedes aegypti.*

See Lee et al. (2012) for full report.

5.1.3.3 Mating competitiveness in regulated environmental releases

All of the sustained regulated environmental releases of OX513A males conducted to date have enabled the estimation of their mating competitiveness. Many factors are compounded in the assessment of mating competitiveness in the field. Environmental and site specific influences, wild *Aedes aegypti* population parameters, timing of release, immigration of wild

Aedes Aegypti, including pre-mated females, from adjacent sites into the release area, and migration of released males and mated females out of the area, are among these factors. Additionally, the process of mass rearing can impact the quality of the insects. Mating competitiveness is enhanced when the insects are sexually competitive and of high quality.

We define mating competitiveness (*C*) as the relationship between the numerical density of wild-type (*N*) and sterile (*S*) insects and the relative mating success, where *P* is the proportion of sterile matings, i.e., proportion of fluorescent larvae detected (Vreysen, 2005; Mayer et al., 1998) such that:

$$C = P*N / [S*(1-P)]$$

The first releases in Cayman Islands, which were to demonstrate the proof of principle of the Oxitec technology in the environment, used low rearing densities, producing high quality OX513A males, and gave a mating competitiveness estimate of 0.56 (95% CI: 0.032-1.97) (Harris et al., 2011) In subsequent studies, with a goal of local *Aedes aegypti* population suppression, much higher rearing densities where used in mass production. Mating competitiveness values in these studies ranged from 0.0004 to 0.059 (Carvalho et al., 2015; Harris et al., 2012;; *Oxitec Internal Research Report* **PH-2013-11**). Data is summarized in **Table 3.** This range is not unexpected given that mating competitiveness as measured by this approach includes any effect of mass rearing, handling and distribution, and the additional external effectors noted above. It may be that at relatively low local *Aedes aegypti* population densities, a significant proportion of the released OX513A males are released in areas that have few or no females; this may further depress the apparent mating competitiveness of the released OX513A males relative to wild males, which are likely to have a similar initial distribution as wild females. This may have been the case in the five latest estimates for the Itaberaba, Brazil study, as the *Aedes aegypti* population had already been suppressed during that period (Carvalho et al., 2015)

Relatively few estimates of mating competitiveness under environmental release conditions have been published, despite the long history of sterile-male release methods. In large-scale, successful Sterile Insect Technique (SIT) programs, field competitiveness of sterile males was estimated at 0.1 for New World screwworm (*Cochliomyia hominivorax*) (Vreysen, 2005; Mayer et al., 1998) and <0.01 for Mediterranean fruit fly (*Ceratitis capitata*) (Shelly et al., 2007; Rendón et al., 2004). Therefore, the mating competitiveness range seen over a variety of different environments with OX513A is predominantly within the range of commercial sterile insect programs. The outlying value of 0.0004 is likely due to releases in areas that are with only low numbers or no females, which depresses the apparent mating competitiveness as described above. It is important to note from the equation above, that as the adaptive release ratio increases, that is the number of OX513A:wild *Aedes aegypti*, the competitiveness value decreases; thus the value *C* must be taken in the context of the program parameters as informed by the local wild *Aedes aegypti* population, and the intent of the release experiment.

Table 3. summarizes data from three different typical habitat for *Aedes aegypti.* The Cayman Islands data (Harris et al., 2011) represent a site that was isolated and untreated with conventional insect control measures; the Itaberaba site data (Carvalho et al., 2015) represent a densely populated site with a high degree of immigration of *Aedes aegypti* from other areas; and the Mandacaru site data (*Oxitec Internal Research Report* PH-2013-11) represent a rural, isolated population with low housing density. This data therefore suggests that there are unlikely to be differences in mating behaviors of OX513A with the local population of *Aedes aegypti,* across different genetic backgrounds and habitat factors such as housing density and site isolation.

Location and date	Cayman Islands 2009	Cayman Islands 2010	Itaberaba, Brazil 2011-2012*												Mandacaru, Brazil 2012
Mating Competitiveness	0.56	0.059	0.031	0.013	0.037	0.025	0.047	0.013	0.003	0.006	0.0004	0.006	0.006	0.023	0.012
- 95% CI from bootstrap**	0.032	0.011	0.0254	0.0089	0.0223	0.0138	0.0399	0.0104	0.0016	0.0031	0.000	0.0039	0.0031	0.0139	0.005
+ 95% CI from bootstrap	1.97	0.21	0.0361	0.0174	0.0546	0.0391	0.0549	0.0152	0.0036	0.0097	0.0008	0.0085	0.0104	0.0352	0.021

*Data points for Itaberaba represent approximate monthly intervals

** 95% confidence intervals were obtained by running a bootstrap statistical analysis (Davidson *et al* 1997, Manly, 2007) on the relative mating success and numerical density of wild-type and sterile insects.

5.2 Response to abiotic factors

Temperature is well studied and is the primary abiotic factor influencing developmental rate and global distribution. Studies are presented which evaluate the response of OX513A to temperature in comparison to a wild-type comparator. Additionally, the genetic modification confers a tetracycline dependant self-limiting trait to the mosquito, thus several studies have been conducted to determine the response of the strain to tetracyclines. Survivability can also be affected by the sensitivity of the modified strain to insecticides used in current vector control programs; this has also been evaluated and included in this section.

5.2.1 Temperature response of OX513A

The temperature response of OX513A has been evaluated in the laboratory in order to determine:

a) If the penetrance of the phenotype of OX513A heterozygotes varies when reared at temperatures different that the laboratory standard; and,

b) If OX513A has altered survival at temperatures outside of *Aedes aegytpti's* natural range.

Aedes aegypti larvae, hemizygous for the OX513A construct, were reared at five temperatures ranging between and including 9°C and 37°C. To mimic environmental conditions larvae were reared in the absence of tetracycline, which as a dietary supplement in the laboratory allows survival of OX513A individuals. Latin wild-type (WT) larvae, the background strain of the OX513A strain, were reared under the same conditions as a control. Five repetitions were conducted for each temperature point. Both OX513A larvae and Latin WT larvae died before pupation when reared at 9°C and 37°C.

These results demonstrate that the presence of the OX513A insertion does not extend the viable temperature conditions for *Aedes aegypti such* that they can develop to functional adults at these temperatures under laboratory conditions. No evidence was found to indicate that OX513A might be able to spread beyond the current temperature-bounded range of wild *Aedes aegypti*. OX513A larvae reared at intermediate temperatures (18°C, 24°C, 30°C) within this range did not show a higher than expected proportion (<5%) of individuals surviving from first instar larvae (L1) to functional adult (range 0-2%) indicating that the OX513A phenotype is stable over the range of temperatures that larvae are likely to encounter in the field.

For the complete study report see *Oxitec Internal Research Report* **PH-2013-5**

5.2.2 Dose response to tetracycline and its analogues

Survival of OX513A is greatly reduced in the absence of tetracycline or its analogues due to the expression of the conditional lethal gene tTAV as described in **Section 4.4.2.** Survival of the progeny of matings of the OX513A male with female *Aedes aegypti* (i.e hemizygous) is also similarly reduced.

The response of OX513A heterozygous larvae to different doses of tetracycline, chlorotetracycline, oxytetracycline, and doxycycline has been evaluated by Curtis et al. (2015) to identify the lowest concentrations which allow for greater survival of heterozygous OX513A as compared to larvae reared in the absence of tetracycline or its analogues. Larvae were reared in increasing concentrations of tetracycline, oxytetracycline, chlorotetracycline and doxycycline. Based on dose response curves, the EC_{50} values (half maximal effective concentration; the concentration that induces a response halfway between baseline and maximum) for each of the compounds tested were determined. **Table 4** summarizes the results of the EC_{50} evaluation.

Table 4. The EC_{50} of OX513A larvae reared in the presence of different tetracycline analogues. Results are displayed for flying adults, modelled using a log-linear model. 95% confidence intervals are also shown.

Compound	*EC_{50} (ng/ml)*	*Confidence Intervals (ng/ml)*
Oxytetracycline	113	89 - 138
Tetracycline	50	34 - 66
Chlorotetracycline	13	9 - 16
Doxycycline	0.48	0.40 – 0.60

Figure 11 shows the fraction of L1 larvae that reach flying adults for the different tetracycline analogues examined and clearly shows that doxycycline is able to increase survival of OX513A at much lower concentrations than the other three drugs examined.

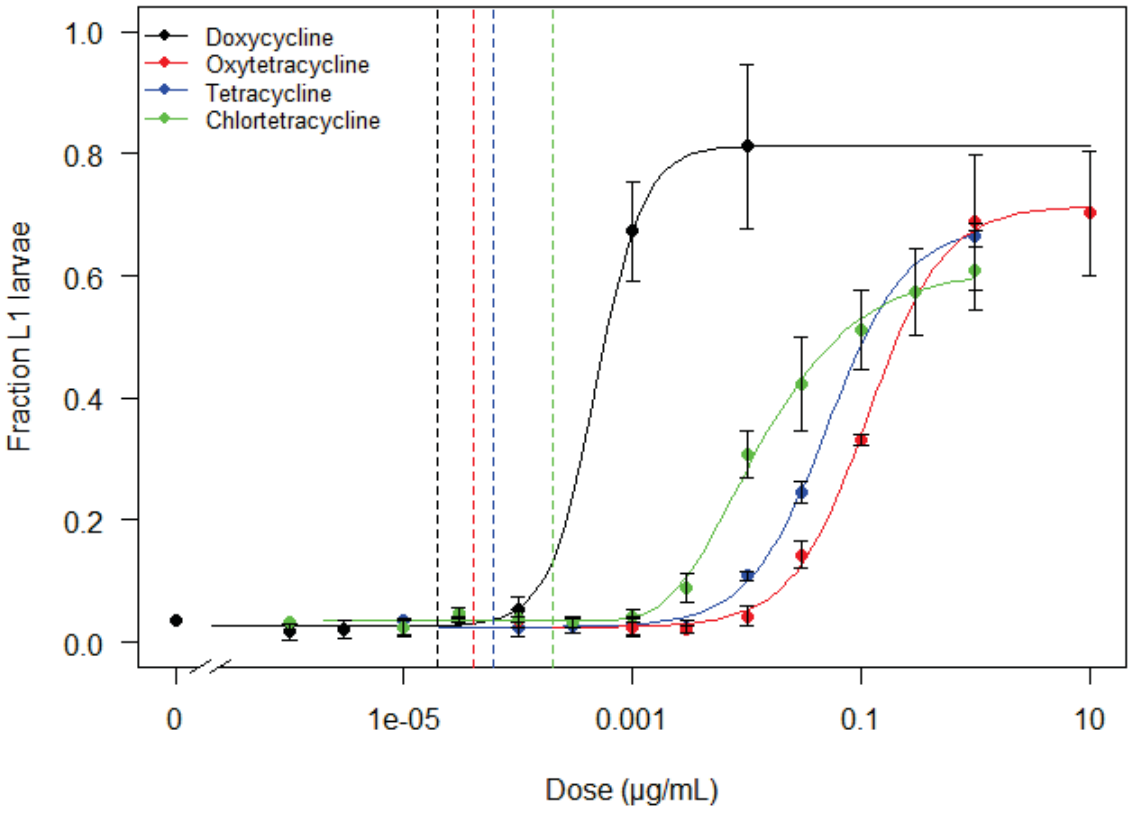

Figure 11. OX513A heterozygous larvae dose response to different concentrations of doxycycline, oxytetracycline, tetracycline and chlortetracycline. Data shown are the fraction of L1 larvae that reach flying adults from initial counts of 200 larvae per repeat, with three repeats/treatment. Lines are a log-linear model. The mean concentrations of each analogue found in environmental bodies of water are represented by dashed vertical lines; if reported values were below the limit of quantification (LOQ), the LOQ value was used to populate the mean

Maximum rescue from the lethal phenotype occurred above 1 ng/mL for doxycycline and above 1 µg/mL for tetracycline, oxytetracycline and chlortetracycline. The experiments reported here have shown that drug concentrations at and below 3 ng/mL for tetracycline, 1 ng/mL for chlortetracycline, 10 ng/mL for oxytetracycline and 0.1 ng/mL for doxycycline do not increase survival of OX513A larvae, i.e. do not increase the proportion of functional adults.

Dashed vertical lines in **Figure 11** represent the mean concentrations of each analogue (in the same colours as the lines) found in environmental bodies of water (mean concentrations calculated from reported field site sampling data reported in Wei et al., 2011; Kolpin et al., 2004; Watkinson et al., 2009; Kemper, 2008; Tong et al., 2014; Gulkowska et al., 2007; Locatelli et al., 2011).

For the complete study report see Curtis et al., (2015)

5.2.3 Susceptibility to chemical insecticides

Susceptibility to chemical insecticides is an important factor in the survivability of the mosquito. An independent study the Liverpool School of Tropical Medicine (LSTM) in 2011 (**Appendix 4**) examined:

a) The susceptibility of the OX513A strain to five commonly used insecticides (temephos, permethrin, deltamethrin, bendiocarb and malathion); and,

b) Screened OX513A for the presence of knock-down (kdr) mutations 1016 and 1534, which are associated with resistance to pyrethroids and DDT.

For the insecticide susceptibility studies, a susceptible laboratory strain (*Aedes aegypti* New Orleans) was used as control. Standard WHO procedures and discriminating doses (WHO, 2006) were used, and 100 insects were assayed in each treatment. Temephos, a larvacide, was tested on 4th instar larvae, and all other insecticides were tested on 2-3 day old adult female mosquitoes. Mortality was recorded 24 hours after exposure. The results of this study are summarized in **Table 5**.

Table 5. Mosquito mortality record 24 hours after exposure to insecticide (Reproduced from Appendix 4.)

		OX513A				*Aedes aegypti* NEWORLEANS strain			
Insecticide	Dose	No. Tested	No. alive	No. dead	% mortalit	No. Tested	No. alive	No. dead	% mortality
temephos	0.012mg/ L	102	0	102	100	n/d	n/d	n/d	n/d
permethrin	0.75%	100	0	100	100	63	0	63	100
deltamethrin	0.05%	100	0	100	100	41	0	41	100
bendiocarb	0.10%	200	106	94	47	100	49	51	51
malathion	0.80%	100	0	100	100	n/d	n/d	n/d	n/d

OX513A was found to be susceptible to discriminating doses of temephos, permethrin, deltamethrin and malathion, and it showed significant survival to bendiocarb; however, the level of resistance to bendiocarb shown was comparable to that seen in the New Orleans (control) strain. Therefore, the study concluded that instead of indicating resistance in the OX513A strain, these results indicate that the WHO-recommended bendiocarb discriminating dose (0.1%) is not appropriate for *Aedes aegypti.*

The results of the OX513A genotype analysis for two *kdr* mutations that are associated with pyrethroid and DDT resistance, showed that these mutations were absent in the OX513A strain.

For the complete study report see **Appendix 4** OX513A insecticide resistance testing.

A separate study was conducted in Malaysia by Nazni et al. (2009). This study compared the susceptibility of the strain MyRIDL-513A[21] and the laboratory strain MyWT. Seven insecticides were tested following standard WHO methods[22]. All of the insects used were 3-5 day old females, and there were 25 adults in each test. The results are summarised in **Table 6**.

Table 6. Susceptibility status of adults of MyRIDL513A and MyWT *Aedes aegypti* against various insecticides. (Table adapted from Nazni et al. (2009)

Insecticide	Dose	24 hours post-exposure mortality (%)	Susceptibility status		24 hours post-exposure mortality (%)	Susceptibility status
		MyRIDL513A			MyWT	
DDT	4%	48	Resistant		33	Resistant
Fenitrothion	1%	98.7	Susceptible		96	Tolerant
Malathion	5%	100	Susceptible		100	Susceptible
Propoxur	0.1%	100	Susceptible		88	Tolerant
Permethrin	0.75%	100	Susceptible		100	Susceptible
Lambdacyhalothrin	0.15%	100	Susceptible		100	Susceptible
Cyfluthrin	0.05%	100	Susceptible		100	Susceptible

There were slight differences in the susceptibility of insecticides between the two strains that were tested, as the MyWT was tolerant to propoxur and fenitrothion, whereas the MyRIDL513A strain was fully susceptible to both chemicals. Additionally, some level of resistance to DDT was detected in both strains, which the authors of the study attributed to the Malaysian genetic background shared by both strains (since use of DDT in the past in Malaysia caused the dissemination of resistance alleles in *Aedes aegypti* populations).

For the complete study report see Nazni et al. (2009)

5.2.4 Behavioural responses of OX513A to insecticides

Kongmee et al., (2010) examined whether the behavioural responses of OX513A mosquitoes (strain OX513A-My, a derivate of the OX513A strain in a Malaysian genetic background) would be comparable to that of wild type strains.

In this study, adult mosquitoes of two age groups (4-5 days old and 8-10 days old) were tested using contact and spatial irritancy assays to three chemical pesticides (alphacypermethrin,

[21] The MyRIDL-513A strain was generated by out-crossing the original OX513A strain to the Malaysian MyWT strain. The resulting offspring (strain MyRIDL-513A) contains the genetic modifications associated with OX513A in a Malaysian genetic background.

[22] WHO guidelines stipulate that mosquitoes with mortality rates ≥98% are considered susceptible; those with mortality rates between 80-97% might be tolerant (and require further testing), and those with mortality rates below 80% are considered resistant.

DDT and deltamethrin). Results indicated that the behavioural responses of OX513A males were overall similar to those displayed by the wild-type strain in both age groups, including significant contact irritancy to pyrethroids and significant spatial repellence to DDT.

For the complete study report see Kongmee et al. (2010).

5.2.5 Tetracycline loaded blood study

The objective of this study was to test the hypothesis that providing high doses of dietary tetracycline to adult female *Aedes aegypti* (either homozygous OX513A transgenic females mated to wild-type males, or wild-type females mated to homozygous OX513A transgenic males) has no effect on the penetrance of the OX513A lethal phenotype observed in their hemizygous offspring. With respect to the administration of tetracycline, the following two scenarios are defined:

"Tetracycline loading" (TL)

To evaluate the effects of the ingestion of high tetracycline concentrations, selected groups of female mosquitoes (henceforth referred to as 'tet-loaded') received both blood and sugar meals containing a pre-determined dose (either 50 µg/ml or 100 µg/ml) of chlortetracycline hydrochloride, which are above the peak concentrations found in human and livestock blood following standard therapeutic doses of tetracycline (see *Oxitec Internal Research Report* **PH-2013-2** for full references)

"Rearing on tetracycline" (ON-tet)

Adult virgin homozygous OX513A individuals (reared ON-tet) were crossed to adult virgin WT individuals. To emulate all potential scenarios in the field following a mass-release of OX513A, both reciprocal crosses (OX513A♀ vs. WT♂, and OX513A♂ vs. WT♀) were performed in tetracycline-loaded cohorts.

Within each group, the following parameters were evaluated:

a) Pupation: Survival from first larval instar to pupation.
b) Adult emergence: Survival from first larval instar to the appearance of adults, regardless of the fitness or longevity displayed by adult mosquitoes.
c) Number of flying adults: Number of adults which were able to fly ≥48 hours after emergence. This category was created to differentiate fully functional adults from those that die soon upon emergence from the puparium (often without being able to leave the rearing water).

Statistical analysis:

Data were analysed using the RStudio software package version 0.97.237 (RStudio, USA). Normality was tested using the Shapiro-Wilk method. For normally-distributed data (survival to pupation, adult emergence), parametric significance tests were carried out using ANOVA and, when required, Tukey's honestly-significant-difference (HSD) tests for post-hoc analysis.

For non-normally distributed data (number of flying adults), non-parametric testing was performed using the Kruskal-Wallis test, followed by post-hoc analysis using the Nemenyi test (Zar, 1999).

Results are summarized in **Figure 12**. No significant difference for any of the three parameters was observed between the non-tetracycline-loaded (NTL) control group (A) and any of the treatment groups (B-E).

Significant differences were only observed in pupation between groups B and E ($p<0.01$), and in the number of flying adults between groups C and E ($0.01<p<0.05$). Values for the ON-tet control groups (F,G) are shown for reference. The fact that no significance was observed when comparing either of groups C or E to their corresponding non tet-loaded (NTL) controls suggests that the observed differences are caused by factors unrelated to the ingestion of tetracycline. Although the exact nature of these factors remains to be described, it is suspected the are probably related to environmental conditions during rearing, and therefore not relevant to the specific objectives of this study.

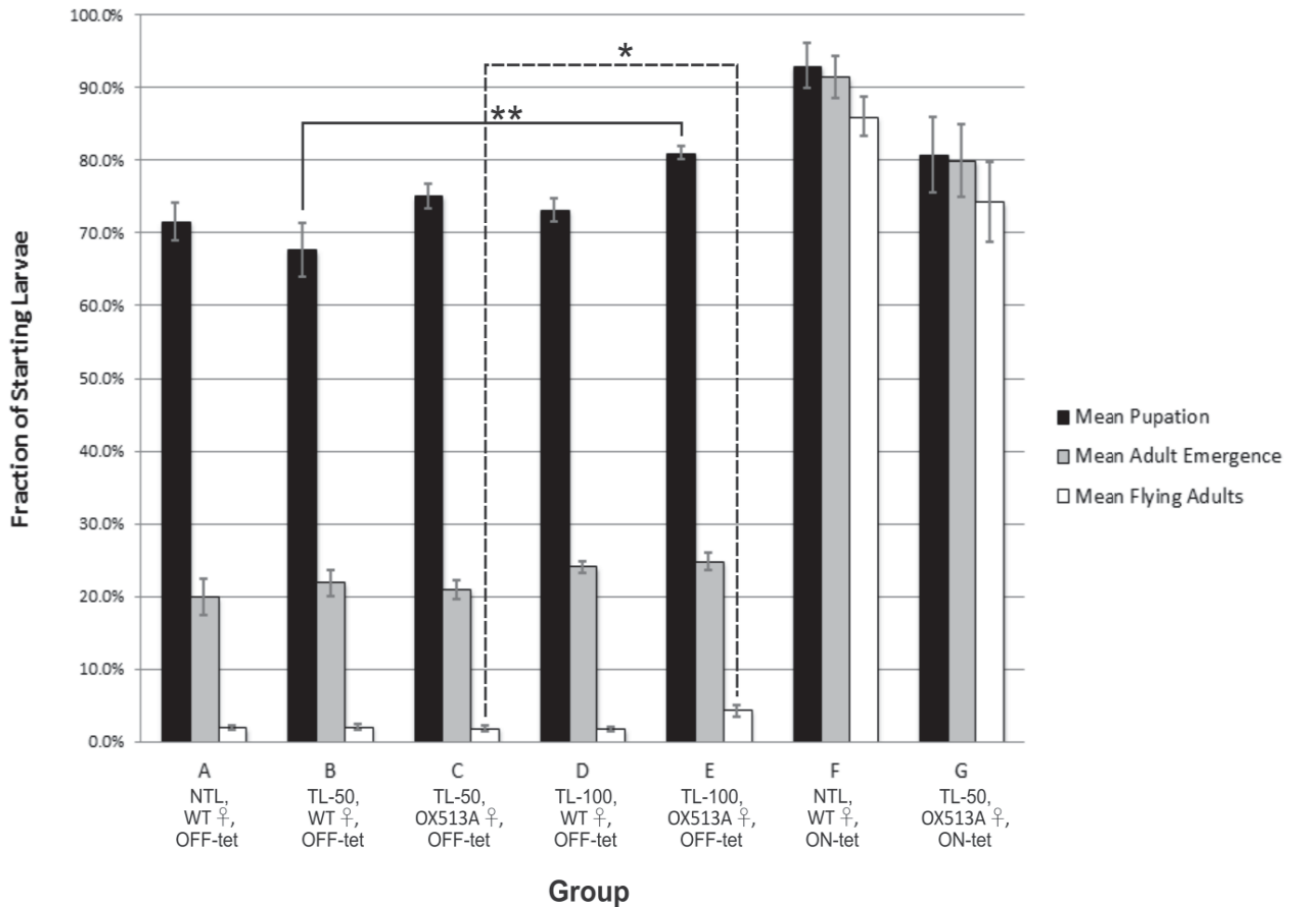

Figure 12. Summary of results of tetracycline-loaded blood study.

NTL: Non tet-loaded; **TL-50**: Tetracycline loaded, 50µg/ml; **TL-100**: Tetracycline loaded, 100µg/ml.
OFF-tet: Larvae reared without tetracycline; **ON-tet:** Larvae reared with tetracycline added to the rearing water.
WT♀: Female of parental cross was wild-type; **OX513A♀**: Female of parental cross was genetically engineered.

These results indicate that the penetrance of the OX513A phenotype in hemizygous offspring of female *Aedes aegypti* which have ingested high doses of tetracycline is not significantly different from that observed in the offspring of females that were not provided with tetracycline in their diet.

For the complete study report see *Oxitec Internal Research Report* **PH-2013-2**

5.2.6 Trait Penetrance

Penetrance can be through of as the probability of a gene or genetic trait being expressed. "Complete" penetrance means the gene or genes for a trait are expressed in all the population who have the genes. "Incomplete" penetrance means the genetic trait is expressed in only part of the population, for example 95% penetrance means that 95% of the population expresses the introduced trait. Under laboratory conditions the observed penetrance of the self-limiting trait in OX513A is always found to be over 95%. This means that less than 5% of the progeny of a cross between OX513A males and wild-type *Aedes aegypti* females ("hemizygous") will survive if reared without tetracycline in the rearing water. This is not considered genetic instability or resistance, as this is seen in the same proportion in each generation and is heritable. Trait penetrance in OX513A described in Phuc et al. (2007) and confirmed through regular quality control testing of OX513A as detailed in ***QD-SOP-00011*** *OX513A Quality Control Protocol for Assessment of Penetrance and Doxycycline Sensitivity.*

5.2.7 Non-penetrant OX513A progeny- Longevity and Fecundity

The longevity and fecundity of the small observed proportion (<5%) of surviving heterozygous progeny of OX513A x wild-type cross has been investigated in the laboratory. Homozygous OX513A was outcrossed to wild-type *Aedes aegypti* to generate heterozygous eggs. These eggs were hatched and reared in the absence of tetracycline as they would be in the receiving environment. As a control, heterozygous eggs were reared in 1µg/ml tetracycline to represent the minimum dose for maximum phenotype rescue. Additionally, homozygous OX513A larvae were reared in 30 µg/ml tetracycline to provide a comparator representative of a colony reared at a well-established dose previously used in experimental rearing.

Of 4000 L1 heterozygous pupae reared in the absence of tetracycline, 4.4% eclosed as flying adults, consistent with the <5% consistently observed and reported (**Section 5.2.6**). The longevity of males and females, and the fecundity of females (87 of the 4000) was assessed over 12 weeks, as both are of interest as measures of potential fitness. The comparator background strain (Latin wild type- LWT) was used as a control.

Substantial mortality was observed within the first few days post-eclosion for the OX513A heterozygous males and females reared without tetracycline (**Figure 13**), although a small fraction (~20%) do survive long enough to take two blood meals and some produced two clutches of eggs. Very little mortality was observed in the week post-eclosion in the LWT strain for both males and females, which contrasts strongly with the OX513A strain (p-value=0). Median survival of both OX513A males and females is 2 days compared to LWT males and females with median survival of 60 and 68 days, respectively.

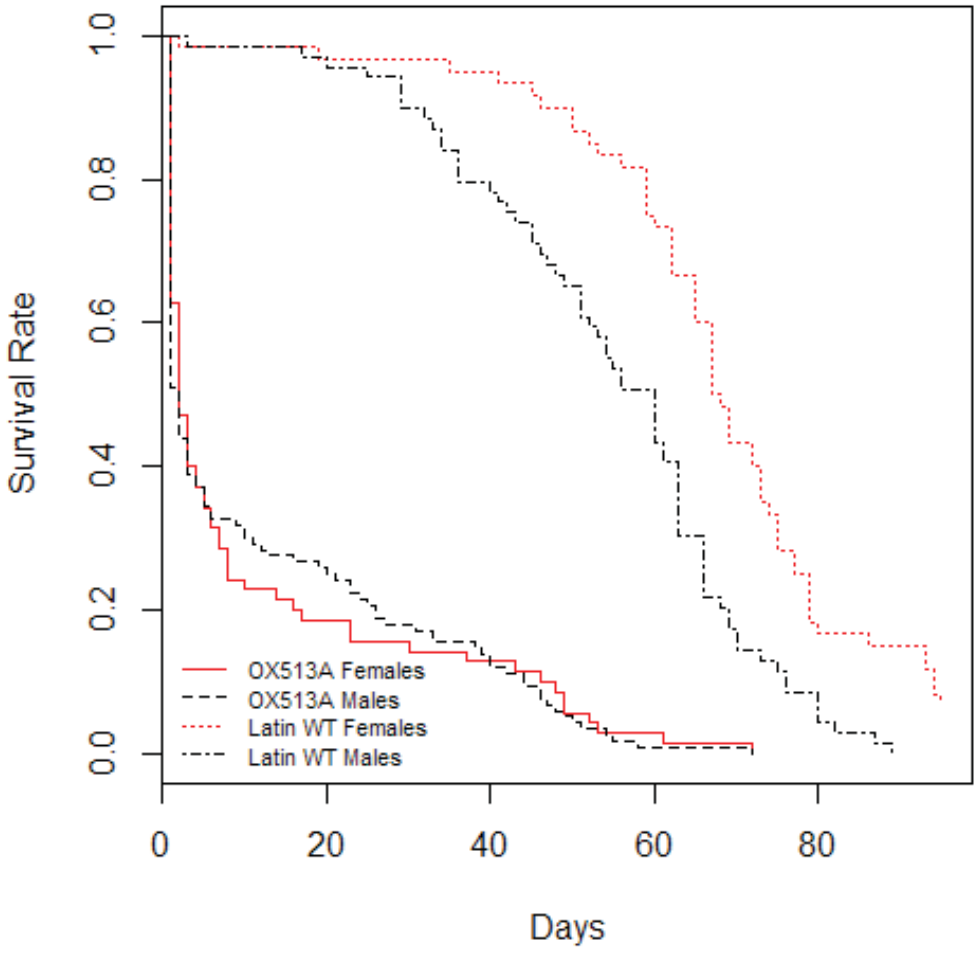

Figure 13. Survival curves for heterozygous OX513A reared in the absence of tetracycline

The mean clutch size for OX513A was 69.9 eggs (S.D. 13.9) and for LWT it was 54.8 eggs (S.D. 12.4). Analysis by t-test revealed a significant difference between average values (p=0.001), indicating that the OX513A strain lays a larger egg clutch during the first gonotrophic cycle, compared to its wild-type background. The mean hatch rates were 92% (S.D. 14) and 82% (S.D. 18) for OX513A and LWT, respectively. Statistical testing did not reveal any significant difference between these values (p=0.089). The non-statistically significant differences that occurred may be accounted for by the fact that the OX513A strain has been intensively mass reared and adapted for over 100 generation equivalents while the LWT strain has not.

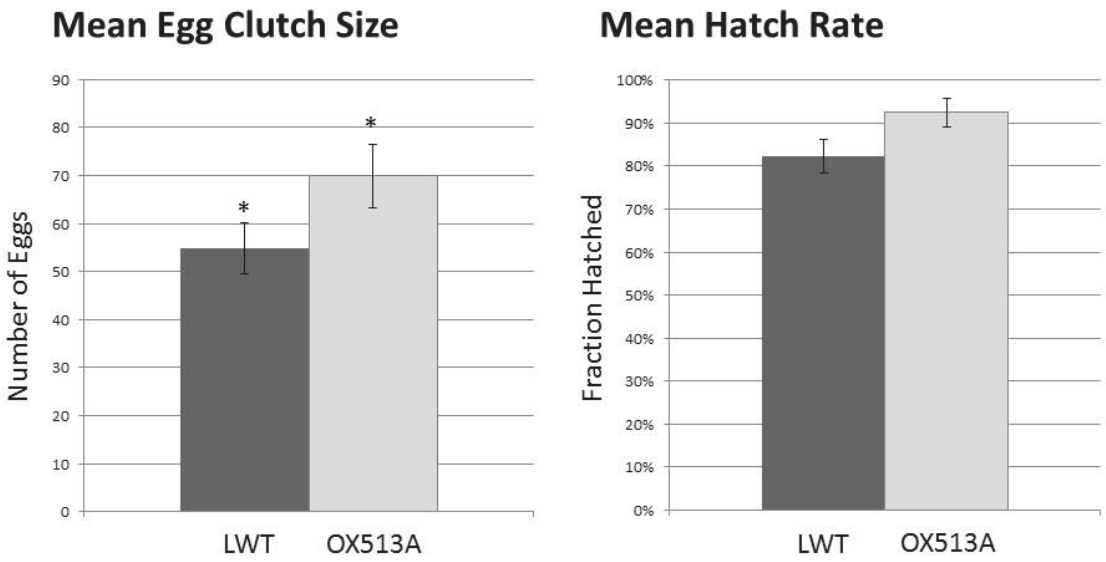

Figure 14. Fecundity study results. * p=0.001. The 95% confidence interval is shown for egg clutch size, and the standard error of the mean for hatch rate.

Fecundity of OX513A heterozygous females reared in the absence of tetracycline was found to be slightly higher than that of LWT females (**Figure 14**).

For the complete study report see *Oxitec Internal Research Report* **PH-2013-3**

5.2.8 Field penetrance

Adult mosquitoes were collected in traps (BG Sentinel type- see **Section 6.2**) as part of multi-component programmes also involving the sustained release of OX513A males in the Cayman Islands (East End) and two release sites in Brazil (Itaberaba and Mandacaru). Samples were genotyped and assigned a genotype of homozygous or heterozygous for the OX513A insertion, or wild–type (WT) for each individual. Real time PCR was also carried out on all of the heterozygous and homozygous individuals identified to confirm the copy number of the transgene. As expected, the majority of the females were wild-type, but a small number of homozygous individuals were detected in each test site.

Overall estimates of percentage incomplete penetrance ranged from 0-4.28%. It should be noted that sample size for some of the studies was low, but taking all studies together it is clear that the figure falls below the ~ 5% reported in laboratory studies. This is to be expected as conditions in field are harsher that the benign conditions in which laboratory studies were conducted. These results support the hypothesis that the lethal phenotype is behaving as expected in the field and that incomplete penetrance is no higher than in the laboratory.

For the complete study report see *Oxitec Internal Research Report* **PH-2013-6**

5.3 Dispersal and longevity- regulated environmental releases of OX513A

Neira et al., (2014) reported that in studies conducted in Panama, marked released WT males had a daily survival probability of 2.3 days. It was anticipated that the dissemination of

OX513A genes into the environment should be limited to the dispersal of released males and their subsequent mating with wild females. Inclusion of a heritable marker as part of the genetic engineering enabled the evaluation of dissemination of OX513A genes resulting from the release of OX513A males. In the project conducted by Carvalho et al. (2015), which was conducted in a typical *Aedes aegypti* urban habitat in Brazil, the dissemination of OX513A genes into the environment was assessed by analyzing the distribution of OX513A eggs recovered from ovitraps in an area adjacent to a site that received sustained release of OX513A males. The mean distance travelled (dissemination) of OX513A genes into the untreated area was estimated at 64 m (95%CI; 55-74) and 79 m (95% CI; 74-86) for the two periods evaluated (**Figure 15**). This differed little from the dispersal of OX513A adult males, and males of the comparator strain (recently colonized *Aedes aegypti*) observed at the same site (mean distance travelled = 39-75 m) and falls in the mid-range of those reported in the scientific literature (mean distance travelled = 12-288 m) for dispersal of *Aedes aegypti*, see **Figure 15**.

Releases of adult male mosquitoes (OX513A and WT) were conducted in an uninhabited forested area of Pahang, Malaysia (Lacroix et al., 2012). This release site represented an atypical environment for *Aedes aegypti,* which is typically associated with urban domestic and peri-domestic settings, thus it provided an opportunity to evaluate dispersal in a regulated environmental release independent of effectors typical to the *Aedes aegypti* habitat. Their longevity and dispersal was assessed by use of a network of traps. Two strains were used, OX513A and a wild-type laboratory strain, to give both absolute and relative data about the performance of OX513A. The two strains had similar maximum dispersal distances (220 m), but mean distance travelled of the OX513A strain was lower (52 vs. 100 m). Recapture rates were high for both strains, possibly because of the uninhabited nature of the site and longevity was similar (2.0 vs. 2.2 days).

In a study in the Cayman Islands (Winskill et al., 2014) four separate mark release recapture studies were conducted with resulting estimates of average life expectancy ranging between 0.1 to 1.6 days. No non GM-comparator was released in this Cayman study.

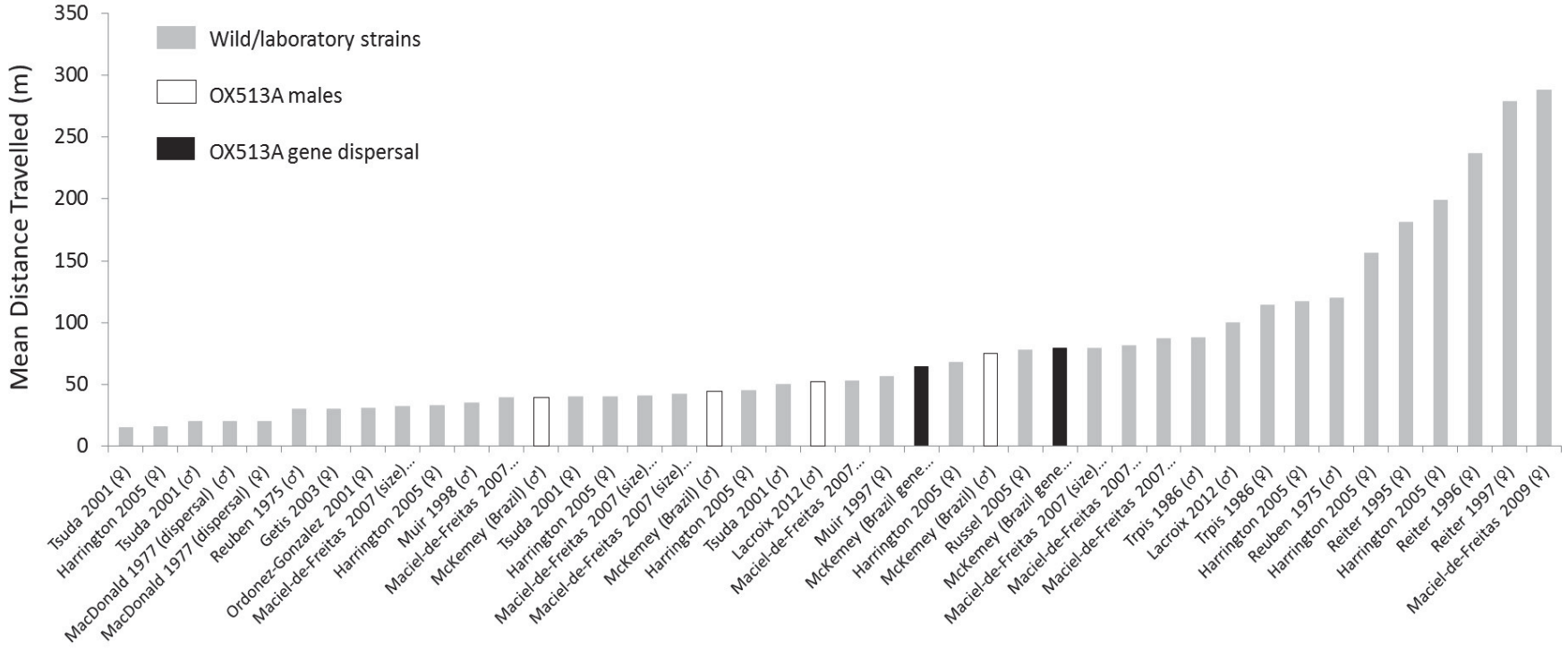

Figure 15. Review of Reported Mean Distance Travelled (m) for Wild-Type and OX513A *Aedes aegypti* and Observed Dissemination of OX513A Gene from Male Release

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5.4 Oral exposure studies

5.4.1 Toxorhynchites spp (predatory Mosquito)

Nordin et al. (2013) investigated the impact of a diet consisting of OX513A larvae on two predatory mosquito species (*Toxorhynchites spp*).

Single *Toxorhynchites* larvae were placed into individual cups and 20 *Aedes aegypti* larvae were maintained in the cup. Eaten larvae were replaced daily. The duration of the developmental stage of the *Toxorhynchites spp*. was recorded daily. T*oxorhynchites* larvae which survived to pupae were placed into cages; female *Toxorhynchites* mosquitoes were presented with 5-8 males from the stock colony and the number of eggs was recorded daily along with survival. After death, the wing length was recorded. In both *Toxorhynchites* species, there were significantly more larvae consumed in the off tetracycline treatments; *Tx. amboinensis* ($t = 9.2$, $p<0.001$) and *Tx. splendens* ($t = 8.3$, $p<0.001$). *Tx. amboinensis* females reared on wild-type larvae consumed significantly more larvae than females fed on OX513A larvae reared on-tetracycline ($t = -3.3$, $p<0.002$). Why this result occurred is unknown but there were no significant differences in any other measured parameters.

Effects on life history parameters of all life stages were compared to being fed on wild type *Aedes aegypti* larvae of the same background strain, any significant differences found were attributed to differences between species and there was no evidence of significant impact on the development, fecundity and longevity on the two Toxorhynchites species.

For the complete study report see Nordin et al. (2013)

5.4.2 *Poecilia reticulate* (Guppy Fish)

A laboratory prolonged toxicity study to determine the effects of ingestion of larvae and pupae of OX513A on the guppy fish *Poecilia reticulata*; according to OECD guideline No. 204 (1984)[23] modified for oral route of exposure, was conducted by SynTech Research France, under GLP conditions.

Guppy fish (20-26mm at the start of the test) were exposed to a mix of freshly defrosted OX513A larvae and pupae and a wild-type (WT) control over a period of 14 days in laboratory conditions. During the study the fish were fed with OX513A or the WT control in the fish diet daily, at the rate of 700g mosquitoes/kg diet, following a rangefinder study. The natural ratio of insects in the diet for this fish species is approximately 50% (500g insects/kg food). The quantity of diet administered daily did not exceed the amount ingested immediately by the fish and was kept constant during the study duration, i.e. 4% of the initial fish weight.

[23] http://www.oecd-ilibrary.org/environment/test-no-204-fish-prolonged-toxicity-test-14-day-study_9789264069985-en (accessed 31/08/2016)

Endpoints assessed were mortality, appearance, size and behavior of the fish, which were observed daily. A toxic reference substance (potassium dichromate) was included to indicate the relative susceptibility of the test organisms and test system. The OX513A mosquito group were analyzed for significant differences compared to the control group using ANOVA ($p \leq 0.05$) and to determine values for the LR50, ER50, Lowest observable effect rate (LOER) and No observable effect rate (NOER).

Table 7. Summary of *P. reticulate* mortality, length and weight after 14-day oral exposure to *Aedes aegypti* OX513A.

Test item	**OX513A mosquito**		
Test organism	***Poecilia reticulata***		
Test medium	**ISO reconstituted water**		
Exposure	Daily oral exposure		
Endpoint	14-day mortality (%)	14-day length (mm)	14-day weight (mg)
Control (700 g non-GM mosquitoes/kg diet)	10	22.44	198.3
OX513A (700 g GM mosquitoes/kg diet)	0	23.2	212.9
LR_{50} / ER_{50} [g GM mosquitoes/kg diet]	> 700		
LOER [g GM mosquitoes/kg diet]	> 700		
NOER [g GM mosquitoes/kg diet]	700		

The results showed that there was no significant difference between mortality, fish length, weight, appearance and behavior in the control and OX513A fed fish, after 14 days. Hence the NOER was found to be 700g GM mosquitoes/kg diet and the LOER and LR50 /ER50 were estimated to be > 700 g GM mosquitoes/kg diet.

For the complete study report see ***Appendix 5*** *Final Report 232SRFR12C1 Poecilia - Aedes OX513A*

5.5 OX513A morphology

Mosquito borne diseases are transmitted through the bite of the female mosquito. The anatomical structure of the mouthparts and stomach in male mosquitoes are incompatible with the ability to bite. In addition, males do not secrete the necessary salivary anticoagulants that are required for blood flow. There are no genes introduced into the male

mosquitoes that are intended to alter the morphology of the insect and no morphological differences between OX513A males and the wild type males have been observed as OX513A has been maintained for over 115 generations.

5.6 Analysis of expression of the introduced proteins in female mosquito saliva

An analysis was conducted to detect the presence of the tTAV and DsRed2 proteins in OX513A female mosquito saliva. Homozygous adult female OX513A were reared to adulthood in the presence of doxycycline. Saliva was collected from these insects as well as from comparator wild-type (WT) *Aedes aegypti* females and two pools (OX513A and WT) created that were used for the entire study. Western blot analysis using a polyclonal tTAV antibody (anti VP16 tag antibody) and a polyclonal DsRed2 antibody was carried out using an Enhanced Chemiluminescence (ECL) method. Sample integrity was confirmed using an antibody detecting a secreted salivary protein, Aegyptin. Aegyptin detection was also used as a basis to determine that equivalent amounts of saliva were loaded in control and sample lanes between the test saliva samples of OX513A and the WT control saliva samples.

The Limit of Detection (LOD) for tTAV and DsRed2 on the Western blots was determined using recombinant tTAV and recombinant DsRed2. Purified tTAV and DsRed2 proteins from OX513A could not be used as sufficient quantity could not be extracted from the insects for this study. Results from Western blot analyses were captured using the ChemiDoc-IT 500 Imaging System (UVP), and signals were quantified by relative densitometry using the VisionWorks LS Acquisition and Analysis Software (UVP). The LOD for recombinant tTAV (rtTAV) was determined to be 0.8 ng, and the LOD for recombinant DsRed2 (rDsRed2) was determined to be between 5.0 and 2.5 ng.

tTAV and DsRed2 were not detected in female OX513A saliva at or above these LODs in the 5 µl of saliva analysed. 5 µl of OX513A saliva equates to the quantity of saliva collected from approximately 5.5 female adult mosquitoes based on the volumes on saliva collected during this study (270 µl of pooled saliva collected from approximately 300 adult OX513A females).

For the complete study report see *Oxitec Internal Research Report* **SR-00004**

5.7 Vertical transmission of Dengue and Chikungunya viruses in OX513A

The vertical transmission of dengue (serotypes 1-4) and chikungunya viruses in OX513A females, and a wild type comparator strain have been evaluated in a study conducted at the Institute for Medical Research, Kuala Lumpur, Malaysia.

The study objective was to assess whether OX513A females were more competent for vertical transmission of dengue and chikungunya viruses than females from a wild type strain to ultimately assess whether the potential for the release of a small proportion of OX513A females due to the mechanical sex sorting thresholds (see Standard Operating Procedure ***TD-***

SOP-00293 OX513A Sex Sorting of Pupae for Release) could enhance the potential rate of vertical virus transmission in the receiving environment.

The study investigated the transmission of the dengue serotypes 1-4 and chikungunya to eggs laid by females of OX513A and the wild-type comparator strain, through hatching of eggs and analysis of larvae. Initial screening of larvae was though molecular analysis (PCR), followed by assessement of virus through cell culture assay. Additionally, the number of eggs laid per individual female was recorded to assess any changes in fecundity between strains.

None of the virus strains had significantly different vertical transmission rates between the OX513A strain and the wild-type comparator, nor were significant differences in fecundity observed.

For the complete study report see Mansor et al. (2016)

5.8 Stability of the insert in OX513A

OX513A has been transformed using the non-autonomous transposon, *PiggyBac* which enabled stable integration of the OX513 construct into the *Aedes aegypti* genome. Non-autonomous transposons required the use of an exogenous transposase source for excision and integration into a genome during the transformation process. This enzyme was supplied on a separate plasmid during the transformation, and was not integrated into the *Aedes aegypti* genome during the process as described in **Section 4.1.** The stability of the trait has been assessed through evaluation of the Mendelian inheritance ratios of the offspring as well as molecular techniques which have confirmed that the insert is a single copy that is stably integrated and behaves in a Mendelian fashion during colony breeding.

See ***Appendix 1*** *Section 3.4 - Molecular characterisation and lineage of Aedes aegypti OX513A Mendelian Inheritance Ratios.*

5.8.1 OX513A quality control

Regular strain integrity quality control assays are carried out on the OX513A colony as detailed in the Standard Operating Procedures below:

- Colony genotyping is performed every fourth generation: ***QD-SOP-00013*** *OX513A Quality Control Protocol for Colony Genotyping*
- Penetrance and tetracycline dose response every sixth generation: ***QD-SOP-00011*** *OX513A Quality Control Protocol for Assessment of Penetrance and Doxycycline Sensitivity*
- Mating competitiveness every twelfth generation: ***QD-SOP-00012*** *OX513A Quality Control Protocol for Assessment of Mating Competitiveness*

OX513A has been maintained in continuous culture in the laboratory since 2002 (>115 generational equivalents[24]) with no observation of genetic or phenotypic instability

5.9 Conclusions regarding the phenotypic characterisation of OX513A

Conclusions drawn from the observations in this section are also presented along with additional conclusions in ***OX513A Environmental Risk Assessment Part C- Section 1.2.2 Phenotypic Characterisation***

No phenotypic changes were detected in OX513A relative to the comparator(s) that suggest unintended effects attributable to the #OX513 construct in examining:

- Life table parameters, including mating competitiveness
- Response to abiotic factors including temperature and insecticides
- Adult dispersal and longevity
- Oral exposure to predators
- Vertical transmission of DENV and CHIKV
- The absence of tTAV and DsRed2 proteins in the saliva of Female OX513A

Additionally, the response to tetracycline and its analogues was assessed through the characterisation of the penetrance of the tTAV trait (i.e. the intended effect). Under laboratory conditions the observed penetrance of the self-limiting trait in OX513A is always found to be over 95% (originally described in Phuc et al. 2007). Thus, less than 5% of the progeny of a cross between OX513A males and wild-type Aedes aegypti females ("hemizygous") will survive if reared without tetracycline in the rearing water. This is not considered genetic instability or resistance, as this is seen in the same proportion in each generation and is heritable.

Trait penetrance and the stability of OX513A is confirmed through the regular quality control testing of OX513A

[24] as of September 2016

6 Detection and identification of OX513A

6.1 Methods and sensitivity for detecting OX513A *Aedes aegypti* in the environment

There are two primary detection methods available, fluorescence-based detection and DNA sequence-based detection.

6.1.1 *Fluorescence-based detection:*

The OX513A construct encodes the fluorescent protein DsRed2 (Phuc et al., 2007) which allows larvae and pupae containing the OX513 construct to be accurately discriminated from wild *Aedes aegypti,* or wild type laboratory strains. This marker has proven to be stable in OX513A over 115 generational equivalents since 2002. The DsRed2 marker is described in ***Section 4.4.1*** *Fluorescent marker DsRed2.*

Fluorescence is visible through a suitable fluorescent microscope (e.g. Leica MZ10F) with a DsRed2 filter set (excitation 520-560 nm and emission 580+ nm) in a darkened room, using trained staff.

For protocol see **SOP *00031_01 OX513A*** *Fluorescence Screening of Larvae*

6.1.2 *DNA sequence-based detection:*

A specific and sensitive Polymerase Chain Reaction (PCR) technique has been developed to detect unique genetic fragments within OX513A, based on the genomic regions flanking the insertion site. This method can be used to confirm the identity of field caught samples if required.

For protocol see ***R_SOP_00001*** *Protocol for the detection of the OX513A transgene by polymerase chain reaction.*

6.2 Monitoring the *Aedes aegypti* population in the environment

The trapping methods used in an OX513A program are described below. Details of how the data generated from trapping informs the monitoring program in the field, along with associated calculations and *Standard Operating Procedures* (SOPs) are covered in ***OX513A Technical Dossier Part B*** in the context of the proposed release.

Trapping methodologies to monitor *Aedes* mosquitoes are well established (Silver, 2008). There are two principle methods used either in combination, or in isolation depending on the phase of the program and the intent of the monitoring.

Ovitrap (egg catch) surveys are the principal monitoring tool deployed in both treated and untreated sites. Ovitraps are a commonly used system for monitoring *Aedes* mosquitoes, mimicking natural breeding sites in which females lay eggs (Hernandez-Avila et al., 2013; Reiter and Nathan, 2001). They were initially developed for the detection of *Aedes aegypti* in the U.S.A. as part of an eradication program (Fay and Eliason, 1966) and have subsequently been adopted as a standard monitoring tool for *Aedes aegypti* populations. The World Health Organization (WHO) recommends use of Ovitraps (WHO, 1997) for *Aedes aegypti* surveillance for;

- Baseline infestation surveys
- Control programs monitoring low infestation levels
- Surveillance against infestation
- Verification of eradication
- Evaluation of control methods

Ovitraps consist of a black plastic pot or cylinder partially filled with clean water with a fibre board paddle, or similar ridged surface protruding above the water-line to provide an oviposition substrate. Ovitraps are to be checked approximately weekly, and eggs hatched and scored for the presence of the OX513A fluorescent marker as referenced in **Section 6.1.1**, or reared to adults for species identification with appropriate taxonomic keys (Bangs and Focks, 2006) as some species are difficult to identify at larval stage. This is required in certain studies to assess the presence of *Aedes albopictus.*

Figure 16. Representations of two types of simple Ovitraps in use.

The BG-Sentinel adult trap[25] was developed specifically targeting *Aedes* mosquitoes. It utilizes a combination of factors to trap live adults. They use a combination of visual and host mimic olfactory cues to attract adult (male and female) Aedes mosquitoes which are captured in a suction trap. The trap incorporates a fan generating a counter flow air stream that produces a plume of kairomones attracting mosquitoes and also uses visual attractant cues; with a black collection tube contrasting against white casing housing the trap. As mosquitoes get close they are caught in the rapid down draft airflow generated by the fan and get trapped in a net bag within the centre of the trap. These traps have been extensively field tested in direct comparison with other live adult sampling methods including the CDC backpack aspirator, human bait landing catch, and Fay-Prince traps and in most direct comparison studies, BG-Sentinel traps generally outperform other types of traps. BG traps collect both males and female adults in comparable numbers and are also effective at sampling *Aedes albopictus*.

BG-Sentinel requires electric power to maintain suction fan. This can be provided by batteries (Lacroix et al., 2012), solar power + recharging battery (Harris et al., 2012), or mains power (Carvalho et al., 2015).

A comprehensive list of supporting literature references on the BG-Sentinel trap can be found at: http://www.bg-sentinel.com/downloads/Publication_List_Biogents_Traps.pdf [26]

Figure 17. Commercially available BG Sentinel trap (http://www.bg-sentinel.com/) used in adult trapping

[25] http://www.bg-sentinel.com/ (accessed 31/08/2016)
[26] Accessed 19/05/2016

7 Regulated environmental releases of OX513A

Section 7.1 describes past *Aedes aegypti* population control projects and demonstrates consistent performance across different urban environments. **Table 8** summarizes the past regulatory decisions on OX513A regulated environmental releases. An overview of the current OX513A *Aedes aegypti* control program proposed is described in ***OX513A Technical Dossier Part B*.**

Table 8. Summary of regulatory decisions for environmental releases of OX513A.

Year	Regulatory body granting approval	Description
2010	Brazil- Comissão Técnica Nacional de Biossegurança (CTNBio), Brazil See more recent commercial approval 2014	*Aedes aegypti* (OX513A) Permit for the purpose of conducting an open field release.
2010	Malaysia- Genetic Modification Advisory Committee (GMAC),Ministry of Natural Resources & Environment (NRE), Government of Malaysia https://bch.cbd.int/database/record.shtml?documentid=101480 (accessed 31/08/2016)	*Aedes aegypti* OX513A - Import for open field use
2009-2010	Cayman Islands- Ministry of Agriculture, Grand Cayman http://data.parliament.uk/DepositedPapers/Files/DEP2011-0053/DEP2011-0053.pdf (accessed 31/08/2016)	*Aedes aegypti* OX513A - Import for open field use
2014	Brazil- Comissão Técnica Nacional de Biossegurança (CTNBio), Brazil https://bch.cbd.int/database/record.shtml?documentid=105831 (accessed 31/08/2016) follow link to *Mosquito Technical Aproach.pdf*	*Aedes aegypti* OX513A- For the purpose of open commercial release
2014	Panama- National Biosafety Commission Panama	*Aedes aegypti* OX513A- For the purpose of conducting an open field release.
2014	Brazil- Comissão Técnica Nacional de Biossegurança (CTNBio), Brazil http://pesquisa.in.gov.br/imprensa/servlet/INPDFViewer?jornal=1&pagina=51&data=23/04/2014&captchafield=firistAccess (accessed 31/08/2016)	*Ceratitis capitata (Medfly)*- Open field trials of an Oxitec strain
2015-2016	Cayman Islands Ministry of Agriculture, Department of Environment Grand Cayman	*Aedes aegypti* OX513A- Import permit and approval for open field use
2016	U.S.A Food and Drug Administration Center for Veterinary Medicine http://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/GeneticEngineering/GeneticallyEngineeredAnimals/ucm446529.htm (accessed 31/08/2016)	Preliminary and Final Environmental Assessment and Fining of no Significant impact In support of a proposed field trial of OX513A in Key Haven, Monroe County, Florida under an investigational new animal drug exemption

7.1 Previous *Aedes aegypti* vector control projects using OX513A

Aedes aegypti has colonised and adapted to human habitats and, most notably outside of its origins in Sub-Saharan Africa, evolved to bite humans and thrive in human habitats (Brown *et al* 2011). Inhabited urban areas where *Aedes aegypti* is present have variables which must be considered in the delivery of vector control programs. Criteria for identifying and evaluating candidate sites for vector control projects have been developed (Brown et al., 2014) and include (among others):

- Human population and housing densities,
- initial infestation levels,
- existing vector control programs,
- potential immigration from untreated adjacent areas (isolation),
- the presence of *Aedes albopictus*; and,
- seasonality

Regulated environmental releases of OX513A males have been conducted since late 2009 in collaboration with partners in both vector control programs and academia.

Releases in 2009 Grand Cayman (Harris et al., 2011), served principally to demonstrate that released OX513A males could successfully compete with wild males in their natural urban setting and inform the environmental risk assessment in demonstrating that the highly species specific nature of mosquito reproduction is not compromised by insertion of the OX513 rDNA construct. Additionally, in Malaysia in 2010 (Lacroix et al., 2012) releases examined dispersal, and longevity and served to demonstrate that the insertion of the rDNA construct in the OX513A strain has not altered the dispersal range of *Aedes aegypti*. Subsequent releases of OX513A conducted to date in Cayman Islands, Brazil and Panama, have served additionally to demonstrate efficacy in the context of a vector control program. The vector control projects also serve to establish a record of environmental, human and animal exposure with no observation of unintended effects. A general description of the methodology and principle considerations in the administration of an *Aedes aegypti* vector control project is provided here. References for projects in Cayman Islands (Harris et al., 2012; Harris et al., 2011), as well as 2 sites in Brazil (Carvalho et al., 2015; *Oxitec Internal Research Report* **PH-2013-11**) may be consulted for additional project specific details. Data is also provided for additional vector control projects in Brazil (Pedra Branca), as well as in Panama (Gorman et al., 2015). Regulated environmental releases conducted to date have all been conducted as originally prescribed under relevant permit conditions. No additional regulatory reporting has been required as a result of adverse or unintended effects with respect to human or animal health or the environment, or as a result of deviation from defined protocols.

Overall the data collected from the studies conducted in Cayman Islands, Brazil and Panama demonstrate that sustainable suppression of wild *Aedes aegypti* populations can be achieved in a variety of urban settings using the OX513A program. Estimates for suppression of wild *Aedes aegypti* populations based on direct egg counts have been made for all vector control

projects to date and serve to normalise the data, as the direct egg count method was not used in all published studies due to methodology evolving with time. See ***OX513A Technical Dossier Part B*- Section 1.7.2.1** for a description of the *Ovitrap Index*, a metric used to measure population suppression which is often cited, and is presented in some published studies. Over 90% reduction of *Aedes aegypti,* based on egg count data was achieved in all program sites (**Figure 18**), representing a variety of infestation levels, human population and housing densities, differing potential immigration from untreated areas, and variable seasonality. The five sites represented have degrees of variability surrounding these parameters, and results of vector control projects have consistently demonstrated population suppression as anticipated.

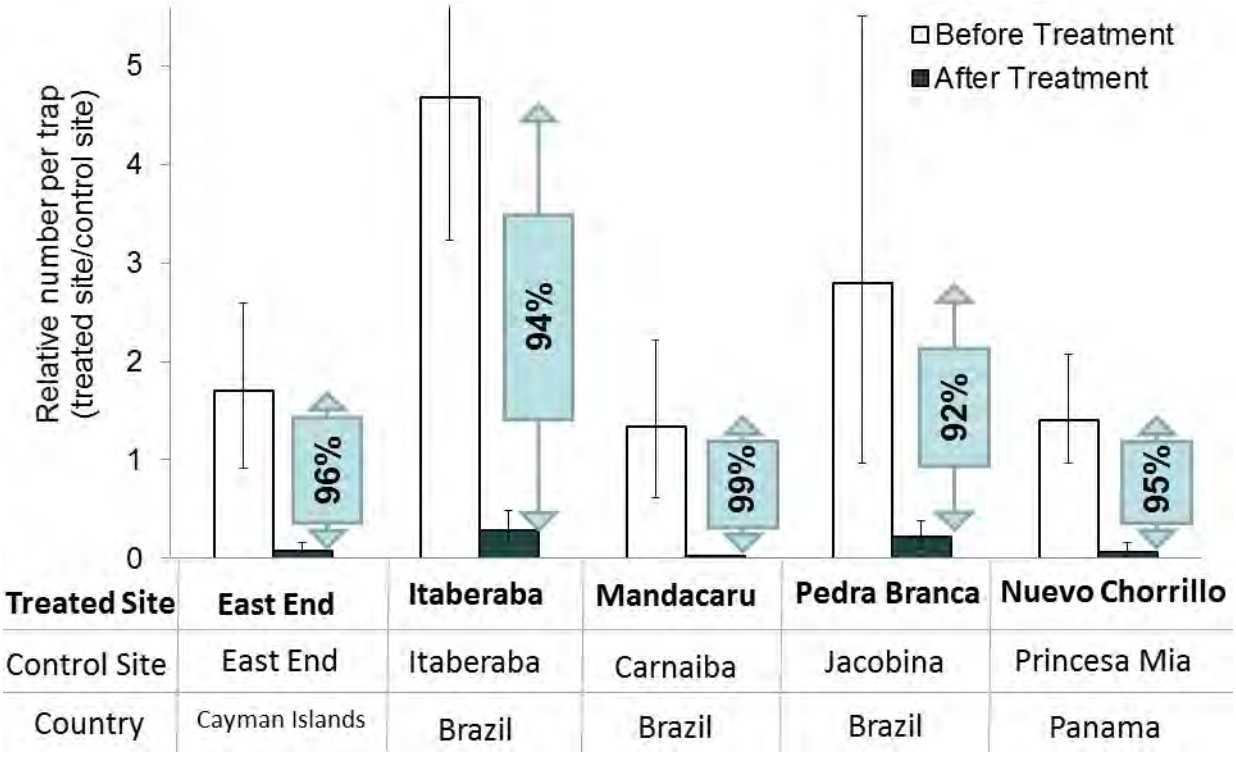

Figure 18. Population suppression based on egg count data. N.B. Data presented are relative to the respective untreated control sites in all cases and represent the highest level of control observed that was sustained for at least 4 consecutive calendar weeks. Two untreated control sites were used during each of the studies in Mandacaru and Nuevo Chorrillo; the data presented represent the higher values of those obtained. (Calculations with alternative control sites however yielded suppression over 90% in both cases).

7.1.1 Cayman Islands

In collaboration with the Cayman Mosquito Research and Control Unit (MRCU), through 2009 and 2010, the first regulated environmental releases of OX513A where conducted. The

chosen site, East End, Grand Cayman is a sparsely populated residential area that has a tropical climate with distinct seasonality of *Aedes aegypti*. Populations are lower during the dry season (November to April) and increase markedly during the rainy season (May to October). This was a challenging coastal environment with a high population of *Aedes aegypti* where no other control methods had been used.

The 2009 releases assessed mating competitiveness and had shown that OX513A males could mate with wild female *Aedes aegypti* and, together with simulation models, indicated a minimum release rate of 3,150 males per hectare (ha) per week to induce population suppression in the absence of immigration of wild *Aedes aegypti* from adjacent sites. Releases in 2010 aimed to suppress wild populations and had the target release rate of >4,000 males per ha per week. Approximately 3.3 million OX513A males were released in a 23-week period between May-Oct 2010 at the East End, Grand Cayman site.

The treated area was not isolated with regard to the *Aedes aegypti* populations as it was adjacent to an untreated area where populations of *Aedes aegypti* remained high. The relatively small size of the treatment site meant it was subject to immigration from neighbouring areas by local *Aedes aegypti* which can typically disperse about 100 metres in its lifetime (5-10 days).

Releases of OX513A were stopped in October 2010, but monitoring continued for a further 9 months. During this time the *Aedes aegypti* population remained supressed relative to the untreated control site. This was despite the fact that the site was relatively small, and susceptible to immigration as discussed above.

These studies are published in Harris et al. (2011) and Harris et al, (2012)

7.1.2 Brazil- Itaberaba

From May 2011 to October 2012 Moscamed Brasil, a Brazilian based biological control company, and the University of São Paulo conducted a project to suppress local *Aedes aegypti* using the strain OX513A in Itaberaba, a neighbourhood in the southeast of the city of Juazeiro, Bahia.

Itaberaba is a densely populated urban setting with a semi-arid climate and limited seasonality of *Aedes aegypti*; populations are present all year-round although lower during the dry season (May to October) and higher during the rainy season (November to April). The site was identified by the local vector control agency as a disease hotspot with a consistently high *Aedes aegypti* population. The primary objective of the project was to achieve substantial suppression of the local *Aedes aegypti* population in this challenging area. The suppression of the wild *Aedes aegypti* population was evaluated through a comparative assessment of a single area treated with OX513A, and an untreated control area.

In the OX513A releases in Grand Cayman, the threshold mating fraction was towards the lower end of the effective range for population suppression, yet significant suppression was still observed at a mating fraction of 12% (Harris et al., 2012). While this does not show that the critical mating fraction was less than 12%, as release rates somewhat below the threshold level may still give significant suppression, it does suggest that this threshold is not at the upper end of the model estimates. A target mating fraction of 50% was established for the Itaberaba releases reasoning that even if the population dynamics were somewhat different relative to Grand Cayman it would likely be sufficient to achieve suppression.

In the initial rangefinder phase, a total 185,000 OX513A were released over a 6-week period, 3x per week, starting in May 2011. To achieve the target mating fraction, release rates were estimated as ranging from 2,800-25,000/Ha/week. Subsequent release numbers increased in line with production capacity of the mass rearing unit and the target mating fraction was consistently achieved from January 2012 with a mean weekly release rate of ~30,000/Ha/week. From mid-February 2012, releases were concentrated in one particular area enabling a further increase in release rate per hectare per week from the fixed production capacity of the mass rearing unit. Substantial suppression of local population followed within 6 months. Following suppression, releases were maintained at a lower level (ca. 5 times lower) designed to counter resurgence of the wild *Aedes aegypti* population. The wild population was then maintained at very low levels despite the treated area being a relatively small suburb within a larger city where populations of *Aedes aegypti* remained high, and thus susceptible to immigration of *Aedes aegypti* from adjacent untreated areas (*Aedes aegypti* which can typically disperse about 100 metres in its lifetime, 5-10 days). A total of over 17.6 million OX513A males were released in this project.

These studies are published in Carvalho et al. (2015).

7.1.3 Brazil - Mandacaru

From March 2012 to March 2013 Moscamed Brasil and the University of São Paulo conducted a project to suppress local *Aedes aegypti* in Mandacaru, a neighbourhood with a largely agricultural landscape, approximately 10km to the west of the city of Juazeiro, Bahia. Comparable villages, Maniçoba and Carnaiba, with similar habitat, *Aedes aegypti* populations and isolation served as control sites for this study.

Mandacaru is a moderately dense rural village comprising 735 houses, equating to a human population of 2 793, surrounded by non-residential land. It has a semi-arid climate and limited seasonality of *Aedes aegypti*. Populations of *Aedes aegypti* are present all year round although lower during the dry season (May to October) and higher during the rainy season (November to April). Due to the relative isolation of the site, migration of *Aedes aegypti* (both into and out of the treated area) was considered to be low. The objective of the project was to achieve substantial suppression of the local *Aedes aegypti* population and subsequently maintain suppression with a reduced release rate.

OX513A was released at the predicted optimal rate for suppression from the outset (12,000-13,000 Ha/week) beginning in March 2012. Initial baseline monitoring indicated a relatively low wild population of *Aedes aegypti* and within four weeks the estimated mating fraction exceeded the target of >50%. Release rates remained relatively constant during the initial four months of releases and substantial suppression of the wild *Aedes aegypti* population was observed. Release rates were subsequently reduced for another four months during which the wild *Aedes aegypti* population was supressed further, and release rates were further lowered in a subsequent maintenance phase. Suppression of the wild *Aedes aegypti* population was successfully maintained for a further seven months, using reduced release rates. A total of over 10.3 million OX513A males were released during this project.

Aedes aegypti populations are often subject to large seasonal fluctuations. In this study treatment with OX513A was initiated and achieved substantial suppression of the wild population, during the low season. Suppression of the wild *Aedes aegypti* population was sustained with reduced release rates during a maintenance phase, which corresponded with the rainy season when *Aedes aegypti* populations are typically higher. This two-phase high-low release rate strategy was successful in this project, indicating that it has potential utility in the context of population suppression program design.

That the observed suppression of the *Aedes aegypti* population was even greater in this study relative to previous studies is likely due to the isolation of the study area and the presumed absence of immigration of *Aedes aegypti* from neighbouring untreated areas.

For the complete study report see *Oxitec Internal Research Report* **PH-2013-11**

7.1.4 Brazil – Pedra Branca

Following the successful projects in other jurisdictions, the government of the state of Bahia, Brazil, decided to apply the OX513A technology to the 50,000 inhabitant city of Jacobina through a project delivered by Moscamed Brasil and the University of São Paulo. To scale up the mosquito production, a dedicated facility with a production capacity of 4 million OX513A males a week was built. The project started in the neighbourhood of Pedra Branca in the northwest of the city and the rest of Jacobina served as a control area except the neighbourhood closest to Pedra Branca.

Pedra Branca is a densely populated urban neighbourhood with a semi-arid climate and limited seasonality of *Aedes aegypti*. Populations of the mosquito are present all year round although lower during the dry season (May to October) and higher during the rainy season (November to April). The site was identified by local vector control agency as a problem hotspot with consistently high *Aedes aegypti* population and dengue incidence.

The release of sufficient numbers of OX513A to target suppression of the wild *Aedes aegypti* population began in July 2013 and a substantial reduction in the population occurred in the following six months. The wild *Aedes aegypti* population was maintained at this low level

despite the fact that the plot was relatively small, and susceptible to immigration from adjacent untreated areas where *Aedes aegypti* populations remained high.

Ovitrap data indicated that local *Aedes aegypti* levels were 13 times higher before suppression by OX513A (based on relative numbers of eggs per trap).

The program in Jacobina was delivered by collaborative partner Moscamed Brasil with the University of São Paulo, and started in June 2013, but the collaboration was subsequently dissolved in early 2016.

Available data is presented in an internal summary report *Oxitec Internal Research Report* ***4pg_PedraBranca_Oxitec***

7.1.5 Panama - Nuevo Chorillo

From April 2014 to October 2014, the Gorgas Memorial Institute for Public Health, under permit from the Ministries of Agriculture and Industry, conducted a vector control project to suppress local *Aedes aegypti* using OX513A in Nuevo Chorrillo, a neighbourhood in West Panama.

Panama has a tropical maritime climate; temperature and relative humidity are generally high with minimal seasonal variation and diurnal ranges are typically low. Rainfall is largely restricted to the wet season, which is usually from May to November. Nuevo Chorrillo has a moderate human population density and marked seasonality of *Aedes aegypti* (Neira *et al*., 2014). Data obtained from the treated site were compared with those for two nearby untreated neighbourhoods, located in residential estates called 'Lluvia de Oro' and 'Princesa Mia'. Vector surveillance over several years had confirmed a predominating *Aedes aegypti* population with an increasing presence of *Aedes albopictus* at all three sites. This was the first OX513A vector control project to be completed in an area where both *Aedes aegypti* and *Aedes albopictus* co-exist.

The period of study was throughout the wet season, when annual *Aedes aegypti* levels were typically high. A target dose rate of 67 OX513A males per person was chosen. As there were 900 inhabitants living in the treated site a requirement of 60,000 per release was determined. To ensure a constant presence of OX513A males a treatment frequency of three times per week was used. A total of over 4.2 million male OX513A were released through the period of the project.

Changes in the wild population of *Aedes aegypti* were estimated for 28 consecutive weeks after OX513A releases began. At week 16, averages across both untreated sites demonstrated significant reductions in the abundance and presence of *Aedes aegypti* at the treated site. The treated population was then maintained at or below these levels for the remaining 12 weeks despite the intense pest pressures evident in both untreated areas as the wet-season progressed.

7.1.5.1 Environmental persistence

An additional component of this project was a post release environmental monitoring survey to assess the persistence of the OX513 genetic construct in the environment. The final releases of OX513A took place 31 October 2014. Environmental monitoring in the control and release sites continued to 138 days post release through a network of 60 ovitraps and fluorescence screening of larvae for the DsRed2 marker. Over 20,000 *Aedes aegypti* larvae where individually screened. Prior to the final release, OX513A fluorescent larvae comprised 100% of the trapped larvae in the treated area, 25 days post-release it was 5%, 84 days post-release it was 0%. Although the absence of OX513A genes could only be confirmed from 12 weeks post-release onward due to a disruption in data collection, the data suggest OX513A genes are unlikely from 6-8 weeks post-release.

For the complete study report see Gorman et al. (2015)

7.1.6 Conclusions taken from previous vector control projects using OX513A

Regulated environmental releases in Cayman Islands, Malaysia, Brazil and Panama have taken place since 2009. Data support the environmental risk assessment of OX513A in evaluating mating competitiveness, adult dispersal and longevity, the dissemination and persistence of OX513A genes into the environment, and in demonstrating stability in expression of the introduced traits across a range of habitat. Releases have taken place in habitat having variability in key site variable parameters such as, human population and housing densities, pre-existing *Aedes aegypti* infestation levels, status of existing vector control programs, potential immigration from untreated adjacent areas (isolation), the presence of other *Aedes* species, and seasonality of the wild population. Despite the variability in these site parameters, the receiving environment in all cases can be defined as urban, and thus sufficiently similar in key parameters relevant to biosafety such that data generated in the urban environment can be applied broadly across urban environments to inform environmental risk assessment. While an internationally recognised definition for urban and rural is not established[27], and country specific definitions are variable[28] for the purpose of biosafety assessment of OX513A, the classification system used by the environmental Administration of Finland is a useful reference[29]. The urban environment is considered to also include peri-urban, which is defined as part of the intermediate zone between urban and rural, which is directly linked to an urban area.

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[27] http://unstats.un.org/unsd/demographic/sconcerns/densurb/densurbmethods.htm#D (accessed 05/09/2016

[28] http://unstats.un.org/unsd/demographic/sconcerns/densurb/Defintion_of%20Urban.pdf (accessed 05/09/2016)

[29] http://www.ymparisto.fi/en-US/Living_environment_and_planning/Community_structure/Information_about_the_community_structure/Urbanrural_classification (accessed 05/09/2016)

As of July 2015, including vector control projects which are ongoing, over 100 million OX513A males have been released worldwide since the initial release programs in Cayman Islands were undertaken. Vector control organisations in areas where OX513A releases have taken place continue to monitor and control wild *Aedes aegypti* populations using existing interventions, and as part of Integrated Vector Management (IVM) programmes. All regulated environmental releases to date have taken place as prescribed under regulatory permit conditions, and no events have been observed which have required additional regulatory reporting, and no unintended or adverse effects on human or animal health or the environment have been reported.

Overall the data collected from vector control projects conducted in Cayman Islands, Brazil and Panama have consistently demonstrated that that sustainable suppression of wild *Aedes aegypti* populations could be achieved in a variety of urban settings using the OX513A release strategy. An over 90% reduction, based on egg counts, of wild *Aedes aegypti* populations was consistently achieved in all program sites **(Figure 18)** demonstrating stability of the OX513A interaction with wild *Aedes aegypti* populations across a range of urban settings having variable site influences.

7.1.6.1 Implications for vector control- Disease transmission threshold

The level of control of wild populations of *Aedes aegypti* demonstrated through the use of OX513A are substantial for vector control operations. The impact of a reduction in vector population density on the transmission threshold of dengue has been estimated by Focks et al. (2000) in relation to pupae per person^{-1} (as a proxy for adult mosquito population), ambient temperature and herd immunity[30]. For a mean temperature of 28°C Focks et al. calculated an epidemic transmission threshold of 0.42, 0.61 or 1.27 pupae per person for initial seroprevalence[31] of 0%, 33% and 67%, respectively. Using calculations and assumptions given in (Focks et al., 2000), we estimate that average pupae person^{-1} decreased in our treated area from 0.7 pre-treatment to 0.04 post-treatment, which in their model would be sufficient to prevent epidemic transmission under these conditions, or indeed under the most adverse conditions modelled for a naive human population with 0% seroprevalence.

Figure 19 represents an example of how the wild *Aedes aegypti* population reduction achieved in Itaberaba Brazil relates to transmission thresholds at 3 levels of sero-prevelance.

[30] Herd immunity describes immunity that occurs when the vaccination of a portion of the population (the "herd") provides protection to unprotected individuals. Herd immunity theory proposes that, in diseases passed from individual to individual, it is difficult to maintain a chain of infection when large numbers of the population are immune.

[31] Seroprevalence is the number of persons in a population who test positive for a specific disease based on serology (blood serum) specimens; often presented as a percent of the total specimens tested or as a proportion per 100,000 persons tested.

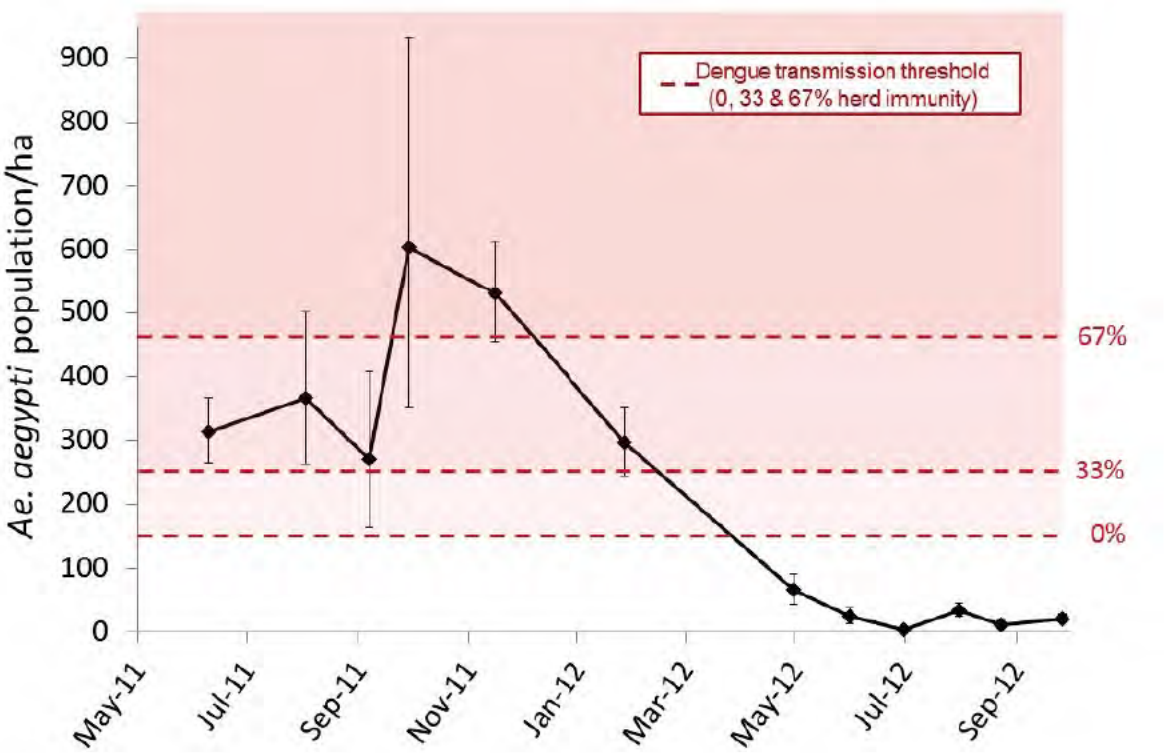

Figure 19. Change in adult *Aedes aegypti* population density throughout the vector control project conducted in Itaberaba, Brazil (Carvalho et al., 2015). Dengue transmission threshold for three seroprevelance values are indicated by red lines. The average temperature during the peak dengue transmission season (January – July was 27.7°C. *Aedes aegypti* population density calculated based on methods of (Focks et al., 2000).

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OXITEC

OX513A Technical Dossier

Part B – Intended use of OX513A *Aedes aegypti* in Saba

Submission to the GMO Office of the National Institute of Public Health and the Environment of the Netherlands (RIVM) for the technical evaluation of the release of *Aedes aegypti* OX513A in Saba.

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Information relating to the intended use *of OX513A*

An overview of the OX513A *Aedes aegypti* control program proposed for Saba is described in **Section 1**, and relevant details of the receiving environment in Saba in **Section 2.**

1 Details of the proposed release on Saba

A general program overview is provided below, and specific details for the proposed Saba island project are outlined in **Section 1.1- 1.7**

Project protocols and evaluation metrics have evolved since the initial regulated environmental releases described in **OX513A Technical Dossier Part A - Section 7.1** and are now standardised through *Standard Operating Procedures* (SOPs) as referenced throughout **Sections 1.1- 1.7.** The entire process from egg production in the UK to adult release at the local site is under a Quality Management System (QMS) and key SOPs relevant to the rearing and release in Saba are provided.

General overview

In the UK egg production facility, eggs are continually produced from a cycling colony of homozygous OX513A and subject to regular quality control checks (See **OX513A Technical Dossier Part A Section 5.8.1**). Male and female pupae are added to a cage and allowed to emerge as adults over a 3-4 day period. Female mosquitoes require a blood meal to provide the nutrients to produce each batch of eggs and thus require a blood meal between each laying cycle. They are fed regularly for 4-6 weeks and approximately three days after blood feeding[1], female mosquitoes develop a batch of eggs and are ready to oviposit. The eggs take about five days to mature, at which time they can be dried and stored under controlled conditions.

Eggs are shipped in regular shipments throughout the course of the program to the facility near the release site where they are reared through to pupae, sex sorted to select male pupae, the males are matured to adults for release. Sexually mature OX513A males are released from specialised release devices in a grid-like pattern from predefined release points to ensure even coverage of the area.

The OX513A programme can be divided into three sequential phases:

1. *Preparation phase:*

   The Preparation Phase involves collating historical climatic, and vector surveillance, and disease incidence data where appropriate. This phase is used to establish production and distribution capacity, and finalise a release plan for the release area, including initial application rates and locations and a community engagement

[1] Feeding is defibrinated horse blood, TCS Biosciences Ltd http://www.tcsbiosciences.co.uk/catalog/product_detail.php?CI_ID=6 (accessed 01/09/2016)

program. The OX513A program is compatible with conventional control programs and exploiting synergy in an integrated approach with local vector control activities is evaluated in the preparation phase. Although the chosen number of OX513A males for releases is relative to the estimated size of the target *Aedes aegypti* population, wild populations of *Aedes aegyp*ti are most closely associated to human populations and therefore the release rate (or dose rate) is often described as 'number of OX513A males per person'.

2. *Intervention phase:*

   The Intervention Phase is when the *Aedes aegypti* population is initially brought under control. If required, this can be timed to take advantage of seasonally low pest pressures or as a follow-on from existing vector management activities. Releases are concurrent with monitoring various parameters, the adult *Aedes aegypti* population and the application rate is evaluated and adjusted as informed by these monitoring activities. Subsequent to the preparation phase, the initial releases of OX513A males take place up to three times a week at a constant release rate and serve a "range-finding" function in order to assess various parameters which inform subsequent releases. The initial release rate is a function of the human population and the estimated wild *Aedes aegypti* infestation level in the treatment area at start of releases. The rate of release is adapted on an ongoing basis as informed by data collected throughout the release, and is generally reduced as control of the wild *Aedes aegypti* population is achieved.

3. *Maintenance phase:*

   Once the *Aedes aegypti* population has been reduced to target levels the programme enters the Maintenance Phase, designed to prevent resurgence of the wild *Aedes aegypti* population. A range of approaches can be adopted and customised to individual control programmes. Re-infestation in this context may be caused by the immigration of wild *Aedes aegypti* into the programme area, perhaps as eggs or adults inadvertently moved by humans. It may also relate to the size of the egg bank (eggs laid at an earlier period remaining in the environment), though the viability of such eggs is expected to decline over time such that this will be a source of re-infestation for a limited period only.

Ongoing monitoring of the releases both during and post release is undertaken using both egg and adult surveillance methods. Identification, detection, and trapping methodologies are described in **OX513A Technical Dossier Part A Section 6** and discussed in detail for the Saba island proposal in **Section 1.7** below.

1.1 Anticipated outcomes for Saba Island

The proposed OX513A *Aedes aegypti* suppression program in Saba aims to suppress the local population of *Aedes aegypti* to very low levels and potentially to elimination with demonstration of continued elimination for a period of a year thereafter. *Aedes aegypti* is an invasive pest in Saba. **Sections 1.4-1.7** provide a description of the facilities and procedures which will be installed and undertaken in this regard.

Sustained releases of OX513A in Saba aims to have several measurable effects on the wild *Aedes aegypti* population, including, in temporal order:

1. An increase of the overall male-to-female ratio for *Aedes aegypti*
2. Wild *Aedes aegypti* females mating with OX513A males; and,
3. Suppression of the target *Aedes aegypti* population leading to potential elimination of *Aedes aegypti* on the island.
4. Demonstration of continued elimination of *Aedes aegypti* for a period of one year.

The OX513A program proceeds with an ongoing evaluation of the population dynamics of the wild *Aedes aegypti* population, and release rates are adapted based on monitoring throughout the release period as control/ elimination is achieved, as described in **Section 1.3.** Once control is gained and the island is effectively free from wild *Aedes aegypti,* continued close monitoring and low- level releases of OX513A at points at risk of potential re-introduction will be required to maintain the wild *Aedes aegypti* free status.

1.2 Location of releases

The proposed release on Saba is island wide throughout the 4 principle human populated areas of The Bottom, Windwardside, Zions Hill and St. Johns, and any minor inhabited areas between. Releases would take place as well in the areas of the port at Fort Bay Harbour, and Juancho E. Yrausquin Airport, as these represent potential areas for the introduction of *Aedes aegypti.*

The release areas will be mapped prior to release including demarcation of inhabited areas, planned OX513A release points, monitoring traps location and incorporated into an appropriate Geographical Information System (GIS). Releases of male OX513A are made typically up to three times a week at predetermined geo-referenced locations generally not more that 100m apart to ensure coverage of the release area.

The island may be subdivided into areas with different release rates depending on the heterogeneity of inhabited areas and corresponding *Aedes aegypti* infestation but the broad area of release will be aligned with road patterns with in the predicted habitat of *Aedes aegypti* in Saba (i.e the areas inhabited by humans) as illustrated in **Figure 1** below.

Figure 1. Principle habitat of *Aedes aegypti* on Saba represented as urban areas to include up to approximately 100m distance from human habitation (shaded in red). Total shaded area equivalent to approximately 3.7 Km^2.

1.3 Determination of release rates- phased approach

As described briefly in the *General Overview* **Section 1**, OX513A releases are administered in a phased program and release rates are informed by ongoing monitoring and evaluation of various metrics described in this section. **Section 1.7** provides additional detail on the monitoring program metrics generally used to inform the adaptive release rates in the phased approach described below.

1.3.1 Preparation phase:

This phase will be used to evaluate the initial densities of *Aedes aegypti* mosquito populations in the treatment areas and optimise the OX513A rearing methodology to local conditions on Saba. A 6-8-week professional community engagement program would also be undertaken with all stakeholders.

Initial densities are monitored by ovitraps and adult trapping methods as well as using best available information including, historical surveillance data, seasonality, epidemiology records, existing mosquito abatement and qualitative factors such as housing type and proliferation of breeding sites. An ovitrap is as device that mimics the preferred breeding sites for container breeding mosquitoes and is routinely used to monitor the presence or absence of mosquitoes in the area of interest (Silver, 2008). Adult traps are designed to directly capture adult mosquitoes using lures, and provide an indirect measure of Aedes *aegypti* adult abundance in the treatment areas and are described in **OX513A Technical Dossier Part A - Section 6.2**.

Based on the densities of wild *Aedes aegypti* determined an initial release rate of the OX513A males is chosen. An initial release rate (IRR), is typically between 100 and 300 male OX513A per person per week in most projects to date.

1.3.2 Intervention Phase

1.3.2.1 Mosquito release and dispersal

The initial release rate (IRR) of OX513A is released in a systematic manner from a pre-determined georeferenced grid of release points at regular time intervals, for even and consistent coverage of the treatment area. Release points will be spaced no more than 100m apart, and releases will occur up to 3 times per week.

There is a minimum time lag of at least one generation (~4 weeks, dependent on temperature) between the start of OX513A release and any initial impact on local *Aedes aegypti* population as control only starts to be observed when progeny of released male OX513A fail to develop due to the action of the self-limiting trait and thus do not contribute to next generation of functional adults. In past projects described in **OX513A Technical Dossier Part A - Section 7** substantial suppression was observed 4-6 months following initiation of releases, but depending on local conditions and mosquito densities this could be up to 12 months.

The key factor governing the rate of *Aedes aegypti* population reduction is the mating fraction of local wild *Aedes aegypti* females mating released OX513A in a greater proportion than local wild *Aedes aegypti* males. The mating fraction attained from a given release rate can be assessed through monitoring the presence of the DsRed2 marker in eggs collected from the field in Ovitraps (as described in **OX513A Technical Part A - Section 6.2**). Alternatively or

concomitantly, if adult trapping is deployed, an early indication of the likely impact of the IRR can be derived from estimated OX513A : local male *Aedes aegypti* ratio. The ratio of OX513A : local male *Aedes aegypti* is calculated based on the change in sex ratio, due to the release of OX513A males, in relation to underlying baseline sex ratio which was 0.7:1 (Carvalho et al., 2015).

Assessment of mating fraction provides an indication of the successes of releases prior to any observed reduction in the local *Aedes aegypti* population. Mating fraction can be used to dynamically adjust release rates to the site specific population dynamics of local *Aedes aegypti*. The ratio of OX513A: local *Aedes aegypti* males resulting from sustained release is critical to the probability of local female *Aedes aegypti* mating with an OX513A male rather than a local wild male *Aedes aegypti* counterpart. Therefore, the release rate necessary to achieve a given mating fraction is proportional to the local *Aedes aegypti* population. The greater the OX513A: local *Aedes aegypti* male ratio achieved, the greater the mating fraction and likely impact on local *Aedes aegypti* population.

Therefore analysis of the ratio of OX513A : local *Aedes aegypti* males can give an indication in advance of determination of the mating fraction of whether the chosen IRR is with a range expected to achieve the target mating fraction (>0.5) and eventual suppression of the wild *Aedes aegypti* population.

1.3.2.2 Adaptive management of release rate

Release rates are dynamically adjusted in response to local *Aedes aegypti* populations which are expected to fall during the period of the intervention. OX513A male releases will continue as the local *Aedes aegypti* population is suppressed, but at a reduced level chosen to maintain the mating faction at >0.5. The release rates will be evaluated every 6-8 weeks during suppression phase and adjusted based on mating fraction. Release rates may be adapted at the discretion of the program manager, as higher rates are expected to achieve suppression more rapidly for a given location/situation.

Assuming target release rates are maintained in sufficient quantity (dependent on mosquito production parameters)) to achieve a ≥0.5 5mating fraction, significant suppression of the local *Aedes aegypti* population is typically expected within 4-6 months of initiation of releases. However, to achieve the effective elimination of *Aedes aegypti* from Saba it is expected to take longer and may tend towards 12 months.

When the local *Aedes aegypti* population drops sufficiently to demonstrate convincing suppression or elimination, the program enters a *Maintenance Phase*, where releases and monitoring will continue, but without estimating the mating fraction. Resurgence of the local *Aedes aegypti* population, as monitored by ovitraps, will trigger an increase in release rates. Resurgence is categorised as 4 consecutive weeks with an ovitrap index >10 %.

1.3.3 Maintenance Phase

Once the wild *Aedes aegypti* population has been effectively eliminated (<10% ovitrap index)- the program enters the Maintenance Phase designed to stop resurgence of *Aedes aegypti* population and to sustain the attained goals. This approach can be applied to contiguous sub-areas of the program as they become well controlled, even while other areas remain in the Intervention Phase.

Resurgence could be from small pockets of residual *Aedes aegypti* population, egg bank and/or immigration. An advantage of the OX513A self-limiting release program is that as the local *Aedes aegypti* population decreases, the efficacy of releases increase as it becomes possible to achieve a higher ratio of OX513A males : local *Aedes aegypti* males. This is contrary to conventional control methods where targeting the remnant local *Aedes aegypti* population becomes increasingly difficult.

The Maintenance Phase protocol is similar to that used in the Intervention Phase – with releases and/or use of other vector management tools being planned and targeted based on monitoring data. The calculation of mating fraction during the Maintenance Phase is not undertaken, as the release rate is informed based on monitoring of the *Aedes aegypti* population overall. A key difference is that in the Maintenance Phase it is possible that releases of OX513A might be reduced significantly in frequency or even stop in low risk areas.

The areas of high risk and the foci of re-infestation will be principally the transport hubs at the port at Fort Bay Harbour, and Juancho E. Yrausquin Airport, which will be further characterized through the monitoring during the Intervention Phase. These areas are at a minimum proposed for sustained releases on an ongoing basis, with program objectives assessed annually in consultation with the Saba Public Health officials and appropriate government and other stakeholders.

In the remaining areas, treatment is anticipated to stop as the *Aedes aegypti* population is controlled, and be subject to ongoing monitoring, with targeted OX513A releases if re-infestation is detected.

1.4 Containment measures prior to release

In the UK, OX513A is regulated by the UK Health and Safety Executive (HSE) under *The Genetically Modified Organisms (Contained Use) Regulations 2014* and is handled under Containment Level 1 (CL1) conditions. Upon export to Saba, OX513A eggs (amounts to be stated in an advanced notification separate from this Technical Dossier) will be packaged according to ***R-SOP-00002 Package and Transport of OX513A Eggs*** which provides for three layers of shatter-proof containment, and shipped by air by commercial courier service transiting through St. Maarten.

Conditions for transit via St Maarten will need to be explored with local regulatory officials of Saba and St Maarten, and has been identified with Saba legal affairs officials. This may require support from RIVM for attestation of adequate containment of OX513A in transit.

1.4.1 Mobile Rearing Unit Overview

OX513A will be imported, under contained conditions, to a locally established Mobile Rearing Unit (MRU) (Example **Figures 2-3**). MRUs are insect production laboratories fabricated within standard shipping containers which conform with relevant ISO (International Organization for Standardization) standards for shipping containers, and are adapted as mobile insectaries as described in this section. MRU design is compliant with CSC (Container Safety Convention) and MRUs are intended to facilitate rapid worldwide deployment of small to medium sized projects, and/or facilitate deployment in areas where pre-existing facilities are unavailable.

Arthropod containment is inherent to MRU design. Compliant features such as insect-proof door seals, smooth walls and ceilings, few, if any, sources of insect harbourage, light-colour interior, sealed lighting units, freezer space (hand wash facilities, and lobby-space combine with established operator protocols to provide compliance up to and including ACL2 (Arthropod Containment Level 2[2]).

The following SOPs detail procedural measures in place relevant to containment:

- **HS-P-00057** **Emergency Procedures**
- **HS-SOP-00053** **Cleaning and Waste Procedures**
- **HS-SOP-00054** **Unit Decontamination**
- **HS-SOP-00059** **Entry and Exit**
- **HS-SOP-00061** **Biosafety**
- **00054_01** **OX513A Hurricane Preparedness Policy (see section 2.1.2.1)**

[2] http://online.liebertpub.com/doi/pdf/10.1089/153036603322163475 (accessed 05/09/2016)

Due to the pre-fabricated nature of MRUs, on-site installation is minimal and consists primarily of constructing four basic concrete pad foundations followed by connection to services (mains water, drainage, mains electricity). Some specific external requirements such as disability access ramps and lighting are also completed during installation.

The MRU for Saba island is proposed to be installed in the area of the port at Fort Bay Harbour in close proximity to electrical and fresh water supply from the desalination plant.

Waste water disposal from the MRU will be consistent with that of other commercial buildings in the area of Fort Bay harbour. The harbour has several commercial and government buildings including a harbour office, customs services, dive shops, fuel stations, restaurants, and electrical generators, among others[3].

1.4.1.1 Design and construction

MRUs are pre-fabricated in the UK to a generic design, which is tailored before shipping according to different project localities and requirements. An assessment of all relevant building codes and installation requirements will be completed for Saba to inform the MRU construction process.

As requirements vary between countries and even within countries, case-by-case information relating to whether the installation of an MRU will require a building permit(s) should be taken into account as early as possible. Issues to consider include (but are not restricted to): the duration of use, overall footprint area or volume, number of workers, whether the installation is considered temporary.

Building codes vary the world over, with countries ranging from having none to enforcing established International Standards. To ensure a safe working environment, Oxitec MRUs have a minimum specification that is compliant with respected codes for structural, mechanical, plumbing, electrical, fire safety, and disability access. List of building codes that govern fabrication and installation of MRUs generally, and was used for the current installation in Cayman Islands:

a. 2009 International Building Code
b. 2009 International Mechanical Code
c. 2009 International Plumbing Code
d. 2005 National Electrical Code (USA)
e. ICC/ANSI 117.1: Accessible and Usable Buildings and Facilities
f. 2009 International Fire Code

[3] http://www.sabatourism.com/sabasport.html (accessed 01/09/2016)

Oxitec will work with local planning officials in Saba to assess applicable code requirements.

Appendix 6 is the MRU design that was approved in Cayman Islands, and provides a general idea of the layout. Specific adjustments to ensure regulatory compliance in Saba may be required. In addition, the MRU design for Saba would incorporate space for analytical fluorescence microscopy for the screening of larvae for the DsRed2 fluorescent marker.

Figure 2. Exterior of Mobile Rearing Unit installation in Cayman Islands 2016

Figure 3. Interior of Mobile Rearing Unit installation in Cayman Islands 2016

1.5 Rearing of OX513A from egg to adult mosquitoes

The process of rearing OX513A eggs to adults is well established in Standard Operating Procedures (SOPs) to provide oversight as part of an overall quality management scheme and additionally serves to support regulatory compliance activities were required.

Table 1 is a list of key SOPs used to guide the production process from the receipt of eggs in the MRU to the eclosion of adults in the release devices. **Figure 4** provides an overview of the process flow and key SOPs referenced are provided.

Table 1. SOPs for the production of OX513A adults for release from eggs

Reference number	Title
R-SOP-00002	Package and Transport of OX513A Eggs
TD-R-00285	OX513A Pre and Post Shipment Checks Record
TD-SOP-00194	OX513A Egg Hatching - For Release
TD-SOP-00195	OX513A Calculation of Aliquot - For Release
TD-SOP-00284	OX513A Pre and Post Shipment Checks Procedure
TD-SOP-00291	OX513A Larval Rearing (Tray System)
TD-SOP-00292	OX513A Larvae Pupae Sorting - For Release
TD-SOP-00293	OX513A Sex Sorting of Pupae for Release
TD-SOP-00294	Male Eclosion in Release Device
TD-SOP-00295	OX513A Releases
TD-SOP-00299	OX513A Pupae Larvae Chemical Sorting

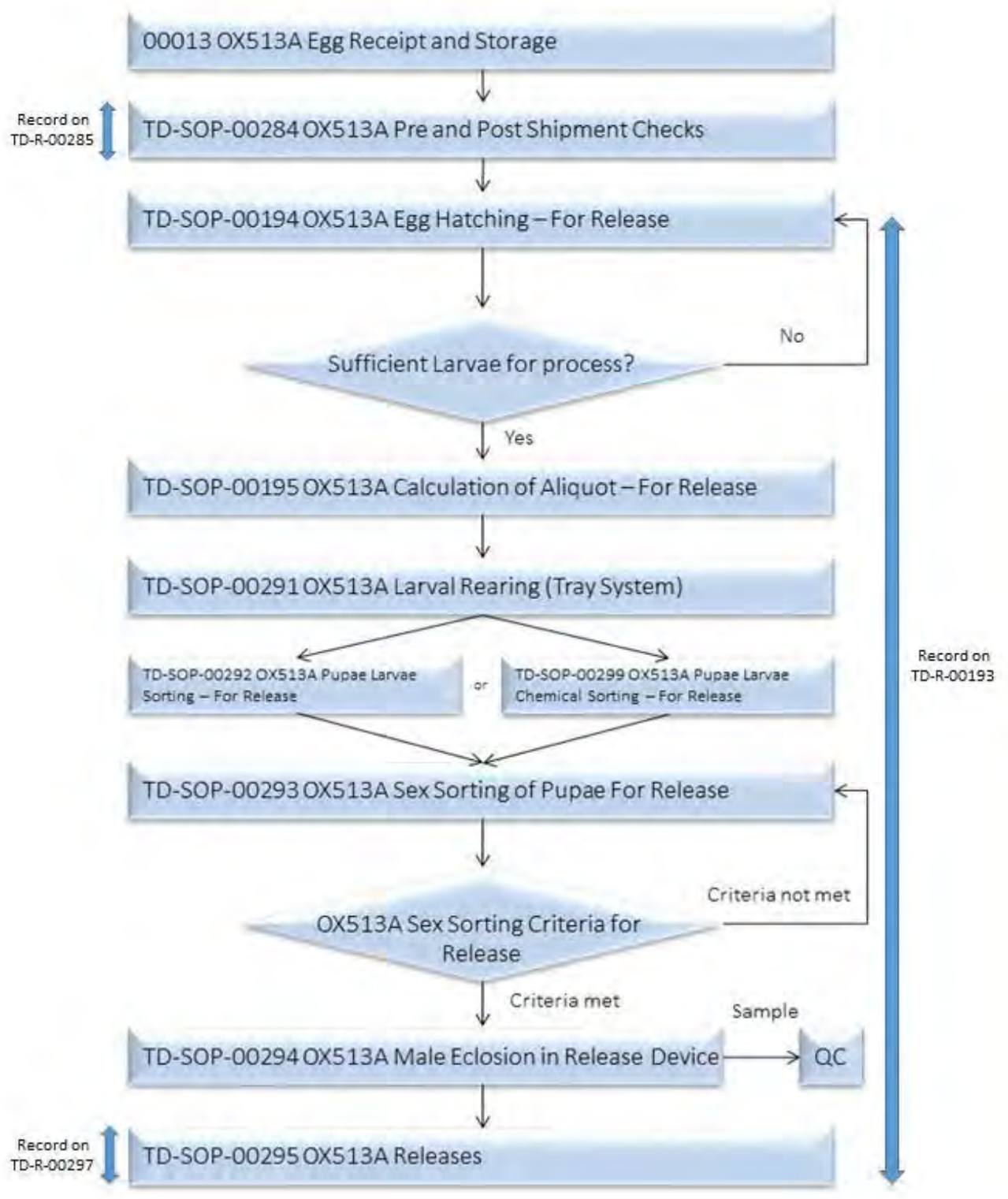

Figure 4. Process flow for OX513A egg rearing to adult

1.5.1 Tetracycline use

As described in ***OX513A Technical Dossier Part A - Section 4.4.2 Self-limiting trait tTAV*** the self-limiting system is repressible in the presence of the antidote tetracycline or its analogues, allowing the switching off of the conditional lethality effect.

As described in ***SOP 00039_02*** *Chlortetracycline HCl Working Solution Preparation* and ***TD-SOP-00291*** *OX513A Larval Rearing (Tray System).* Chlortetracycline is used at a final concentration of 30mg/litre final concentration at the larval rearing stage. The amount of tetracycline generated from the rearing facility and present in the final waste water can thus be estimated based on the initial input and reduction in concentration which occurs through the rearing process.

The number of OX513A adult males required to be released is calculated based on the parameters described in ***Section 1.3*** *Determination of release rates- phased approach*. In order to provide an estimate of tetracycline use, a target release rate must be identified. For illustrative purposes, and based on the typical release rates for past projects the maximum value of OX513A 300 males /person/ week is used in the calculation below.

The human population figure used for the example is 2000, consistent with the approximate population of Saba for the past 5 years as noted in ***Section 2*** *Receiving environment.*

- 300 males/pp/wk X 2000 people = 600 000 males to produce/ week
- Per OX513A male pupae for release, 5 larvae are needed (**TD-SOP-00194**)
- There are ~2000-2500 larvae / litre in production (**TD-SOP-00291**)

Thus;

- 600 000 OX513A males needed X 5 larvae per male = 3 000 000 larvae reared/week
- 3,000,000 larvae/ 2500 larvae/litre = 1200 litres / week
- 1200 litres/week X 30 mg/ litre = **36 grams of chlortetracycline/week input**

A study was conducted to analyse how the concentration of tetracycline changes in rearing water and in mosquitoes during their aquatic life stages, in order to assess the potential quantities of tetracycline that would be generated in production. The chosen method of analysis was adapted from the Association of Analytical Communities *(AOAC) Official Method 995.09 Chlortetracycline, Oxytetracycline, and Tetracycline in Edible Animal Tissues* (**Appendix 12**). Initial studies on the amount of material required showed that at least 0.5g of each biological sample (mosquitoes without any water) would be necessary for analysis. The number of mosquitoes which comprises 0.5g depends on the life stage being sampled. For water samples 1.5ml (1.5g) of the rearing water was required.

Six time points representing different life stages were sampled and a total of 5 biological samples, and 5 rearing water samples taken at each time point. **Table 2** below shows the time points and mosquito life stages sampled. Estimations of the numbers of mosquitoes required for each time point are based on measurements of the weight of different life stages. The rearing schedule was 3 larvae per ml, set up in the standard rearing trays (20x35x5cm). All biological samples had excess rearing water removed and were stored at -20°C; samples were sent on dry ice for chlortetracycline concentration analysis at CEM Analytical Services (Bracknell, Berkshire, UK).

Table 2. Sample collection for OX513A taken at different life stages for chlortetracycline analysis

Time Point	Life stage	Day of rearing	Numbers required per repeat	Numbers required total
0	Eggs	-	41667	208333
1	L1/L2	1	83333	416667
2	L3	4	813	4065
3	L3/L4	6	499	2496
4	L4	8	186	928
5	Male pupae	9	250	1250
6	Female pupae	9	125	625

The analytical report is referenced in (Curtis et al., 2015) and demonstrates the concentration of chlortetracycline reduced by about 21x the original concentration added to the rearing water (30 to 1.43µg/ml).

The figure of **36 grams of chlortetracycline/week input** for the production of 300 males/pp/week, for a population of 2000 people, can this be divided by a factor of 21 to give an approximate maximum indication of the expected concentration of chlortetracycline in the output waste water from the rearing facility; i.e. ~ **1.7 grams / week** generated in waste water output.

1.6 Transport and adult release

OX513A male releases are conducted in accordance with **TD-SOP-00295 Releases.** OX513A adult males are transported in release devices, typically with 500 or 1000 males, using an adapted van or open-box truck. A GPS-based system both informs the driver of the route to follow and the points at which to release mosquitoes. While different release methodologies are used in larger scale projects underway (e.g. Brazil), the small scale of the Saba project is amenable to hand release from an open truck or van, similar to **Figure 5**.

A chain of custody protocol would require release devices to be signed out of the MRU, and signed for upon receipt by authorized personnel prior to transport to the release site.

Figure 5. Release devices used in previous OX513A projects; similar equipment is foreseen for Saba

1.7 OX513A program monitoring

Species specific monitoring of *Aedes aegypti* is required in the OX513A program to evaluate the change in the target *Aedes aegypti* population to further inform the adaptive management of release rates and program decisions. Ovitraps are the primary monitoring tool for monitoring changes in *Aedes aegypti* abundance and to assess the mating fraction, while adult traps can be used to provide an additional metric for evaluation local *Aedes aegypti* populations in addition to allowing estimation of the OX513A: local male *Aedes aegypti* ratio. **Section 1.3** provides details on how monitoring information is used to inform

program decisions, while the details of the monitoring activities and description of the metrics are provided below.

The primary focus of monitoring will be for *Aedes aegypti*, however due to the closely related nature and behaviour of *Aedes albopictus*, monitoring tools available for *Aedes aegypti* also generally target *Aedes albopictus* (e.g. ovitraps) where it is present. For operational programs, routine ovitrap monitoring is conducted for both *Aedes aegypti* and *Aedes albopictus* populations. *Aedes albopictus* is not however reported in Saba (see **Appendix 9**).

Depending on the trap type, other mosquito species may be captured and data recorded if deemed relevant to project.

A description of the ovitrap and adult traps used is provided in **OX513A Technical Dossier Part A - Section 6.2.**

Figure 6 provides an overview of the field monitoring process and key SOPs referenced are included in the technical dossier. These SOPs and record keeping documents are listed in **Table 3**.

Table 3. SOPs used in the field monitoring in the OX513A program

Reference number	Title
00027_01	OX513A Identification of Trap Sites
00028_01	OX513A Placing and Collection of Ovitraps
00030_01	OX513A Hatching Larvae from Ovitrapped Eggs
00031_01	OX513A Fluorescence Screening of Larvae
00032_01	OX513A Species screening of ovitrapped larvae
00057_01	OX513A Placing and Collection of BG-Sentinel traps
00058_02	OX513A Identification of BG-Sentinel catches

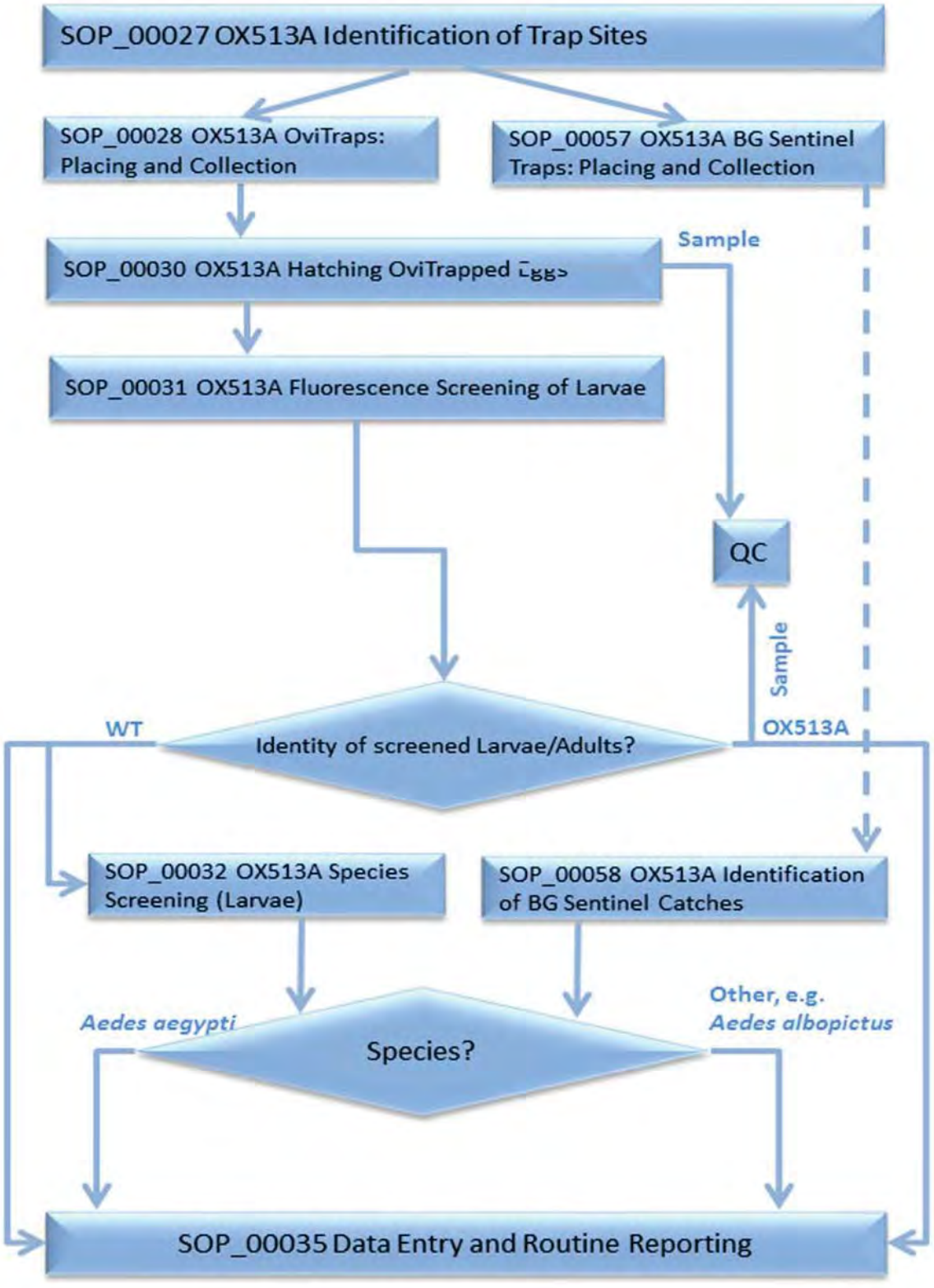

Figure 6. Field monitoring process overview

1.7.1 Ovitrap monitoring

Ovitraps provide an indirect measure of female abundance allowing assessment of the local *Aedes aegypti* population, without interference from released OX513A males. Ovitraps will be used for two distinct parameters:

- Local *Aedes aegypti* Population Monitoring: Provide the initial assessment of the infestation level and subsequently track changes in local *Aedes aegypti* population levels.
- Assess Mating Fraction: Ovitraps will be used to recover eggs from which mating fraction (fraction of local females mating with OX513A/local male *Aedes aegypti*) will be assessed.

1.7.1.1 Ovitrap density, location and servicing

Refer to SOPs;

- **00027_01 OX513A Identification of Trap Sites**
- **00028_01 OX513A Placing and Collection of Ovitraps**

Ovitraps are to be located predominately by domestic dwellings, although non- residential sites such as commercial or industrial buildings may be included. Consent will be sought for the placing and servicing of traps and each trap is identified with a unique code and a location reference by GPS. A minimum number of 30 traps, with a minimum trap density of 30 per Km2 are will be deployed for each assessment area.

Ovitraps need to be serviced at intervals ranging between 6-8 days. At trap servicing the water is topped up/replaced as needed and the oviposition substrate replaced. Alternatively, the entire trap may be replaced. The oviposition substrate is transferred to the laboratory for further processing and analysis. Oviposition substrate will be transported individually in containers designed to stop substrate from different ovipots touching, and therefore mitigate risk of cross contamination. Ovitrap substrate will be labelled with date and receive unique code corresponding to GPS location for trap location allowing spatial and temporal tracking of data.

1.7.1.2 Species Identification

Refer to SOPs:

- **00030_01 OX513A Hatching Larvae from Ovitrapped Eggs**
- **00031_01 OX513A Fluorescence Screening of Larvae**
- **00032_01 OX513A Species Screening of Ovitrapped Larvae**

Ovitraps are selective for clean water container breeding mosquitoes. In some areas this will be limited to *Aedes aegypti*, however other species may be present that lay eggs which cannot easily be distinguished from *Aedes aegypti* (e.g. *Aedes albopictus*). Identification of different species at the egg stage is not feasible, so identification should be conducted on the larval or adult stage.

Eggs collected from ovitraps will be matured, by allowing them to dry at room temperature (20-30°C) for a minimum of 2 days (with upper limit of 14 days) before hatching. Hatch in clean water and maintain, providing food ad libitum (e.g. Tetramin® Fish food), until species identification at larval and/or adult stage using appropriate taxonomic keys (for example http://fmel.ifas.ufl.edu/key/ (accessed 01/06/2016). Larvae positive for expression of the DsRed2 fluorescent marker (**OX513A Technical Dossier Part A - Section 6.1.1**) will be scored as *Aedes aegypti*, without need for further rearing and identification by taxonomy.

1.7.1.3 Species Composition

Ovitrap collections from all sites will initially be processed assuming presence of non-*Aedes aegypti*, with confirmation at species level. A sample of 100 egg positive traps will be assessed, with sampling duration continued until the 100 traps are recovered.

Thereafter, presence of non-*Aedes aegypti* will be checked every 4 months for the first year and annually thereafter; eggs from a minimum of 100 egg positive traps will be reared to larva/adult stage an identified to confirm *Aedes aegypti* is the only species recovered from ovitraps. If non-*Aedes aegypti* are detected, routine screening for species in ovitrap processing is re-initiated as described below in **Table 4.**

Table 4. Recommended trap processing for assessing mating fraction and monitoring local *Aedes aegypti* population abundance.

Assessment Parameter	Trap Possessing; according to species composition	
	Non-*Aedes aegypti* recovered from ovitraps	***Aedes aegypti* only recovered from ovitraps**
Aedes aegypti population	Hatch eggs and positively identify *Ae aegypti* species at larval/adult stage. Count number of *Ae aegypti* larvae recovered from each trap	Hatch eggs. Count number of larvae recovered from each trap (all larvae assumed to *Ae aegypti*)
Mating Fraction	Hatch eggs and screen larvae for fluorescence. Process and identify non-fluorescent larvae to establish ratio of *Ae aegypti* to Non-*Ae aegypti*.	Hatch eggs and screen larvae for fluorescence (no species identification, assume all larvae are *Ae aegypti*)

1.7.2 Ovitrap analysis

Following egg maturation and hatching, ovitrap catches will be processed according to **Table 4**, with species identification in areas where non-*Aedes aegypti* are recovered from ovitraps. Larvae are maintained until positive species identification can be conducted either at late larval stage or as adults using morphological features and taxonomic keys.

The suppression of *Aedes aegypti* will be estimated by the calculation of Ovitrap Index (O_I), and Ovitrap *Aedes aegypti* Abundance (O_A).

Ovitraps are a useful and effective tool for demonstrating the presence or absence of *Aedes aegypti* in the treatment areas and a good indicator in changes of abundance of the mosquito population. As the size of the adult *Aedes aegypti* population decreases, the number of positive ovitraps and number of eggs per ovitrap is likely to decrease as well. Therefore, from the ovitraps the abundance can also be calculated.

1.7.2.1 Ovitrap Index:

$$[1] \quad O_I = \frac{L}{T}$$

Where:

O_I = Ovitrap Index
L = number traps from which one or more eggs confirmed as Ae aegypti
T = total traps recovered

1.7.2.2 Ovitrap *Aedes aegypti* abundance calculation:

$$[2] \quad O_A = \frac{A}{T}$$

Where: O_A = *Ovitrap Ae aegypti Abundance*
A = number of eggs confirmed as Ae aegypti recovered from Ovitraps
T = total traps recovered

1.7.3 Estimating mating fraction

Following egg maturation and hatching, larvae will be visually screened by trained staff for the presence of the fluorescent marker and each larva scored as fluorescent or non-fluorescent.

Refer to SOPs:

- **00030_01 OX513A Hatching Larvae from Ovitrapped Eggs**
- **00031_01 OX513A Fluorescence Screening of Larvae**

1.7.3.1 Mating Fraction calculation – Only *Aedes aegypti* recovered from Ovitraps.

In populations where only *Aedes aegypti* are recovered from ovitraps the Mating Fraction (M) is calculated as the fraction of fluorescent larvae:

$$[3] \quad M = \frac{F}{N + F}$$

Where: M = Mating Fraction
F = Number of Fluorescent larvae
N = Number non-Fluorescent larvae

1.7.3.2 Mating Fraction calculation – Non-*Aedes aegypti* recovered from ovitraps.

In populations where non-*Aedes aegypti* species are determined or suspected to be present the Mating Fraction is calculated as:

$$[4] \quad M = \frac{\mathrm{F}}{\mathrm{F} + \mathrm{N} * \frac{Ae}{Ae + Al}}$$

Where: F = Number of Fluorescent larvae
N = Number non-Fluorescent larvae
Ae = Number of non-fluorescent larvae identified as *Aedes aegypti*
Al = Number of non-fluorescent larvae identified as non-*Aedes aegypti* (e.g. *Ae albopictus*)

1.7.4 Adult Trapping

Direct sampling of adults has the advantage that adults are readily identified enabling species specific differentiation for closely related *Aedes albopictus* and other species. This is contrary to Ovitrap sampling where eggs need to be hatched reared before confirmation of species identification. Adult females caught are easily differentiated from males. The number of female *Aedes aegypti* can be used as metric for assessing the local population of *Aedes aegypti* without interference from released males as OX513A can not easily be visually differentiated from local wild counterparts (Molecular tools are needed as described in **OX513A Technical Dossier Part A - Section 6.1.2**).

Adult trapping will be conducted using the BG-Sentinel trap is described in (**OX513A Technical Dossier Part A - Section 6.2**)

Traps will be located predominately by domestic dwellings, although alternative sites such as schools, shops may also be included. Appropriate consent for the placing and servicing of the traps will be sought. All traps will receive unique number and be georeferenced. A minimum of 15 BG Sentinel traps is anticipated for adult monitoring in Saba.

Traps will normally be installed for the duration of the Intervention Phase as a minimum and will be checked weekly. When serviced, caught mosquitoes are retained for identification. *Aedes aegypti* will be counted and sexed with aid of dissecting microscope. Other species may be identified and counted. Adult abundance will be calculated from adult traps.

2 Receiving environment

A generic description of the habitat and origins of *Aedes aegypti* are provided in ***OX513A Technical Dossier Part A - Section 1 Recipient organism- Aedes aegypti.*** This section provides site specific elements for Saba.

The human population on Saba as of January 2015 as reported by Statistics Netherlands[4] was 1811, and was between 1811 and 1991 in the 5 consecutive years to 2015.

2.1 Geography and Climate

2.1.1 Geography

A general description of the geography of Saba is provided below for the purpose of this technical dossier. Excerpts of text have been taken from the following sources, and have generally been reproduced directly without significant edit:

- The Dutch Caribbean Nature Alliance[5]
- The Dutch Caribbean Biodiversity Explorer[6]

Saba belongs to the group of the Windward Islands of the Dutch Caribbean (17°37' - 17°39' N: 63°13' - 63°15' W). The Windward group is about 900 kilometres north, east of the Virgin Islands, and consists of the islands St. Martin (of which half is French territory), St. Eustatius and Saba (About 18° North and 63° West).

Saba is located about 50 km south of St. Maarten and rises steeply from the sea. Saba is the youngest island of the Windward group and from studies on tree remains, found intercalated in volcanic tuffs, it is known that the volcanic activity stopped only about 500 years ago. Today, warm water springs indicate ongoing magmatic activity and Saba is considered to be a 'sleeping' volcano, rather than a 'dead' one.

The highest point, Mount Scenery, rises 840.4 m above sea level. Weathering and erosion of the old volcano have strongly cut the sides, leading to numerous long radially expiring deep fissures, called "guts". After rainfall water runs rapidly through these guts towards the sea. The seaboard slopes of the volcanic island are steadily undermined and eroded by the sea, and as a result they are very steep. Saba has no natural harbor. Only at a few locations it is possible to land with small boats. Fort Bay and Ladder Bay, respectively to the south and the west coast, are the only natural landing spots on the island. Large ships have to remain several

[4] http://statline.cbs.nl/Statweb/publication/?DM=SLEN&PA=80534eng&D1=0&D2=0&D3=a&D4=3&D5=9-13&LA=EN&VW=T (accessed 01/09/2016)
[5] http://www.dcnanature.org/islands/saba/ (accessed 01/09/2016)
[6] http://www.dcbiodata.net/explorer/info/islands (accessed 01/09/2016)

hundred meters offshore. The other bays are unimportant for shipping and provide only difficult access to the island. Saba has no fully developed beaches.

The vegetation of Saba is mainly composed of woodland forest with ferns and damp soil, and many introduced fruit trees like the mango (*Mangifera indica*). Once there had been forests of mahogany trees (*Swietenia mahagoni)* until a hurricane in the 1960s destroyed most of the trees. The mahogany tree appears to be recovering on the island. Visitors refer to the forests of the top of Mount Scenery as "the Elfin Forest" because of its high altitude mist, dwarfed growth and mossy appearance.

Saba's terrestrial park stretches from Great Hole on the northeastern shoreline and the Pirate Cliffs in the northwest, up to the cloud forest at the peak of Mount Scenery. The park contains everything from arid coastal vegetation to rich cloud forest as well as the culturally important site of the island's former sulphur mine. **Figure 7** shows the boundaries of the terrestrial park and protected zones on Saba.

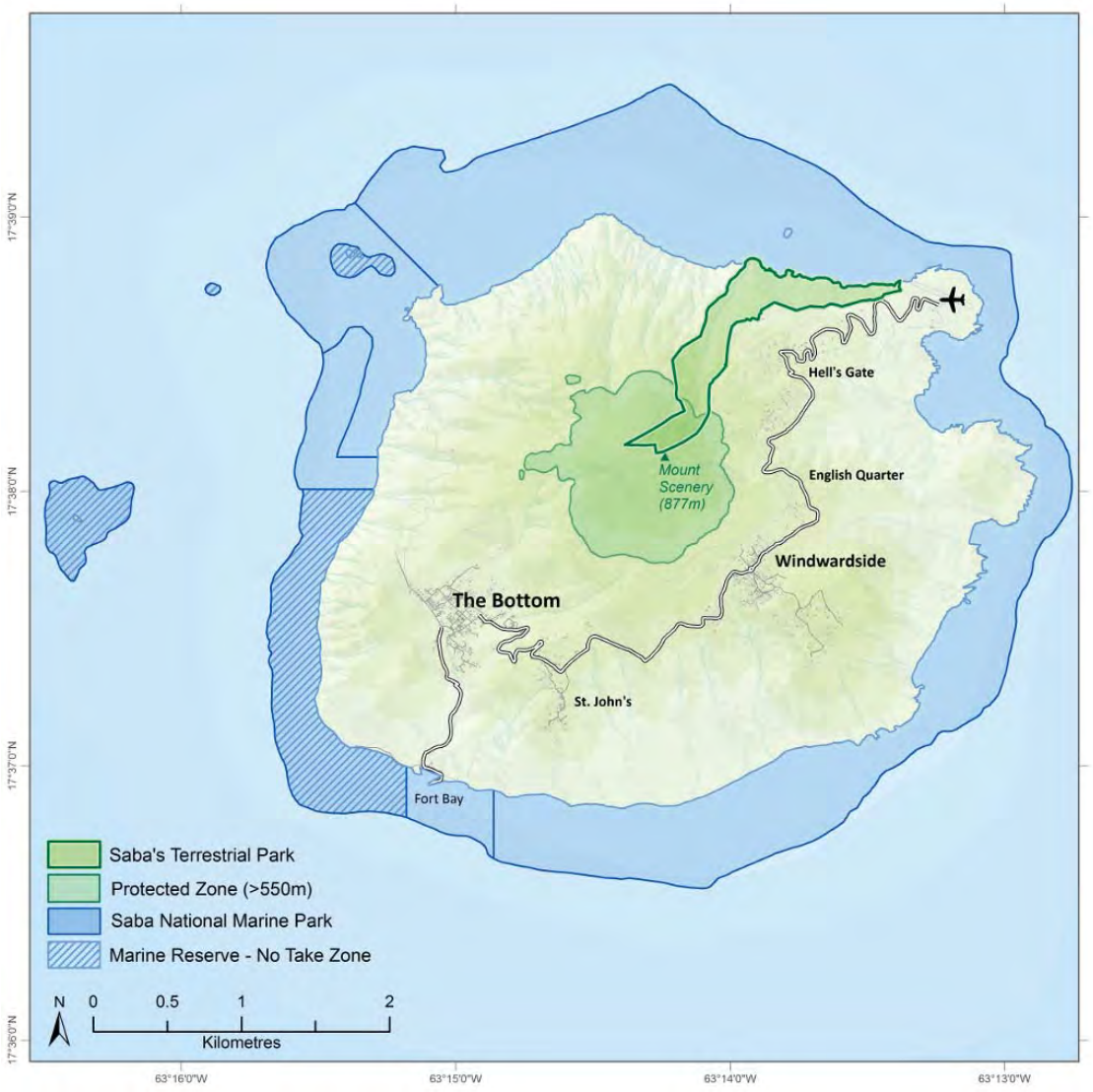

Figure 7. Areas of interest to ecological conservation on Saba reproduced from (http://www.dcnanature.org/islands/saba/) (accessed 01/09/2016)

Comprehensive data sets related to the geology, soil makeup, topography and contour data, roads, hydrology and watersheds, buildings, landcover among other data, are available through the GeoServer[7] platform. GeoServer an open source server for sharing geospatial data and publishes data from any major spatial data source using open standards. Data sets for the Dutch Caribbean islands is maintained by Alterra, Wageningen UR (http://www.wageningenur.nl/en/Expertise-Services/Research-Institutes/alterra.htm) (accessed 01/09/2016)

[7] http://geoserver.org/ (accessed 01/09/2016)

Complete data sets for Saba can be found at (pages 5-6 in Link):

https://dcbd.services.geodesk.nl/geoserver/web/?wicket:bookmarkablePage=:org.geoserver.web.demo.MapPreviewPage (accessed 01/09/2016)

Additional characterisation of the geography, climate, geology and geomorphology, and flora and vegetation is available in ***Appendix 6.1 - A landscape ecological vegetation map of Saba (Lesser Antilles)***

2.1.2 Climate

The Köppen Climate Classification System[8] is the most widely used system for classifying the world's climates. Its categories are based on the annual and monthly averages of temperature and precipitation. The system was first presented in 1900, and further updated in the 1950s, and the classification concept has been broadly applied across disciplines in physical geography, hydrology, agriculture and biology. Kottek *et al (*2006) published an updated digital world map of climate classification based on recent data sets from global climactic research resources.

Under the Köppen system, Saba is classified as type "Am", which is designated "Equatorial monsoon". This may also be referred to commonly as tropical monsoon climate, tropical wet climate or tropical monsoon and trade-wind littoral climate. Equatorial monsoon climates have monthly mean temperatures above 18 °C in every month of the year and feature wet and dry seasons. The driest month sees less than 60 mm of precipitation but *more* than 100 − [total annual precipitation (mm)/25]. See (Peel et al., 2007; Kottek et al., 2006).

The Meteorological Department of Curaçao, had been responsible in recent years, up to January 2016[9] for meteorological services and has produced a climate summary for Saba which captures temperature and rainfall, key abiotic factors for the survival of *Aedes aegypti.* The climate is described as characterized by a relatively dry season (January-April) and a rainy season (August-December) with moderate to fresh east to north easterlies. **Figure 8** is reproduced from the Meteorological Department of Curaçao climate survey of the Dutch Windward islands[10] during the period 1971 - 2000 and illustrates the monthly rainfall and temperatures on Saba. Sint Eustatius and St Maarten are included for comparison as the seasonality of the rainfall of the Dutch windward islands is more pronounced in these neighbouring islands, as local effects are noted to be especially significant on Saba. Average daily high temperatures are generally near 27°C with August as the warmest month. Cooler conditions are common in the higher elevations of the island.

[8] http://koeppen-geiger.vu-wien.ac.at/ (accessed 03/06/2016)
[9] http://meteo.cw/Data_www/pdf/pub/Press%20Release_EndServicesBES.pdf (accessed 01/09/2016)
[10] http://meteo.cw/climate.php?Lang=Eng&St=TNCC&Sws=R11 (accessed 01/09/2016)

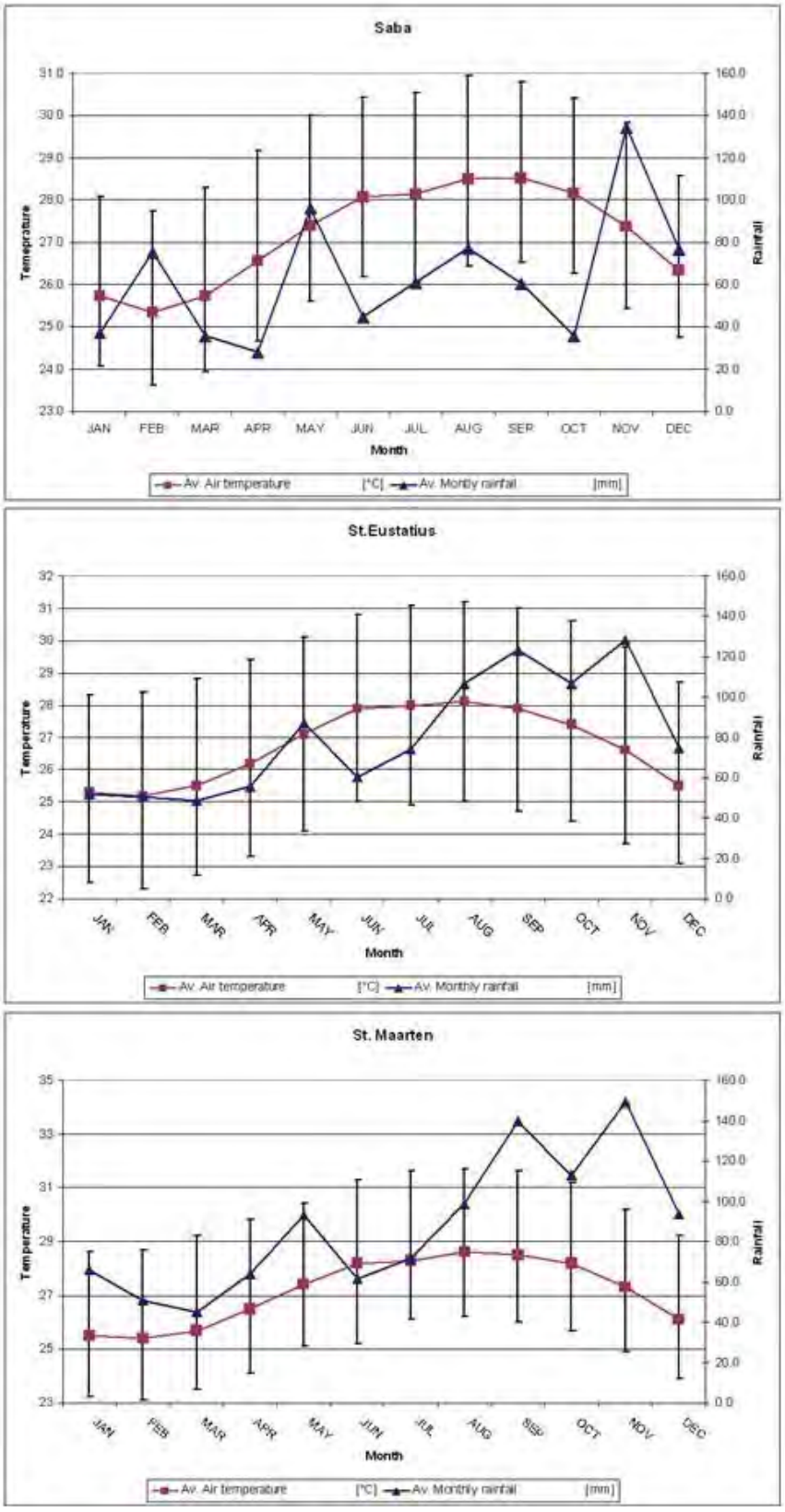

Figure 8. Climate summary of the Dutch Windward islands (The Meteorological Department of Curaçao)

2.1.2.1 Adverse weather

Tropical cyclones are reasonably well documented back to the 19th century, and the history of the tropical cyclones of the Dutch Caribbean up to 2014 has been well described by the Meteorological Department Curaçao. **Appendix *7*** *Hurricanes and Tropical Storms in the Dutch Caribbean* provides a comprehensive history of the tropical cyclones of the Dutch Caribbean as far back as records would allow. In an average year, greater than 100 tropical disturbances with hurricane potential, are observed in the Atlantic, the Gulf of Mexico and the Caribbean, but less than 25 develop into a tropical depression. Of these tropical depressions, fewer than ten reach the tropical storm stage and only about six mature into hurricanes. Saba, St. Eustatius and St. Maarten (SSS) are located within the hurricane belt and almost every year at least one tropical cyclone occurs within a range of 100 miles of the SSS islands and on the average once every 4-5 years hurricane conditions are experienced. The most recent hurricanes to cause considerable damage to the islands were the hurricanes Omar (2008), José (1999), Lenny (1999), Georges (1998), Luis (1995), Marilyn (1995), Hugo (1989), Donna (1960) and Dog (1950).

While flash flooding is expected to be associated with the rains experienced in hurricane conditions, it has been noted (***Appendix 6.1*** pg 40) that extensive prolonged flooding was experienced in Saba associated with hurricanes Eloise (1975) and Frederic (1979).

Although the Meteorological Department Curaçao had been responsible in recent years for disaster preparedness organization in the Dutch Caribbean as outlined in **Appendix 7**, from January 1, 2016, the Royal Netherlands Meteorological Institute (KNMI) has been responsible for Bonaire, St. Eustatius and Saba to prepare the weather forecasts and warnings for the general public as well as services to the aviation and marine sectors in Bonaire.

In the event adverse weather is anticipated steps will be followed as described in a site specific Hurricane Preparedness Policy for Saba. Oxitec SOP ***00054_01 OX513A*** *Hurricane Preparedness Policy* outlines steps identified for a proposed trial in the USA, and similar principles will be implemented for the MRU in Saba once installation is finalised.

2.2 *Aedes aegypti* in Saba

2.2.1 Habitat of *Aedes aegypti* in Saba

Aedes aegypti is a peri-domestic species closely associated with human habitations and has been well described in this regard (**Oxitec Technical Dossier Part A - Section 1** Recipient Organism- *Aedes aegypti*). Breeding is tied to artificial water containers, such as potted plant holders, water tanks, tires, discarded plastic and metal containers such as soda cans, drains and roof guttering as well as ephemeral containers, such as puddles. Once eclosed the adult *Aedes aegypti* mosquitoes live in and around houses where females have easy access to the blood meal necessary for egg development. The “domestication” of *Aedes aegypti* is well

described. (Powell and Tabachnick, 2013) provide a review of the history of domestication and spread of *Aedes aegypti.*

In Saba, quality drinking water is supplied by desalination facilities in Fort Bay and is considered costly. Rain water collection in urban areas means that there is an abundance of cisterns serving both private households and larger shared public areas. Local vector control services have identified cisterns as principal *Aedes aegypti* breeding sites and vector control activites target cisterns among other water containers (Saba vector control team personal communication). The *Aedes aegypti* habitat on Saba thus has the unique addition of cisterns, not typically as abundant in other urban areas with less costly municipal water supply. See https://www.facebook.com/sabapublichealth/ video posting April 5th, 2016- (accessed 01/09/2016) for a description of *Aedes aegypti* habitat on Saba by the Head of the Department of Agriculture, Hygiene and Vector Control Randall Johnson.

Figure 1 - Section 1.2 provides an overview of the main urban areas in Saba which represent the primary habitat for *Aedes aegypti.* The area noted is likely an overestimate as population levels of *Aedes* across the four key communities on Saba were recorded in late 2013/2014 as part of an assessment of the strategies in place on Saba for larval source management. **Appendix 8** *Final Report of the Application stage of the Biological Vector Control Project in Saba Island (Dutch Caribbean)* highlights the differences in *Aedes* population across communities, with Zion's Hill (denoted Hill gate in the report) having a consistently lower *Aedes* population count as measured by the three parameters; The Breteau Index (BI) which is the number of positive containers per 100 houses inspected; the Household Index (HI) which is the percentage of houses that are positive in the test area, and; the Water Deposit Index (WDI) which is the percentage of water deposits (i.e. Cisterns) positive in the test area.

Local vector control officials noted in a site visit in early 2016, that area of Zions Hill is subject to higher winds than other areas on the island and it was suggested that the constant winds, and relative geographical isolation from the other communities may serve as a barrier to migration from other communities on Saba with higher *Aedes* populations.

2.2.2 Functions of *Aedes aegypti* in the ecosystem of Saba

As has been well described, *Aedes aegypti* is considered an invasive species globally apart from its origins in Sub-Saharan Africa (**OX513A Technical Dossier Part A - Section 1**). For Saba the *Aedes aegypti* has been identified as invasive and a high priority for both control and research, with good prospect for control by the Ministry of Economic Affairs, Agriculture and Innovation of the Netherlands; the Institute for Marine Resources & Ecosystem Studies (IMARES) was contracted to produce the 2011 document *Introduced agricultural pests, plant and animals diseases and vectors in the Dutch Caribbean, with an "Alert species" list* (**Appendix 9).** A total of a total of 47 species including molluscs, diplopods, fungi, ants, cockroaches, ticks and mites, mosquitoes, moths, weevils, among other insects, and an additional 21 "Alert Species" are identified which pose a risk to the economy and ecology.

For *Aedes aegypti* the report highlights in its summary: "*By far the most economically costly invasive species is the yellow fever mosquito Aedes aegypti, a pest and disease vector closely associated with man. In a few cases, biological control and eradication has been successful."*

Invasive species is defined in the IMARES report as *"Invasive species are non-indigenous species (or exotic species) introduced by historic human actions, whose introduction causes, or is likely to cause, economic or environmental harm or harm to human health."*

Aedes aegypti is not native to Saba and existing interventions attempt to control *Aedes aegypti* populations through biological control approaches involving the use of larvivorous fish in breeding sites, as well as the application of products based on *Bacillus thuringiensis*. Suppression or elimination of the wild *Aedes aegypti* population is thus consistent with current protection goals for vector control, and is thus not expected to alter population dynamics of non-target organisms as it is not a keystone species in the local food chain.

2.3 Flora and Fauna

The most recent comprehensive systematic review of the flora and fauna of Saba can be found in **Appendix 11** *Biological Inventory of Saba- 1997.*

520 species of plant are identified which is practically the same number of species of wild plants as the much larger island of St. Maarten.

In contrast Saba's fauna is noted as having relatively few species and among the vertebrates, birds form the largest group, represented by 26 local and breeding species. Terrestrial species are more likely to have habitat overlap with *Aedes aegypti* and include the Green-throated Carib (*Eulampis holosericeus)* and the Antillean Crested Hummingbird (*Orthorhyncus cristatus*), The Scaly-breasted Thrasher (*Allenia fusca*), the Pearly-eyed Thrasher (*Margarops fuscatus*) the Trembler *(Cinclocerthia spp.)*, the Lesser Antillean Bullfinch (*Loxigilla noctis*), the Blue-crowned Euphonia (*Euphonia occipitalis*), The Zenaida Dove (*Zenaida aurita*) and Common Dove (*Columbina passerina*), Bridled Quail Dove *(Geotrychon mystacea)* and the Red-necked Pigeon *(Columba squamosa),* the Red-tailed Hawk *(Buteo jamicensis jamaicensis),* the American Kestrel *(Falco sparverius caribaearum).*

Five species of Seabirds are reported to nest in Saba, and additionally 36 migrating species are reported as present every year on a temporary basis. A large number of these migratory species have broad distributions as such, the majority of these species are classed as Least Concern according to the International Union for Conservation of Nature (IUCN). There is little opportunity for *Aedes aegypti* to overlap with birds which live and feed on the islands coast, *as Aedes aegypti* lives and breeds in fresh water held in artificial containers in and around human habitation and is highly unlikely to be present near saline waters. The potential exists for incidental aerial exposure during periods of OX513A release near coastal areas such as in the vicinity of the Airport, but this scenario seems highly unlikely as sea birds would need to

fly through a swarm of released OX513A prior to the released mosquitoes finding their preferred resting habitat.

Bats are the only mammals on Saba that were not introduced by humans. Five species have been reported: the St.Vincent Fruit-eating Bat *(Brachyphylla cavernarum)*, Free-tailed Bat *(Tadarida brasiliensis antillularum)*, Mastiff bat *(Molossus molossus debilis),* Mexican Funnel-eared Bat *(Natalus stramineus stramineus)*, and the Jamaica Fruit-eating Bat *(Artibeus jamaicensis).*

Amphibians and reptiles are the second largest group of vertebrates with 10 species of reptile, and one amphibian (the piping frog *Eleutherodactylus Johnstonei*). There is one island endemic among the vertebrates: the lizard *Anolis sabanus*. One species, the Red-bellied Racer *Alsophis rufiventris* is limited to Saba and neighbouring St. Eustatius and is listed on the "Red List of Threatened Animals" of the IUCN.

An analysis of protected, charismatic and valued species that may occur in the release areas on Saba has been conducted using detailed online searches from source such as the IUCN Red List[11] as well as biological inventory reports from the Caribbean Research and Management of Biodiversity foundation[12], and information from the Dutch Caribbean Nature Alliance[13]. IUCN searches were thus applied broadly to include near threatened, vulnerable, endangered, and critically endangered species, found in terrestrial, and relevant freshwater[14] habitats identified from 1996 to 2014. Results are summarised in **Table 5**. Only generalist insectivores, with potential habitat overlap, which have been included in the IUCN red lists that could act as predators have been listed. While a comprehensive IUCN Redlist review for the Netherlands is available in the 2013 publication *The Netherlands' biodiversity at risk*[15], and provides an overview of the conservation status of species in the Netherlands, the geographical scope does not include the Dutch Caribbean.

[11] http://www.iucnredlist.org/search (accessed 01/09/2016)
[12] http://www.carmabi.org/ (accessed 18 January 2016)
[13] http://www.dcnanature.org/ (accessed 01/09/2016)
[14] Excludes fast moving water such as cascades and streams as these are not habitat for *Ae. Aegypti.*
[15] https://cmsdata.iucn.org/downloads/netherlands_biodiversity_at_risk_fact_sheet_may_2013.pdf (accessed 01/09/2016)

Table 5. Summary of protected, charismatic or valued species present in Saba and classified by the International Union for Conservation of Nature (IUCN) classifications, Near Threatened, Vulnerable, Endangered, or Critically Endangered, and potential for exposure to OX513A in the receiving environment.

Animal	Habitat/Distribution	IUCN level	Potential exposure[16]
Red-bellied Racer (*Alsophis rufiventris*)	Exclusively on Saba and Sint Eustatius. Favours rocky environments offering plenty of refuge which are also the preferred habitat of prey species. Known to predate upon young rats and small lizards. It is most active during the morning and late afternoon sliding between rocks and through leaf litter, with a lull around midday to avoid the high temperatures. Rear-fanged and are known to use venom to subdue their prey. Individuals have also been observed consuming lizard eggs, probably belonging to the same Anolis lizards which they prey upon.	Endangered (1996)	Negligible potential for exposure to OX513A due to and non-insectivorous feeding habits. A low level of habitat overlap may exist in small freshwater or rainwater pools in close proximity to human habitation
Saban Anole (*Anolis sabanus*)	Sabas only true endemic species may be considered rare because of its limited geographical distribution. Within Saba it is widespead and common, found in all terrestrial habitats and at all altitudes feeding mostly small insects. In towns, it usually rests on warm rocks and on walls of houses, while in forest areas it is typically found perched on trees and leaves	Not Listed	Moderate potential for exposure to OX513A due to. habitat overlap around human habitation and opportunistic feeding

The *Biological Inventory of Saba* also makes reference (pg 38) to additional sources for other animals reported to be present on Saba. The Exotic Species Ordinance Saba AB2000 (**Appendix 10**) which was enacted in the past to serve the purpose of "*protection of indigenous flora and fauna, to establish regulations concerning the import and introduction of exotic species of flora and fauna*", establishes the *Biological Inventory of Saba* as the reference for native species protected under the ordinance. Thus for the purpose of this technical dossier the *Biological Inventory of Saba* has served as the principle reference in defining the flora and fauna of Saba.

[16] See Table 2 Section 1.3 Part C- *OX513A Environmental Risk Assessment* for definitions

2.3.1 Insects

While comprehensive assessments of the conservation status all European butterflies[17] , and Dragonflies[18] have been undertaken independently following the Red List methodology developed by the International Union for Conservation of Nature (IUCN), these assessments have not included the Dutch Caribbean within the geographical scope.

The *Biological Inventory of Saba- 1997- Appendix IV therein* provides a list of the Butterflies identified on Saba, and additionally makes reference to external sources for reference on insect species in Saba generally. References provided below for convenience and all references for *Studies on the Fauna of Curaçao and other Caribbean Islands* are accessible at the following:

http://repository.naturalis.nl/cgi/b/bib/bib-idx?c=naturalis;size=10;type=simple;rgn1=journal;q1=Studies%20on%20the%20Fauna%20of%20Cura%C3%A7ao%20and%20other%20Caribbean%20Islands (accessed 01/09/2016)

70 insect species:

Weber, N.A., 1948. Ants from the Leeward Group and some other Caribbean Localities. Stud.Fauna Cur.& Car.Isl. III: 78-88.

Kuyp, E. v.d., 1953. Culicinae from the Netherlands Antilles and some other Caribbean Localities. Stud.Fauna Cur.& Car.Isl. IV: 144-148.

Kuyp, E. v.d., 1954. Mosquitos of the Netherlands Antilles and their Hygienic Importance. Stud.Fauna Cur.& Car.Isl. V: 37-114.

Cobben, R.H., 1960. The Heteroptera of the Netherlands Antilles-I. Forword. Gerridae, Veliidae,Mesovelliidae (Waterstriders).-II. Saldidae (Shorebugs). Stud.Fauna Cur.& Car.Isl. XI: 1-97.

Cobben, R.H. & D. Wygodzinsky, 1975. The Heteroptera of the Netherlands Antilles- IX. Reduviidae (Assasin Bugs). Stud. Fauna Cur.& Car.Isl. XLVIII: 1-62.

Drake, C.J. & R.H.Cobben, 1960. The Heteroptera of the Netherlands Antilles -V. Tingidae (Lace Bugs). Stud.Fauna Cur.& Car.Isl. XI: 67-97.

Forrest Gilmour, E., 1963. Some Caribbean Coleoptera Carambycidae. Stud.Fauna Cur.& Car.Isl. XVIII: 75-102.

Marcuzzi, G., 1977. Further Studies on Caribbean Tenebrionid Beetles. Stud.Fauna Cur.& Car.Isl. LII: 1-71.

Marcuzzi, G., 1962. Tenebrionid Beetles of the West Indies. Stud.Fauna Cur.& Car.Isl. XIII: 21-48.

Doesburg, P.H., 1970. Records of Surphidae (Diptera) from the Lesser Antilles. Stud.Fauna Cur.& Car.Isl. XXXIV : 90-101.

[17] http://ec.europa.eu/environment/nature/conservation/species/redlist/downloads/European_butterflies.pdf (accessed 01/09/2016)

[18] http://ec.europa.eu/environment/nature/conservation/species/redlist/downloads/European_dragonflies.pdf (accessed 01/09/2016)

Simonthomas, R.T. 1984. Notes on the Hymenoptera Aculeata from St. Martin, Saba and St. Eustatius. Stud.Fauna Cur.& Car. Isl. LXVII: 92-97.

Stusák, J.M. & R.H. Cobben, 1975. The Heteroptera of the Netherlands Antilles - X Berytinidae (Stilt Bugs). Stud.Fauna Cur.& Car.Isl X 63-78.

Gillett and Gillett, (2015) conducted a survey of the Dynastinae (Rhinoceros beetles) of Saba over several years and across all months and identified three species: *Cyclocephala mafaffa* Burmeister; *Tomarus cuniculus* (Fabricius); and *Phileurus valgus antillarum* Prell

2.3.2 Other organisms

Slowik and Sikes, (2011) conducted a survey of the spiders of Saba and identified 18 families, representing 76 species, of which, six were undescribed, and 27 could only be identified to generic or family level. A high species richness on Saba was noted. Spiders consume their prey using venom to paralyze their victims. Spider venom usually begins dissolving the prey from inside so the spider can simply suck up the liquefied creature.

2.4 Tetracycline in the environment in Saba

The primary routes of exposure to tetracycline's in the environment in general are well defined and are briefly discussed below to provide context for the proposed release on Saba.

In an agricultural setting, the most likely sources of tetracycline are from application of manure contaminated with tetracyclines used in prophylactic or therapeutic veterinary applications (Martinez-Carballo et al., 2007; Kim et al., 2011). There is no large scale agriculture on Saba currently principally due to the geology of the island as it has been noted (**Appendix 11**) that historically only ~216 of the 1300 hectares were usable for agriculture and cattle breeding. **Figure 9** shows the distribution of land use in 1980, and agriculture has diminished since that time Local agriculture played a more prominent role locally in the past, predominantly as subsistence farming, up to the 1970's, and efforts are underway to re-invigorate the sector locally (***See Appendix 13 Reforming Saba's agricultural sector for increased food-security***).

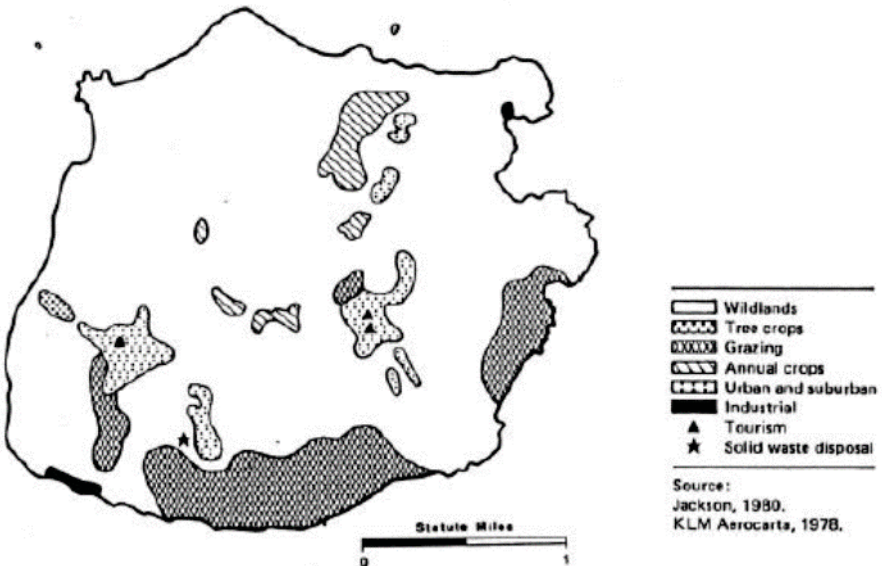

Figure 9. Reproduced from *The Biological Inventory of Saba* (Appendix 11). Land use distribution map from 1980 showing land use at the time.

Although there is no large scale agriculture on Saba, with much of the food imported, fruit trees exist on the island, along with small cohorts of chickens as well as companion/feral populations of animals (dogs, cats). The use of tetracycline's in companion and other animals maintained by humans may contribute to tetracycline in the in the environment through shedding in urine and faeces. The absorption rate of the antibiotics within the animals is known to be small with up to 72 % of the antibiotic being excreted in faeces and urine within 2 days of antibiotic application (Kim et al., 2011).

As well, direct application of antibiotics to crop plants is permitted as a treatment for bacterial infections in various jurisdictions around the world, however residue limits are generally in place and application is only permitted on a small number of crops (Maia et al., 2009). There are a number of pesticides that contain tetracycline as active ingredients in small amounts mostly in the form of oxytetracycline. This antibiotic controls bacteria, fungi, and mycoplasma-like organisms including Erwinia amylovora or Fire Blight. Oxytetracycline is predominantly used on pear trees however other crops that can be treated with antibiotics include peaches, nectarines, and apples. Use of tetracycline complexes on apples has been approved under emergency exemption where streptomycin resistant strains of E. amylovora have been documented (McManus and Stockwell, 2000). This pesticide is also registered for use on forest trees such as Elm and ornamental trees especially palms, shrubs, and vines.

Another potential source of antibiotics in receiving environments generally is via the presence of waste water (e.g. irrigation, sewage) which has been contaminated with veterinary or human therapeutic tetracyclines (Sarmah et al., 2006). A review of environmental antibiotic degradation indicated that in general the highest sources of environmental tetracylines (in the µg/L range) were from hospitals and municipal wastewater, whereas surface waters, sea and ground waters were in the ng/L range (Homem and Santos, 2011). Waste water

treatment plants can reduce the amount of tetracycline present in the effluent as part of the treatment process (Brown et al., 2006; Gulkowska et al., 2008). Tetracylines are well known to degrade rapidly in sunlight (photolysis) in the presence of catalysts (iron and hydrogen peroxide, both of which can occur naturally in sunlit water) where degradation of tetracycline was complete after 1 minute (Bautitz and Nogueira, 2007). The rate of degradation is dependent on the initial concentration and the pH of the water. It is also reported that in natural water samples the rate of photo -degradation is higher than in pure waters due to aquatic matrix effects (López-Peñalver et al., 2010). Homem and Santos (2011) report that with tetracylines over 80% reduction can be rapidly achieved by photo-degradation using advanced oxidation processes (1 -300 minutes depending on whether a catalyst was used and the pH of the reaction). These data have largely been generated from examination of tetracycline levels from wastewater treatment plants and their downstream flow as they are expected to have particularly high levels, along with the efficiency of removal of tetracylines during treatment.

Additionally, Curtis et al. (2015) reports water samples were collected from *Aedes aegypti* breeding sites in Brazil to determine the environmental concentrations of tetracyclines that OX513A larvae are likely to encounter in the field. Water sampling sites were selected based on their potential to contain high concentrations of tetracyclines (close to sewage plants or intensive livestock operations) or for being an *Aedes aegypti* larval habitat, as determined by the presence of larvae. The concentration of each tetracycline tested; tetracycline, oxytetracycline and chlortetracycline, was below the limit of quantification for each of the field samples. The limit of detection was 1.0 pg mL-1 for tetracycline and chlortetracycline and 2.5 pg mL-1 for oxytetracycline.

The likelihood of residual tetracycline's in the environment currently attributable to the agriculture sector can thus be considered negligible. Additionally, due to the steep and rocky nature of Saba and the watershed patterns[19], it is unlikely that agricultural run-off, if it were to exist, would accumulate to any degree.

As the waste treatment system in Saba is essentially via private cesspits in residential areas there is the possibility that individuals receiving therapeutic doses of antibiotics may contribute to isolated concentrations of tetracycline's in individual residential cesspits. The A.M. Edwards Medical Center hospital on Saba in The Bottom could be expected to generate waste containing a higher concentration of tetracyclines than private residences, however waste cesspits are not the preferred habitat of *Aedes aegypti,* as it is well characterised as preferring clean standing water in an around human habitation (**OX513A Technical Dossier Part A -** ***Section 1 Recipient organism- Aedes aegypti***). The possibility of damaged or cracked septic tanks or covers on cesspits does exist and reports have suggested that *Aedes aegypti*

[19] https://dcbd.services.geodesk.nl/geoserver/web/?wicket:bookmarkablePage=:org.geoserver.web.demo.MapPreviewPage (accessed 01/09/2016)

can breed in septic tanks, usually where they are cracked or broken (Mackay et al., 2009); (Barrera et al., 2008) but this tends to be in the clear water at the top of the tank, whereas tetracyclines tend to bind to the sediment which collects at the bottom (Watkinson et al., 2009); (Brown et al., 2006) (thereby making any tetracycline's less accessible in the clear surface layer of water).

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OXITEC

OX513A Environmental Risk Assessment

Part C - Prepared for the release of OX513A *Aedes aegypti* in Saba in accordance with the provisions of Directive 2001/18 EC – Annex II

Submission to the GMO Office of the National Institute of Public Health and the Environment of the Netherlands (RIVM) for the technical evaluation of the release of *Aedes aegypti* OX513A in Saba.

Companion to *OX513A Technical Dossier- Part A and Part B*

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Introduction

This environmental risk assessment (ERA) has been carried out consistent with the general principles and methodology as described in Annex II to Directive 2001/18/EC of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms. Additional direction has been taken from Commission Decision of July 24, 2002, establishing guidance notes supplementing Annex II to Directive 2001/18/EC.

Interpretation specific to genetically modified insects has been taken from the EFSA publication *Guidance on the environmental risk assessment of genetically modified animals* (EFSA, 2013), the ESFA technical report *Defining Environmental Risk Assessment Criteria for Genetically Modified Insects to be placed on the EU Market* (Benedict et al., 2010), as well as the EFSA Scientific Opinion *Guidance on the environmental risk assessment of genetically modified plants* (EFSA, 2010).

Risk assessment methodologies, such as the ones developed by the Australian Office of the Gene Technology Regulator (OGTR, 2013) are also considered. Through the problem formulation step, potential hazards and pathways to exposure may be identified and systematically evaluated against protection goals. The process of developing a risk hypothesis, and assessment and measurement endpoints, in most cases reveals that there is adequate information to formulate a conclusion of negligible risk, and that risk characterisation for the particular hazard or pathway to exposure is complete.

The scope of this ERA is for the deliberate environmental release of OX513A in the context of a vector control project for *Aedes aegypti* in Saba, a special municipality of the Netherlands.

1. Approach of the environmental risk assessment

Environmental risk assessment (ERA) in the present context, forms part of the overall risk analysis process in order to make informed decisions regarding the deliberate release into the environment of OX513A. The ERA is carried out using published data, study reports and other data generated through evaluations both in the laboratory and in contained use, and through regulated environmental releases. Additionally, scientific literature reviews and independent expert analysis have been considered in order to develop scientifically sound rational in the overall assessment of risk.

A structured and systematic approach has been taken following the six steps of ERA described in Directive 2001/18 EC to enable an individual case assessment of the potential effects of the deliberate release into the environment of OX513A. The six steps of ERA are represented in **Figure 1**.

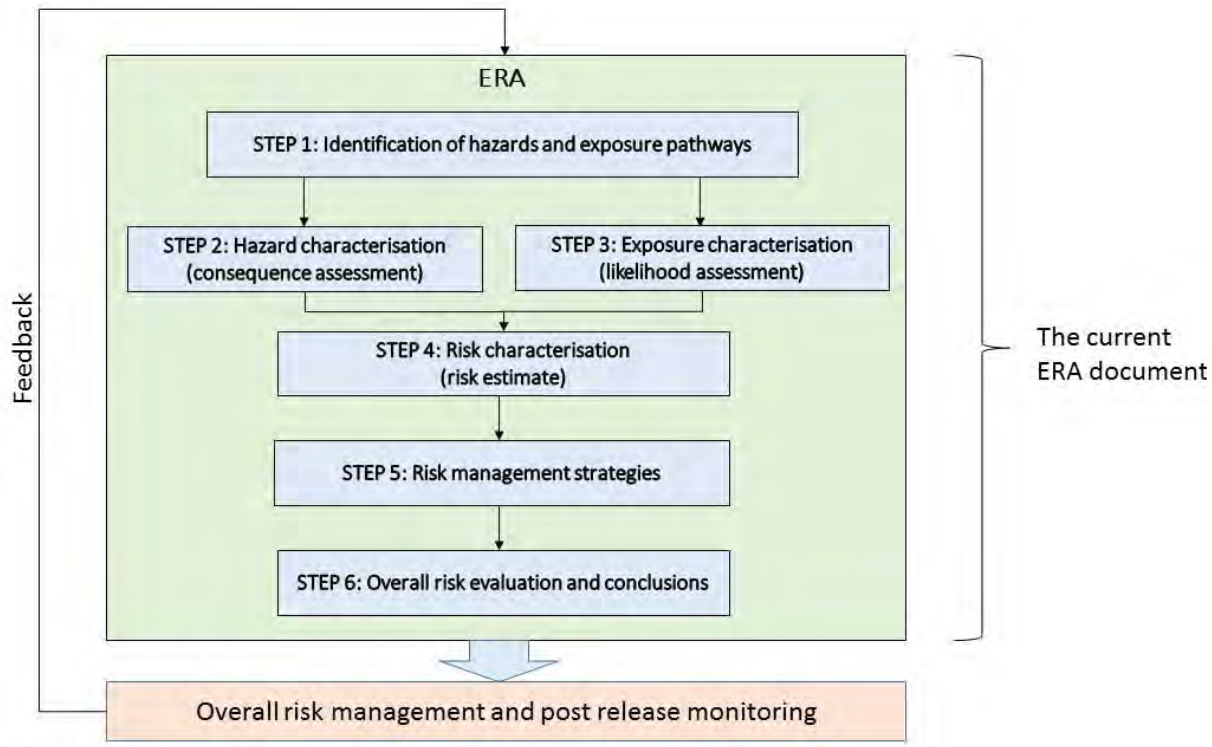

Figure 1. Six steps of environmental risk assessment (ERA) as presented in (EFSA, 2013) and interpreted from Directive 2001/18/EC. The relationship to risk management and post release monitoring are presented. Steps 2, 3 and 4 include in parenthesis the corresponding terminology presented in section 2.4 taken from the *Australian Office of the Gene Technology Regulator - Risk Analysis Framework (OGTR, 2013).*

Using the six steps of the ERA, areas of risk identified in Directive 2001/18/EC Annex II D1 have been evaluated for OX513A, as interpreted in the EFSA guidance for GM insects (EFSA, 2013) by means of seven specific risk areas:

1. Persistence and invasiveness of GM insects, including vertical gene transfer
2. Horizontal gene transfer
3. Pathogens, infections and diseases

4. Interactions of GM insects with target organisms
5. Interactions of GM insects with non-target organisms
6. Environmental impact of the specific techniques used for the management of GM insects
7. Impacts of GM insects on human and animal health

In addition, generic cross-cutting considerations have been taken into account throughout the ERA as appropriate and may include: receiving environments, experimental environment, the choice of comparators, use of non-GM surrogates, statistics and experimental design, long-term effects, modelling, and uncertainty analysis. It should be noted that much of the EFSA 2013 guidance has been conceived with the environmental risk assessment of insects for commercial releases, and that smaller scale demonstration projects will have different considerations. Additionally, the EFSA 2013 guidance considers GM insects broadly, encompassing potential applications for insect vector control, agricultural pest management as well as the enhancement of production systems (e.g. honey bees, silk worms). Within the category of insect vector control, which is the scope of application for OX513A, EFSA (2013) further identifies potential applications using GM insects for both population suppression or population replacement. OX513A is designed to be self-limiting in the environment as it is deployed to effect population suppression of the local wild *Aedes aegypti* population. The self-limiting trait is non persistent by design (Alphey, 2014), thus elements of EFSA (2013) which broadly consider all GM insect applications must be balanced within the context of the intended non-persistent design of the self-limiting trait in OX513A to assess their applicability. **Figure 2** has been adapted from the EFSA 2013 guidance and represents how the current ERA document has been structured in order to systematically ensure appropriate coverage of the requirements of Directive 2001/18/EC Annex II.

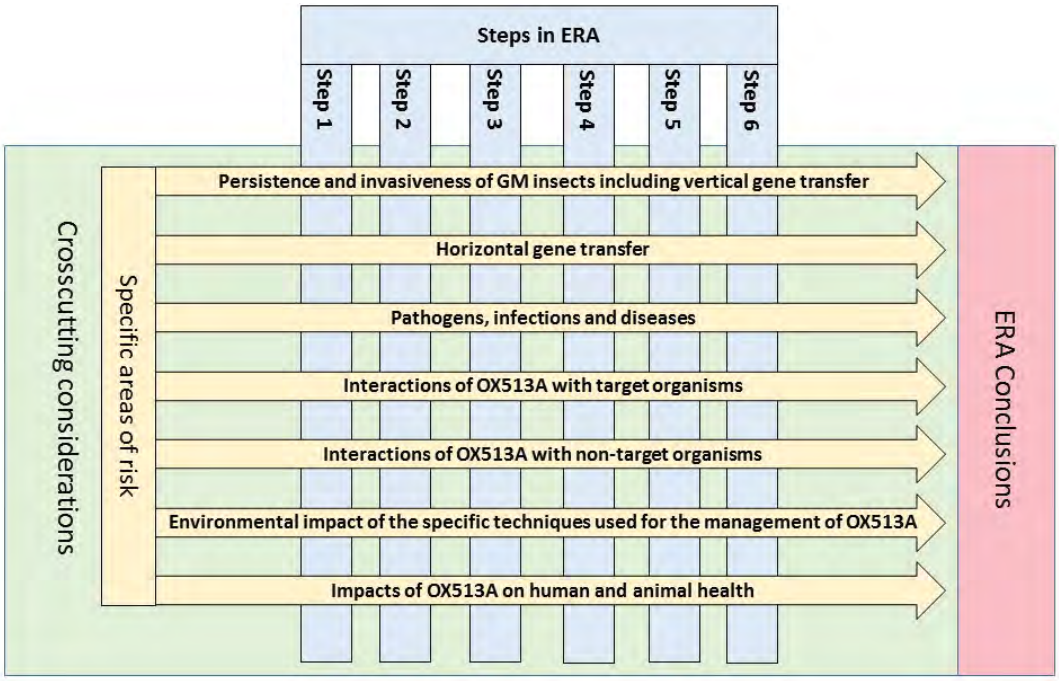

Figure 2. Structural representation of the ERA and inter-relation between the different elements.

1.1 Problem formulation: Identification of hazard and exposure pathways.

Problem formulation is a systematic and transparent methodology for identifying potential hazards, and potential exposure pathways which could ultimately lead to harm being caused to the entities that require protection. The problem formulation provides a direction for the risk assessment and indicates where further data may be required. It is used in a variety of risk assessment scenarios such as for chemicals and genetically modified crops.

A hazard is defined for the purpose of this ERA consistent with EFSA glossary- taxonomy terms[1] and is *a substance or activity which has the potential to cause adverse effects to living organisms or environments.* This is consistent with the EFSA Scientific Opinion on Risk Assessment Terminology (EFSA, 2012), which in comparing definitions from international standard setting organisations (CAC, OIE, IPPC) identify the common element in defining a hazard is something that has *a potential to cause an adverse health effect or to be injurious to target populations*. A hazard thus considers the characteristics of the potential adverse effect, independent of the likelihood of exposure. Whereby harm in the context of this ERA may be defined as an *adverse outcome or impact* (OGTR, 2013) and accounts for the exposure pathway.

A problem formulation has been carried out as Step 1 in each of the seven specific areas of risk in accordance with Annex II of Directive 2001/18 EC.

In order to evaluate the magnitude of potential harm, they must be linked to assessment endpoints, derived from protection goals in the receiving environment. Assessment endpoints allow the formulation of a risk hypotheses, which can be tested through a systematic, methodological approach.

The broad protection goals and selected assessment endpoints which have been evaluated have been selected considering the EFSA 2013 ERA guidance. These protection goals are;

1. Protection of human and animal health - ensuring that humans or non-target animals are not harmed by the deliberate release of OX513A.
2. Protection of biodiversity and ecosystem services - ensuring that irreversible harm, or harm that cannot be mitigated does not occur due to the deliberate release of OX513A to:
    a. Populations of charismatic or protected species which are likely to inhabit or have overlapping habitat with the areas where OX513A deliberate releases will take place.
    b. Populations of key species that are the sole providers of, or key contributors to, ecosystem services in the areas where OX513A deliberate releases will take place.

Broad protection goals are examined individually in the context of each of the specific areas of risk and ultimately refined into assessment endpoints. A risk hypothesis may then be formulated, and tested towards defined measurement endpoints, which are preferably quantifiable, and which can act as indicators of change, and thus measures of hazard and

[1] https://www.efsa.europa.eu/en/glossary-taxonomy-terms (accessed 07/09/2016)

exposure. **Figure 3** illustrates this principle with a theoretical example for a generic GM insect examined under the persistence and invasiveness area of risk.

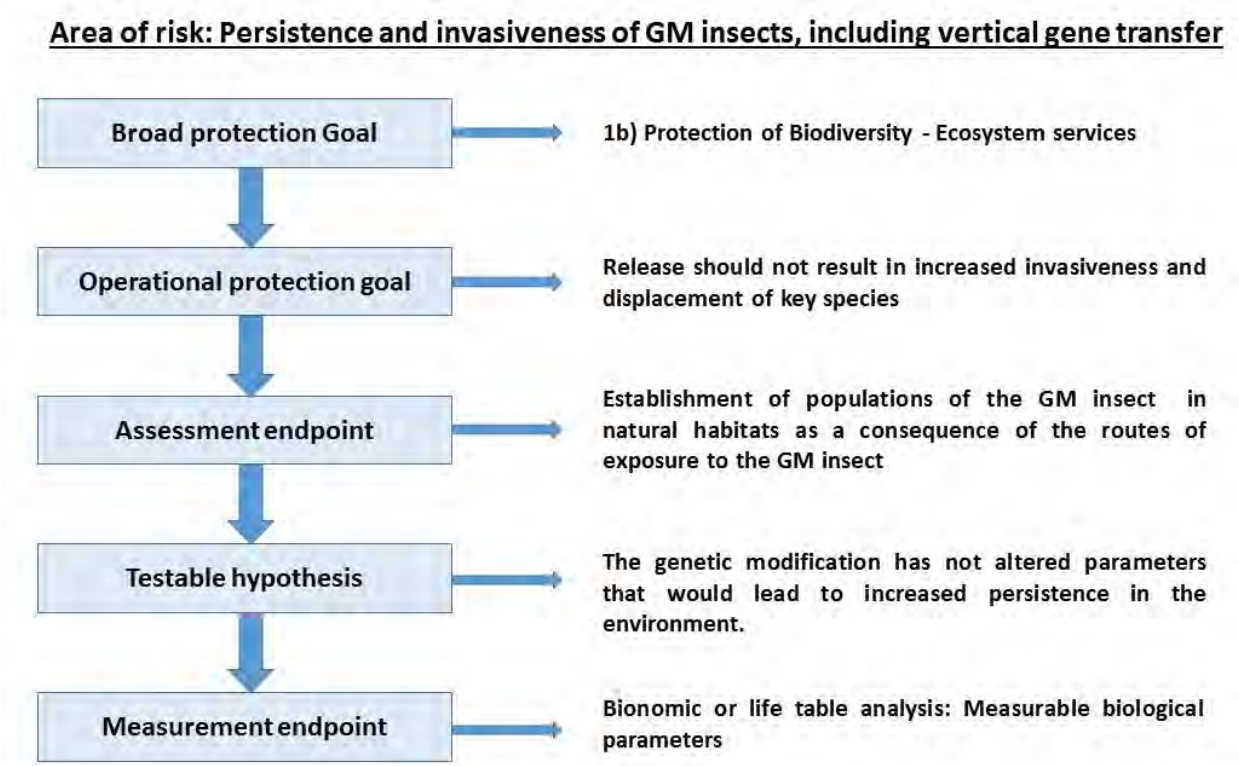

Figure 3. Process of deriving measurement endpoints from broad protection goals in risk assessment. Theoretical example provided for a generic GM insect in the defined risk area of persistence and invasiveness.

The overarching risk hypotheses in this assessment for OX513A is that there are no unmanageable adverse effects to human health, animal health, and the environment associated with the deliberate release of OX513A relative to an appropriate comparator. The appropriate comparator depends on the particular assessment endpoint identified.

Through the problem formulation (Step 1), potential hazards and pathways to exposure may be identified and systematically evaluated against protection goals. The process of developing a risk hypothesis, and assessment and measurement endpoints, in some cases reveals that there is adequate information to formulate a conclusion that there is no plausible pathway to harm, and that further hazard and exposure characterisation (Steps 2 and 3) is not required. The following questions are considered in identifying where further characterization is needed (adapted from OGTR 2013):

- Is the hazard or exposure pathway attributable to the genetic modification in OX513A?
- Is there a plausible and observable pathway linking OX513A to the potential hazard or pathway to harm?
- Is the potential hazard or pathway to harm substantive? (i.e. can the magnitude be estimated in the problem formulation step?)

The structure of the problem formulation which has been carried out in this assessment considers elements of the guidelines proposed by Wolt et al. (2010).

1.1.2 Choice of comparators

EFSA guidance deems it appropriate to draw on previous knowledge and experience with non-GM animals (e.g. irradiated sterile insects; mark release recapture[2] (MRR) of wild-type) and from previous applications for similar GM and non-GM traits and GM events. Accordingly, this ERA for the deliberate release of OX513A has been conducted using appropriate comparators relevant to the specific area of risk under evaluation. Comparators may include one or several of the following:

a) Wild-type *Aedes aegypti* (unmodified laboratory strains of similar genetic backgrounds to the modified strain)

b) Wild *Aedes aegypti* (wild local populations)

c) Existing control measures for *Aedes aegypti*

1.2 Molecular characterisation and phenotypic characterisation

Molecular and phenotypic descriptions provide the baseline for identification of hazards and exposure pathways, and the evaluation against appropriate comparators and are thus presented.

The genetic transformation of OX513A involved the stable integration of exogenous DNA into the genome through the use of non-autonomous transposons. The non-autonomous transposons are incorporated into a gene construct along with the other genetic elements required to effect the desired phenotypic changes.

OX513A is characterised by the expression of two intended traits: a self-limiting trait (as conferred by expression of the tTAV protein) and a fluorescent marker trait (as conferred by expression of the DsRed2 protein) (see **OX513A Technical Dossier Part A Section 4.4 - Nature of the inserted traits DsRed2 and tTAV**). In addition to expression of the intended traits, EFSA (2013) has identified the potential for unintended effects in GM insects. The characterisation of both the intended effects, and the identification and characterisation of any unintended effects in relation to protection goals must be addressed. In this section, both molecular and phenotypic characteristics of OX513A are described in order to aid in the identification of hazard and exposure pathways through the problem formulation to inform the assessment across the seven specific areas of risk.

1.2.1 Molecular characterisation

Molecular characterisation supports both specific and cross cutting considerations across the seven defined areas of risk.

The following evidence derived from molecular characterisation (**OX513A Technical Dossier Part A Section 4 - rDNA Insert and characteristics of modification** and **Appendix 1** referenced therein) has been used to corroborate the hypothesis that for OX513A in comparison to wild type *Aedes aegypti*, the insertion of the #OX513 genetic construct or the transformation process itself, did not result in molecular changes in OX513A which could lead to a plausible pathway to harm:

[2] http://web.anglia.ac.uk/numbers/functions_and_models/markRelease/mark_release.pdf (accessed 02/09/2016)

- The sequence of the construct in OX513A is as intended without re-arrangements.
- Based on flanking sequence analysis, the insertion is not known to disrupt endogenous gene function and no proteins other than those intended are likely to be produced.
- OX513A does not contain vector backbone sequences from the plasmid used for transformation, including antibiotic resistance genes or origins of replication, verified by as verified by molecular analysis.
- No contaminating materials such as viruses, cells or chemicals were introduced during the transformation process only the relevant parts of the rDNA construct intended to express the desired genotype and phenotype
- The non-autonomous transposable element used in the transformation is stable under a wide variety of conditions; published evidence is available to indicate that it would be refractory to movement, even if exposed to exogenous transposases.
- The insert has been shown to be stable and a complete single copy insertion.
- No sequences have been inserted that encode for pathogens, toxins, or allergens as evidenced by both literature searches and bioinformatics studies.
- The expression pattern of the inserted trait is as expected for a single insertion event.
- The insert has remained stable in the breeding population for over 115 generations including periods of mass-rearing where it could be conceivably argued that there is likely to be more pressure on the genetic stability of the strain and the potential mutation rate could increase.
- Regular genotyping of the OX513A colony, and quality assurance procedures have showed that the genotype has been consistently maintained.

No molecular changes were detected in OX513A relative to the wild type comparator that suggest unintended effects attributable to the genetic construct. The full molecular characterisation of OX513A is described in **OX513A Technical Dossier Part A Section 4 rDNA Insert and characteristics of modification,** and **Appendix 1** referenced therein. Additionally OX513A was first described in Phuc et al., (2007).

Additionally, bioinformatics and literature analysis on the molecular components used in OX513A presented in **OX513A Technical Dossier Part A Section 4.5 Potential for toxicity and allergenicity of the introduced proteins** suggests:

- No sequences have been introduced that encode for pathogens, toxicants, allergens or are likely to have other potential adverse effects on the animal with the exception of the intended effect.
- Evidence has been provided from the literature and bioinformatics studies on the lack of allergenicity and toxicity of the gene sequences in the rDNA construct from the donor organisms.
- Information from searches of the scientific literature on pathogenicity has been provided for the sequences in the rDNA construct, which indicates there is unlikely to be any adverse effect on human health, animal health or the environment.

1.2.2 Phenotypic characterisation

Phenotypic characterisation supports both specific and cross cutting considerations across the seven defined areas of risk.

Unintended effects may be detected through the comparison of the phenotypic (e.g. morphological, physiological and behavioural) characteristics of OX513A with its conventional counterpart comparator(s) (e.g. wild or wild-type *Aedes aegypti*). The following evidence derived from phenotypic characterisation been used to corroborate the hypothesis that for OX513A in comparison to an appropriate comparator, the insertion and expression of the #OX513 genetic construct did not result in phenotypic changes in OX513A which represent plausible pathway to harm, apart from the intended effect of lethality of the mosquito itself in the absence of tetracycline:

- Life table parameters
- Adult size and longevity
- Species-specific mating
- Response to temperature
- Response to insecticides

No phenotypic changes were detected in OX513A relative to the comparator(s) that suggest unintended effects attributable to the #OX513 construct. Reference to specific phenotypic characterisation evidence in **OX513A Technical Dossier Part A Section 5 Characterisation of OX513A** is made in this ERA as required in each of the seven specific areas of risk.

Additionally, the response to tetracycline and its analogues is assessed through the characterisation of the penetrance[3] of the tTAV trait (i.e. the intended effect). Under laboratory conditions the observed penetrance of the self-limiting trait in OX513A is always found to be over 95% (originally described in Phuc et al. 2007). Thus, less than 5% of the progeny of a cross between OX513A males and wild-type *Aedes aegypti* females ("hemizygous") will survive if reared without tetracycline in the rearing water. This is not considered genetic instability or resistance, as this is seen in the same proportion in each generation and is heritable. Trait penetrance in OX513A is confirmed through regular quality control testing of OX513A as referenced in **OX513A Technical Dossier Part A Section 5.2.6 Trait Penetrance.**

1.2.2.1 Description of the tTAV- self-limiting trait

The self-limiting trait (via tTAV expression) confers a strong selective disadvantage when expressed in OX513A progeny in the absence of tetracycline or its analogues. The tTAV protein is non-toxic but deleterious to the development of OX513A progeny as they will not survive to functional adults due to tTAV expression. The mode of action of tTAV is described in **OX513A Technical Dossier Part A Section 4.4.2 Self-limiting trait tTAV.**

[3] Penetrance can be through of as the probability of a gene or genetic trait being expressed. "Complete" penetrance means the gene or genes for a trait are expressed in all the population who have the genes. "Incomplete" penetrance means the genetic trait is expressed in only part of the population.

1.2.2.2 Description of the DsRed2- fluorescent marker trait

The expression of the *DsRed2* fluorescent marker gene allows OX513A and its progeny to have a fluorescent phenotype when excited by illuminations of a specific wavelength of light and viewed under a fluorescence microscope and facilitates detection in the laboratory and in the environment. The expression of the protein continues throughout the mosquito larval development making DsRed2 a clear marker in both larval and pupal stages. The DsRed2 system is described in detail in **OX513A Technical Dossier Part A Section 4.4.1 Fluorescent marker DsRed2**.

DsRed2 has been used in a variety of insects which survive in a range of conditions (temperature and humidity) (e.g.; Harvey-Samuel et al., 2015; Leftwich et al., 2014; Ant et al., 2012; Harris et al., 2012). Studies of the longevity of the DsRed2 marker in Pink Bollworm show that as a marker for identifying test insects from the field it has a robust performance and would be acceptable for use in a SIT programme (Simmons et al., 2011).

1.3 Hazard characterization

If specific hazards are identified in the problem formulation step and deemed as requiring further characterisation they can be subject to quantitative and/or qualitative evaluation as appropriate. A quantitative approach is possible mostly where parameters can be readily measured and compared to baseline data from an appropriate comparator; this is most often the case where there is a history of accumulated data. A qualitative approach is however applied frequently when dealing with interactions between biological systems due to their complex and dynamic nature.

The potential for harm in relation to protection goals is evaluated against appropriate comparators in relation to assessment endpoints, and are expressed on a scale of magnitude. Ordered categorical descriptions *high, moderate, low,* or *negligible* are defined in Table 1 using language adapted from both (EFSA, 2013) and (OGTR, 2013).

Table 1. Descriptive scale for characterization of the magnitude of hazards posed to human and animal health and the environment, of a GM insect relative to a comparator.

Hazard characterization category	Nature of potential harm
Negligible (EFSA) Marginal (OGTR)	**Human and animal**: No significant increase in illness/injury to people. **Environment**: No significant increase in harm to any of the populations in the environment or ecosystem
Low (EFSA) Minor (OGTR)	**Human and animal**: Non-significant increase in illness/injury to people that is readily treatable. **Environment**: Non-significant harm to non-endangered, non-beneficial species that is reversible and limited in time and space or numbers affected. Changes in population densities may be minor but not result in the total eradication of a species or have negative effects on the functioning of the ecosystem.
Moderate (EFSA) Intermediate (OGTR)	**Human and animal**: Significant increase in illness/injury to people that requires specialised treatment. **Environment**: Significant harm to non-endangered, non-beneficial species, but not resulting in the total eradication of a species. This may include an increase in damage that is widespread but reversible or of limited severity. No significant effect on charismatic, protected, or other key ecosystem service species is observed.
High (EFSA) Major (OGTR)	**Human and animal**: Significant increase in severity of illness/injury to people, or large numbers of people affected, and generally not treatable. **Environment**: Significant harm to whole ecosystems, communities or an entire species, including endangered or charismatic species. Harm would not be readily reversible and would persist over time. Harm includes the entire eradication of a species leading to a negative effect on the functioning of the ecosystem.

In evaluating potential harm, it is important to consider that effects may be direct or indirect, immediate or delayed. The definitions below provide a basis for the hazard characterization process in the context of a deliberate release into the environment of OX513A:

Direct effect refers to the primary effects that the deliberate release could have on the environment; there is no causal chain of events that could lead to the effect.

Indirect effect refers to a causal chain of events being established whereby the effect is reached though mechanisms not directly related to the deliberate release, such as interaction with other organisms, transfer of genetic material or changes in use or management at the deliberate release site.

Immediate effect refers to one that would be expected to be seen throughout the timescale of the deliberate release

Delayed effects may not be observed in the deliberate release period but might become apparent as a direct or indirect effect at a later stage.

1.4 Exposure scenarios

Specific exposure pathways may be identified in the problem formulation step and deemed as requiring further characterisation. A qualitative approach may be taken to characterise exposure of OX513A in the release environment. The assumption made is that exposure to OX513A could occur throughout the release site and surrounding area, as well as at the rearing facilities and transport routes between the release site and rearing facility.

The following general exposure routes have been considered:

- Environmental exposure in an urban/semi-urban environment due to the deliberate release.
- Environmental exposure through the inadvertent release of viable insects, during transport, storage, handling, or rearing.
- Human exposure through rearing procedures for OX513A.
- Exposure of the local human and animal population in the vicinity of the release.

The exposure routes are further characterised giving consideration to various parameters surrounding the design of the deliberate release programme. Such considerations include but are not limited to:

- Temporal considerations such as:
    - timing of the overall release period,
    - phases within the programme
    - daily timing of release activities.
- The size of the release area
- Buffer zones or untreated comparator sites (where appropriate)
- Climatic and seasonal weather patterns
- Seasonality and population patterns of the insect
- Local geography such as the presence of rivers, lakes, forest, geological features
- Human population density within the release area
- Parameters surrounding the release procedure such as the rate and number of OX513A released

- Parameters surrounding the rearing procedure such as the male/female physical sorting
- Containment practices at the rearing facility and in transport and handling

It is recommended in EFSA 2013 that, for mass releases (e.g. continued augmentative release of sufficient numbers to ensure that sterile males are likely to mate with the majority of wild females), the numbers of GM insects released, the frequency of releases and the proportion of fertile, female or other off-type individuals should be assessed taking into consideration all aspects of the mass release and associated mitigation measures. As, these numbers will vary within different insect vector control programmes exact numbers are not established as it is a function of existing vector population, seasonality, existing controls, among other variables. An OX513A release will usually start with a range-finding phase to assess the existing population, followed by a suppression phase aimed at reducing the target population, followed by a maintenance phase to prevent population resurgence. OX513A Release rates are adapted as the program progresses based on a continual monitoring of the population. Program parameters are described in **OX513A Technical Dossier Part B- Section 1 - Details of the proposed release on Saba.**

For reference, a brief description of OX513A previous releases are presented here for five sites: *i)* Cayman Islands - East End; *ii)* Brazil- Itaberaba ; *iii)* Brazil - Mandacaru; *iv)* Brazil – Pedra Branca; and *v)* Panama - Nuevo Chorillo. Additional details on release program methodology, and literature references are provided in **OX513A Technical Dossier Part A Section 7.1 Previous *Aedes aegypti* vector control projects using OX513A**.

i) Cayman Islands - East End-

In collaboration with the Cayman Mosquito Research and Control Unit (MRCU), the first regulated environmental releases of OX513A where conducted through 2009 and 2010 and aimed to demonstrate that OX513A males could mate with wild female *Aedes aegypti,* and served to inform the minimum release rate for suppression trials. Subsequent releases in 2010 aimed to suppress wild populations and had the twin targets of >4,000 OX513A males per ha per week. Approximately 3.3 million OX513A males were released in a 23-week period between May-Oct 2010 at the East End, Grand Cayman site.

ii) Brazil- Itaberaba

In conjunction with Moscamed Brasil and the University of São Paulo the initial rangefinder phase, a total 185,000 OX513A mosquitoes were released over a 6-week period, 3x per week, starting in May 2011. To achieve the target mating fraction[4], release rates were estimated as ranging from 2,800-25,000/Ha/week. Subsequent release numbers increased from January 2012 with a mean weekly release rate of ~30,000/Ha/week. Following suppression, releases were maintained at a lower level (ca. 5 times lower) designed to counter resurgence of population. A total of over 17.6 million OX513A males were released in this project.

[4] **OX513A Technical Dossier Part A - Section 7.1.2 Brazil- Itaberaba**

iii) Brazil - Mandacaru

OX513A was released from the outset at the rate of 12,000-13,000 Ha/week beginning in March 2012. Initial baseline monitoring indicated a relatively low wild population of *Aedes aegypti*. Release rates remained relatively constant and substantial suppression was observed within four months. Release rates were subsequently reduced during the following phases of the program and suppression of the wild *Aedes aegypti* population was successfully maintained for a further seven months. A total of over 10.3 million OX513A males were released during this project that was conducted in conjunction with Moscamed Brasil and the University of São Paulo.

iv) Brazil – Pedra Branca

The release of sufficient numbers of OX513A to target suppression of the wild *Aedes aegypti* population began in July 2013 and a substantial reduction in the population occurred in the following six months. Ovitrap data indicated that local *Aedes aegypti* levels were 13 times higher before suppression by OX513A (based on relative numbers of eggs per trap).

The wild *Aedes aegypti* population was maintained at this low level despite the fact that the plot was relatively small, and susceptible to immigration from adjacent untreated areas where *Aedes aegypti* populations remained high.

The program in Jacobina was delivered by Moscamed Brasil and started in June 2013 but the collaboration has subsequently been dissolved.

v) Panama - Nuevo Chorillo

From April 2014 to October 2014, a vector control project to suppress local *Aedes aegypti* using OX513A in Nuevo Chorrillo, a neighbourhood in West Panama, was conducted. A target dose rate of 67 OX513A males per person was chosen. As there were 900 inhabitants living in the treated site a requirement of 60,000 per release was determined. To ensure a constant presence of OX513A males a treatment frequency of three times per week was used. A total of over 4.2 million male OX513A were released through the period of the project. This project was conducted with the participation of the Instituto Conmemorativo Gorgas de Estudios de la Salud (ICGES) in Panama.

Exposure characterisation includes considerations of the nature, magnitude, frequency and duration of exposure to the hazard. The likelihood of the entities identified in the protection goals being exposed to hazards either directly, or indirectly, may be evaluated for each of the seven areas of risk and assigned a level of magnitude if deemed necessary following the problem formulation and hazard characterisation. Magnitude can be expressed qualitatively using an assessment adopted from the approach taken by the OGTR 2013 (**Table 2**).

Table 2. Likelihood assessment scale adopted from OGTR (2013). Magnitude terminology is taken from EFSA 2013, with OGTR 2013 terminology in parenthesis.

Magnitude	Likelihood of harm
Negligible (Highly Unlikely)	May occur only in very rare circumstances
Low (Unlikely)	Could occur in some circumstances
Moderate (Likely)	Could occur in many circumstances
High (Highly likely)	Is expected to occur in most circumstances

In conducting the likelihood assessment, all steps in a causal pathway are considered in order to determine the overall likelihood of exposure. One step may be highly likely to occur, whereas another step in the pathway may be highly unlikely; similarly, many steps may be unlikely within a causal pathway. In both cases, the result may be a highly unlikely probability of exposure occurring.

1.5 Risk characterisation

It is the combination of the hazard characterisation, and the likelihood of exposure that serves to provide the characterization of risk in a semi-quantitative manner (EFSA, 2013). A strict quantitative measurement of the level of risk is unlikely to be possible in dealing with complex biological systems. In this regard EC guidance (2002/623/EC) recommends ordered categorical descriptions with clear definitions be used consistently within the ERA. Levels of risk have been directly adopted from OGTR 2013 (**Table 3**) for this ERA. EFSA 2013, and EC guidance in this respect is less prescriptive.

Table 3. Levels of risk: product of hazard and exposure characterisation (OGTR, 2013)

Level of risk	Risk evaluation definitions
Negligible	Risk is of no discernible concern and there is no present need to invoke actions for mitigation
Low	Risk is of minimal concern, but may invoke actions for mitigation beyond standard practices.
Moderate	Risk is of marked concern and will necessitate actions for mitigation that need to be demonstrated as effective.
High	Risk is of considerable concern that is unacceptable unless actions for mitigation are highly feasible and effective

The level of risk is considered semi quantitative in the sense that it is a direct product of the magnitudes derived in the hazard characterisation, and in the exposure characterisation. **Figure 4** is the matrix used to estimate risk in the OGTR 2013 guidance, and has been used to inform the current ERA for OX513A.

		LEVEL OF RISK			
LIKELIHOOD ASSESSMENT	Highly likely	Low	Moderate	High	High
	Likely	Low	Low	Moderate	High
	Unlikely	Negligible	Low	Moderate	Moderate
	Highly unlikely	Negligible	Negligible	Low	Moderate
		Marginal	Minor	Intermediate	Major
		CONSEQUENCE ASSESSMENT			

Figure 4. **Risk matrix used to estimate the level of risk (OGTR, 2013)**

The final evaluation has also considered the overall uncertainty associated with the identified risks stemming from any assumptions or extrapolations, issues which remain unresolved in the scientific literature, or explicit unknowns. In this regard, consideration was also given to various scenarios beyond those which would be encountered through planned program activities, which would lead to high levels of exposure, such as accidental releases or breeches of containment.

1.6 Risk Management strategies

The risk characterisation step provides the basis for advancing to the overall risk evaluation and conclusions (Step 6) through the determination of feasible risk management options to mitigate any risks identified. Risk management strategies must relate to the initial protection goals established and be proportionate to the characterised risk, taking into account any uncertainties identified throughout the hazard and exposure characterisation processes. Where uncertainty exists, for example, control strategies which minimize exposure scenarios may be put in place at particular steps in relation to containment or confinement procedures. For the rearing and transport of OX513A, risk management measures are followed to prevent unintended releases as this is a route of exposure relevant to multiple areas of risks evaluated in this ERA. These measures are described in detail in **Section 2.6** ***Environmental impacts of the specific techniques used for the management of OX513A.***

2. Specific Areas of Risks

Consistent with EFSA 2013 guidance on GM insects, seven specific areas of risk associated with the deliberate release of OX513A have been assessed according to the six steps adapted from Directive 2001/18 EC.

2.1 Persistence and invasiveness, including vertical gene transfer

Step 1: Problem formulation (including identification of hazard and exposure pathways)

It is important to note that *Aedes aegypti* is already an invasive species in Saba as described in **OX513A Technical Dossier Part B Section 2.2.2 Functions of Aedes aegypti in the ecosystem of Saba,** whereby reference is made to ***Appendix 9*** *Introduced agricultural pests, plant and animal diseases and vectors in the Dutch Caribbean, with an "Alert species" list w*hich notes:

> *"The yellow fever mosquito Aedes aegypti was introduced from Africa. It can also transmit dengue fever. It is most active at dusk and dawn, but will also bite during the day, especially in shady areas. This may be the most economically costly and dangerous invasive species introduced to date in the Dutch Caribbean. It is present on all islands of the Dutch Caribbean and is a known vector for the human viral dengue fever, for which outbreaks on the island are a regular occurrence…."*

Aedes (Stegomyia) aegypti (L.) mosquito belongs to the genus Diptera: Culicidae. It is considered predominantly an urban vector, utilising artificial containers around human habitations as larval sites, and feeding almost exclusively on humans (Powell and Tabachnick, 2013). In addition to climate (specifically temperature), urbanization, water storage and the availability of breeding sites, are the main factors that influence the distribution, survival and establishment of *Aedes aegypti.* Additional information on the origins and distribution of *Aedes aegypti* can be found in ***OX513A Technical Dossier Part A Section 1 Recipient organism- Aedes aegypti.***

Under this area of risk, the assessment endpoint is the environmental persistence of OX513A, or of sexually compatible counterparts containing the #OX513 construct as a result of vertical gene flow, within semi-natural or natural habitats in the receiving environment.

The hypothesis that OX513A or its progeny, will be no more persistent or invasive in semi-natural or natural habitats than the existing wild population has been tested.

The key considerations for this risk area which have been taken in evaluating measurement endpoints to test this hypothesis are (based on EFSA 2013):

1. The potential to persist or invade the receiving environment.
2. The extent to which can OX513A reproduce and hybridise with non-GM insects of the same or different species under conditions in the receiving environment to produce viable and fertile offspring.

3. Whether the traits introduced into OX513A confer increased fitness to the resulting population that could allow it to persist or invade more than wild *Aedes aegypti*.

4. The potential for the introduced traits to alter the habitat and/or geographic range of OX513A or hybrid populations.

In addressing these considerations, EFSA (2013) guidance suggests evaluating the fitness effect associated with the genetic modification of the GM insect itself or its hybrid offspring. In this context, two considerations must be noted.

a) The purpose of the self-limiting trait in OX513A is functional genetic 'sterility', whereby OX513A males are released and mate with wild females and their offspring die before reaching the adult stage. The effect is intended to reduce the target wild *Aedes aegypti* population, and by design ensures that OX513A or offspring carrying the #OX513 genes cannot become established in the environment (i.e. it is self-limiting as described in Alphey, 2014).

b) *Aedes aegypti,* is a disease vector, already subject to vector control measures in Saba. Vector control aims to effectively reduce the incidence of vector borne disease (WHO, 2012). *Aedes aegypti* is also considered as an invasive species in Saba as noted in **OX513A Technical Dossier Part B Section 2.2.2 Functions of Aedes aegypti in the ecosystem of Saba**

In evaluating the fitness effect, specific measurement endpoints describing the biology of *Aedes aegypti* have been examined for OX513A in comparison to unmodified wild-type comparators. The evaluation also includes sensitivity to tetracycline and tetracycline analogues as this is a fundamental component of the genetic system used in the self-limiting trait (**OX513A Technical Dossier Part A Section 4.4.2 Self-limiting trait tTAV**).

Additionally, site specific features may help define non-biological parameters which may limit the capacity of OX513A to become persistent or invasive. These include physical features of the receiving environment, as well as human activities.

Dispersal, gene dissemination and longevity

Spontaneous flight of adult *Aedes aegypti* is limited to around 200 m (650 ft) depending on availability of breeding sites, and hosts from which to take a blood meal (Facchinelli et al., 2011; Maciel-de-Freitas et al., 2010; Suwonkerd et al., 2006) although there are reports of females travelling further, even in urban environments (Halstead, 2012). It was anticipated that the dispersal of adult male OX513A would be similar to an unmodified counterpart, and that dissemination of OX513A genes into the environment should be limited to the dispersal of released males and their subsequent mating with wild females.

The DsRed2 marker (**OX513A Technical Dossier Part A Section 4.4.1 Fluorescent marker DsRed2**) as part of the rDNA construct enables the evaluation of dissemination of OX513A mosquito genes resulting from the release of OX513A mosquito males and their subsequent mating with wild females. The dispersal and longevity of OX513A has been assessed in both an urban habitat, typical to *Aedes aegypti*, as well an uninhabited forested area representing a non-typical *Aedes aegypti* habitat:

1. Regulated environmental releases were conducted in Brazil to examine the dispersal of OX513A genes into the environment through the examination of eggs recovered from ovitraps adjacent to an area which had received a sustained release of OX513A males. The dissemination of OX513A mosquito genes, differed little from the dispersal of OX513A adult

mosquitoes, and males of the comparator strain (recently colonized *Aedes aegypti*) observed at the same site, and falls in the mid-range of mosquito dispersal reported in the scientific literature. See **OX513A Technical Dossier Part A Section 5.3 Dispersal and longevity- regulated environmental releases of OX513A.**

2. Adult male OX513A mosquito and wild-type *Ae. aegypti* were released into an uninhabited forested area of Pahang, Malaysia, and survival and dispersal was assessed by use of a network of traps. OX513A showed a similar dispersal pattern to the unmodified comparator strain in dispersal experiments and falls within the midrange of the reported distances of *Aedes aegypti* flight from the literature. See **OX513A Technical Dossier Part A Section 5.3 Dispersal and longevity- regulated environmental releases of OX513A.**

The results suggest that OX513A does not have an extended range of dispersal in comparison to an unmodified comparator.

Temperature response

Aedes aegypti has an ecological temperature range of 14-30°C (Brady et al., 2014; Brady et al., 2013; Hemme et al., 2009). Global historical collections and laboratory experiments on this well-studied vector have suggested its geographical distribution is limited by the 10°C winter isotherm[5] (Christophers, 1960) while a more recent stochastic population dynamics model analysis suggests the temperature's limiting value to be more towards the 15°C yearly isotherm (Otero et al., 2006). At temperatures lower than 15°C, *Aedes aegypti* becomes torpid, unable to fly, or moves its limbs only slowly (Yang et al., 2009; Rowley and Graham, 1968; Christophers, 1960). The effect of temperature on larval development of *Aedes aegypti* has been well studied. Larval development is a function of temperature, which affects adult size, dry weight and ovariole number, all of which fall as the temperature rises (Rueda et al., 1990; Christophers, 1960).

Lower temperatures can slow development time to such a degree that the species is prevented from establishing itself, egg to adult cycles of longer than 45 days are likely to prevent establishment. *Aedes aegypti* does not appear to enter a true diapause, although the eggs are able to survive in dry conditions for several months. Scholte et al., (2010) indicated that *Aedes aegypti* could not survive winter temperatures in Northern Europe, while Thomas et al. (2012) found that a tropical strain of *Aedes aegypti* eggs could only survive at a threshold of -2°C for 24 hours before hatching broke down completely.

High temperatures alone (>40°C) are unlikely to limit the species while temperatures below the 15°C isotherms are likely to severely limit the geographical range, although the protection of human habitations may afford some protection from lower temperatures. Survival at temperatures below freezing is extremely unlikely for *Aedes aegypti.*

The climate in Saba is described in **OX513A Technical Dossier Part B Section 2.1.2 Climate.**

Temperature response of OX513A

Temperature is a key abiotic factor in the consideration of the survivability of OX513A mosquito, although this can be complicated by the interaction with diet and larval density dependent effects (Couret et al., 2014). The temperature response of OX513A has been

[5] An isotherm is a line on a map or chart of the earth's surface connecting points having the same temperature at a given time or the same mean temperature for a given period.

evaluated in the laboratory through rearing OX513A at different temperatures and evaluating survival. The results are described in **OX513A Technical Dossier Part A Section 5.2.1 Temperature response of OX513A** and demonstrated that in comparison to an unmodified counterpart:

- OX513A did not survive at temperature extremes outside its normally reported range in controlled laboratory conditions (9°C and 37°C).
- OX513A larvae reared at intermediate temperatures within this range did not show a higher than expected proportion (<5%) of individuals surviving from first instar larvae (L1) to functional adult (range 0-2%) (i.e. trait penetrance remained stable)

The results suggest that the phenotype of OX513A is stable over a range of temperatures that Aedes aegypti larvae are likely to encounter in the environment, and no unintended adaptability to temperatures outside the range published in the scientific literature has been observed in OX513A.

Lifetable parameters

With multiple generations per season, *Aedes aegypti* is considered as a multivoltine species It prefers mammalian hosts and in particular human hosts, even in the presence of alternatives (Saifur et al., 2012) and feeds on multiple hosts during one gonotrophic cycle. Its activity is both diurnal and crepuscular (ECDC[6]).

Aedes aegypti has a complex life-cycle with changes in shape, function, and receiving environment (see CDC[7] for brief description). The female mosquitoes will lay their eggs close to water and larvae will hatch when water reach the eggs. After that, larvae will feed on microorganisms and organic matter. Metamorphosis is triggered at the fourth instar. Pupae do not feed, only changing form until reaching the adult stage. The adult will emerge from the water after breaking the pupal skin. The life cycle lasts typically 8-10 days, but this is temperature dependant. *Aedes aegypti* reproductive biology also detailed in the literature (Lees et al., 2014; Oliva et al., 2014).

Various lifetable parameters have been examined for OX513A compared to *Aedes aegypti* of different genetic backgrounds including both laboratory reared wild-type strains (i.e. established strains for laboratory use), and laboratory reared wild-caught. Details are provided in **OX513A Technical Dossier Part A Section 5.1 Life table parameters,** which support the following conclusions:

1. OX513A compared to wild-type *Aedes aegypti* laboratory strain

In comparing OX513A to a wild type *Aedes aegypti* in two separate studies (Bargielowski et al., 2011; Lee et al., 2009b), the following parameters examined were statistically indistinguishable.

- the number of eggs laid,
- the number of unhatched eggs,
- the egg-hatching rate,
- the duration of larval period in all four instars,

[6] http://ecdc.europa.eu/en/healthtopics/vectors/mosquitoes/Pages/aedes-aegypti.aspx (accessed 02/09/2016)

[7] http://www.cdc.gov/dengue/entomologyecology/m_lifecycle.html (accessed 02/09/2016)

- pupation,
- Adult eclosion rate,
- gonotrophic cycle,
- adult fecundity,
- adult lifespan
- offspring sex ratio.
- adult size (i.e., wing length),
- longevity.

Statistically significant differences were found between OX513A and the wild type in the following areas:

- Larval survival was 5% lower for OX513A than WT *Aedes aegypti*
- Reduced adult longevity (20 days for OX513A vs 24 days for WT mean lifespan).
- OX513A pupated approximately one day sooner than WT *Aedes aegypti* resulting in statistically smaller OX513A adults than the wild-type; this effect was more pronounced in females than in males.

These differences are likely to be attributable to the WT strains and OX513A being laboratory adapted over different time frames and under different conditions. The WT used in the above noted studies originated from a Malaysian back ground laboratory reared since 1975, whereas OX513A has been laboratory adapted since 2002.

2. OX513A compared to laboratory reared *Aedes aegypti* of wild origin (wild caught)

OX513A was compared to wild-caught laboratory reared *Aedes aegypti* originating from two regions of India. Patil et al. (2015) found the following parameters examined were statistically indistinguishable.

- Blood meals per female
- Oviposition events per female
- Eggs laid per female
- Hatch rate (%)
- Pupation rate (%)
- Adult emergence (%)

OX513A developmental time from first instar to adult emergence was found to have a statistically significant difference compared to the wild-caught Indian strains, with a slightly longer developmental time for OX513A (1.3-1.6 days longer). These differences are likely to be attributable to attenuation to laboratory based rearing for OX513A since 2002, whereas the Indian strains were wild caught in 2011 and minimally reared in laboratory conditions prior to the studies being undertaken.

The results of the examination of specific lifetable parameters suggest that OX513A does not have increased fitness compared to a wild-type or wild comparator.

Mating competitiveness

Mating competitiveness is a key parameter in the assessment of the fitness of insects for use in population control programs using self-limiting genetic control strategies. A detailed description of mating competitiveness studies, and parameters used to measure mating

competitiveness is covered in **OX513A Technical Dossier Part A Section 5.1.3 Mating competitiveness**. Successful mating competitiveness of OX513A compared to wild-type *Aedes aegypti* of various genetic backgrounds has been demonstrated in laboratory conditions with no statistically significant differences observed. In addition, semi-field studies have been conducted (Lee et al., 2012) in a purpose built field house in Malaysia whereby males of two different genetic backgrounds carrying the #OX513 genetic construct where found to compete equally successfully with wild type males of similar background. Mating competitiveness with wild populations has also been assessed during regulated environmental releases in the Cayman Islands (Harris et al., 2012; Harris et al., 2011) and Brazil (Carvalho et al., 2015).

The regulated environmental releases conducted to date (See **OX513A Technical Dossier Part A Section 7.1 Previous Aedes aegypti vector control projects using OX513A**) represent three distinct yet typical habitats for *Aedes aegypti.*

- Urban community isolated and untreated with conventional insect control measures (Cayman);
- A densely populated urban site with a high degree of immigration of *Aedes aegypti* from adjacent areas (Brazil-Itaberaba)
- A rural, isolated community with low housing density (Brazil-Mandacaru)

The equivalent mating competitiveness of OX513A against both wild-type and wild *Aedes aegypti* from different genetic backgrounds, both under laboratory conditions and in several natural habitats with variable conditions such as housing density and site isolation, implies that the capacity of OX513A to react to the specific mating signals of the wild females and reproduce and hybridise in the receiving environments has not been compromised. This suggests that there are unlikely to be differences in mating behaviours of OX513A with the local *Aedes aegypti* populations across different genetic backgrounds and habitat factors in the receiving environment.

The results suggest that the highly species specific nature of mosquito reproduction is not compromised in OX513A and the introduced traits are stable, and express as intended. The ability of the OX513A mosquito to produce viable and fertile offspring is thus only limited by the self-limiting trait as intended.

Vertical gene transfer- Interspecies compatibility

In mosquitoes, mating is extremely species-specific. Exchange of genetic materials between different insect species in the natural environment rarely happens as insects exchange gametes internally and have complex mating behaviours and structures which prevent interspecies mating (Oliva et al., 2014; Cator et al., 2009). *Aedes albopictus* is the most closely related species to *Aedes aegypti* which is likely to be encountered in the receiving environment (Shepard et al., 2006). The existence of multiple mating barriers between *Aedes aegypti* and *Aedes albopoictus* are well established (Leahy and Craig, 1967) namely; mating behaviour e.g. wing beat harmonic convergence (Cator and Harrington, 2011; Cator et al., 2009), structural incompatibility of genitalia, sperm inactivation, reduced oviposition in crossed females, and genetic incompatibility. Additionally, *Aedes aegypti* does not form part of a species complex, which can be described as a group of insects of similar form that are often indistinguishable at the species level.

Despite multiple barriers to mating, interspecific mating between *Aedes aegypti* and *Aedes albopictus* has been observed both experimentally in caged conditions (Marcela et al., 2015; Nazni et al., 2009a) and at a very low frequency in the field (e.g. Tripet et al., 2011; Nasci et al., 1989) but is not reported to result in viable offspring. The subject has been reviewed by (Bargielowski and Lounibos, 2016) in the context of competitive displacements of invasive mosquito species by satyrization. Satyrization is a form of mating interference in which males of a species mate interspecifically, with a female of a closely related species but produce no viable offspring. The body of evidence for this effect between *Aedes aegypti* and *Aedes albopictus* serves to affirm the existence of a biological mating barrier, even when insemination occurs.

Thus while interspecific mating can be forced under laboratory conditions, and low frequencies have been observed in the field, many barriers to the mating exist, and when mating and insemination does occur between *Aedes aegypti* and *Aedes albopictus* no viable offspring result. Additionally, Lee et al., (2009a) found no evidence for successful interspecific cross-mating of OX513A of Malaysian background and wild type *Aedes albopictus* under laboratory conditions.

In addition to existing established mating barriers, OX513A does not have an altered capacity for interspecies mating, and thus does not have a greater capacity to enable the sexual transfer of genes to a closely related species in the receiving environment, if they were present.

Penetrance of the self-limiting trait

Penetrance can be thought of as the probability of a gene or genetic trait being expressed. "Complete" penetrance means the gene or genes for a trait are expressed in all the population who have the genes. "Incomplete" penetrance means the genetic trait is expressed in only part of the population, for example 95% penetrance means that 95% of the population expresses the introduced trait.

The expression of the self-limiting tTAV trait in OX513A is intended to confer a strong selective disadvantage, i.e. cell death in the larval stage as described in **OX513A Technical Dossier Part A Section 4.4.2 Self-limiting trait tTAV**. In the absence of tetracycline, it has been consistently observed that >95% of the hemizygous progeny of a mating between OX513A and wild-type die though expression of the tTAV trait. The penetrance of the introduced self-limiting trait in OX513A is therefore approximately 95%, meaning that in the laboratory <5% of the progeny will survive if reared in the absence of tetracycline or its analogues (Phuc et al., 2007). The >95% trait penetrance is heritable and is consistently evaluated through quality control procedures performed every 6th generation (see **OX513A Technical Dossier Part A Section 5.8.1 OX513A quality control**) Additionally penetrance has been found to be consistent across a range of temperatures from 18-30 °C (**OX513A Technical Dossier Part A Section 5.2.1 Temperature response of OX513A**)

The length of time non-penetrant OX513A survives in the absence of tetracycline is a key factor in evaluating the capacity for persistence and invasiveness in the receiving environment. To assess environmental persistence, monitoring for the #OX513 genetic construct in the environment after a regulated environmental release in 2014 in Nuevo Chorrillo, Panama was undertaken (**OX513A Technical Dossier Part A Section 7.1.5.1**

Environmental persistence). The data suggested that OX513A genes are unlikely to persist in the environment from 6-8 weeks post-release.

Additionally, PCR analysis of field collected adults was undertaken as part of OX513A regulated environmental releases in the Cayman Islands (East End) and two release sites in Brazil (Itaberaba and Mandacaru) in order to evaluate trait penetrance in the field. Reference to the studies can be found in **OX513A Technical Dossier Part A Section 7.1 Previous Aedes aegypti vector control projects using OX513A.**

Overall estimates of percentage incomplete penetrance ranged from 0-4.28%. It should be noted that sample size for some of the studies was low, but taking all studies together it is clear that the figure falls below the ~ 5% reported in laboratory studies. This is to be expected as conditions in field are harsher that the controlled conditions in which laboratory studies were conducted. These results support the hypothesis that the self-limiting phenotype is behaving as expected in the field and that incomplete penetrance of the self-limiting trait is no higher than in the laboratory.

The results suggest that the trait penetrance is sufficient to prevent the persistence of OX513A in the environment as observed in regulated environmental releases.

Longevity of non-penetrant OX513A under laboratory conditions

A laboratory assessment of hemizygous non-penetrant OX513A gives an indication of the potential for persistence in the absence of tetracycline in the receiving environment.

Experiments described in **OX513A Technical Dossier Part A Section 5.2.7 Non-penetrant OX513A progeny- Longevity and Fecundity** examined the longevity of hemizygous OX513A which survived rearing in the absence of tetracycline in the rearing medium (i.e. non-penetrant). The "non-penetrant" individuals represented 4.4% of an initial cohort of 4000 pupae, consistent with the reported <5% (Phuc et al., 2007). Additionally, the experiment examined the fecundity of the female surviving offspring through egg clutch size and hatch rate. The longevity and fecundity data are also examined in the context of **Section 2.3** *Pathogens, Infections and Diseases* with respect more specifically to the persistence of females as the biting vector of disease.

The lifespan of non-penetrant OX513A hemizygotes was found to be significantly reduced relative to wild-type comparators. Both male and female surviving hemizygous progeny where found to have a with a median lifespan of two days relative to a wild-type median lifespan of 60 and 68 days for males and females respectively. A fraction (~20%) do survive long enough for females to take two blood meals and some females produced two clutches of eggs; this however did not result in longer lifespan that the wild type comparator. The reduction in longevity of non-penetrant hemizygous OX513A mosquito males and females is even lower than one would expect simply by considering survival to adulthood when compared to wild-type.

Statistical testing of the data for the egg clutch size and hatch rate revealed a statistically significant difference between non-penetrant OX513A females and the wild type comparator only for the mean clutch size. OX513A females had 69.9 eggs (S.D. 13.9) and for LWT it was 54.8 eggs (S.D. 12.4). The apparent slight fecundity increase is likely a consequence of selection under mass-rearing conditions for early egg production, as this would be a desirable trait in a mass rearing production context.

Taken together, although a slight increase in fecundity was observed in non-penetrant OX513A females, the reduced longevity suggests that overall the capacity of non-penetrant individuals to persist in the environment is not enhanced in OX513A compared to a wild type counterpart.

Dose response to tetracycline and its analogues

Colonies of OX513A are reared on a diet with a concentration of tetracycline(s) sufficient to supress tTAV expression and thus enable rearing of both males and females for mass egg production. As a function of penetrance of the self-limiting trait, survival of the hemizygous OX513A progeny is greatly reduced (to < 5%) in the absence of tetracycline(s). Hence, the response to tetracycline or it's analogues in the environment can affect survivability and thus persistence of OX513A in the receiving environment. This has been examined in detail and the results are evaluated in light of potential exogenous tetracycline concentrations that might be encountered in the environment.

The response of OX513A hemizygous larvae to different doses of tetracycline, chlorotetracycline, oxytetracycline, and doxycycline has been evaluated in the laboratory to identify the lowest concentrations which allow for greater survival of hemizygous OX513A as compared to larvae reared in the absence of tetracycline or its analogues (see **OX513A Technical Dossier Part A Section 5.2.2 Sensitivity to tetracycline and its analogues**). These results have been published in Curtis et al. (2015).

Key results are summarised below:

- Maximum rescue from the phenotype occurred at:
  - >1 ng/mL for doxycycline
  - >1 µg/mL for tetracycline, oxytetracycline and chlortetracycline.

- Concentrations which did not increase the proportion of functional adults:
  - ≤0.1 ng/mL for doxycycline
  - ≤1 ng/mL for chlortetracycline
  - ≤3 ng/mL for tetracycline
  - ≤10 ng/mL for oxytetracycline

Determining the lowest concentrations of tetracycline analogues which could give rise to a greater than the nominal (fraction at 0 ng/mL) percentage of functional adults allows an assessment of the likelihood that larvae will develop to adulthood in habitats containing tetracycline or one of its analogues at or above this concentration.

The mean concentrations of each tetracycline analogue assayed, found in environmental bodies of water, has been calculated from reported field site sampling and compared to dose response curves for each analogue. In all cases the minimum concentration for each analogue required to increase OX513A survival, is higher than the mean concentrations found in environmental water bodies such that concentrations of tetracyclines likely to be encountered in environmental water bodies would not be sufficient to increase survival.

Reported concentrations sampled from field sites around the world, were recorded as follows (Curtis et al., 2015);

- Tetracycline: 0.096 ng/mL to 1.3 ng/mL
- Chlortetracycline: 0.04 ng/mL to 0.97 ng/mL

- Oxytetracycline: 0.7 ng/mL to 1.34 ng/mL
- Doxycycline: 0.07 ng/mL to 0.4 ng/mL

These data have largely been generated from the scientific literature. They examine of tetracycline levels in wastewater effluent from treatment plants and their downstream flow in evaluating the efficacy of treatment in removing antibiotics from waste water. Waste water treatment environments are not typical *Aedes aegypti* larval habitats which include artificial containers such as used car tires, flower vases, water storage vessels and discarded materials in domestic/peri-domestic environments. *Aedes aegypti* larvae are found in clean, still water, not flowing river systems and are rarely found in collections of water in the ground such as borrow-pits or earth drains (Dieng et al., 2012; Morrison et al., 2006; Christophers, 1960). Thus while environmental concentrations of doxycycline that would fall within the range that could rescue the OX513A phenotype (0.1 ng/ml), have been reported (see references in Curtis et al. 2014), these locations are not associated with the *Aedes aegypti* typical habitat, nor are these environmental conditions present on Saba.

It is important to note that members of the tetracycline class are well known to degrade rapidly in sunlight (photolysis) in the presence of catalysts (iron and hydrogen peroxide, both of which can occur naturally in sunlit water) where degradation of tetracycline has been observed to be complete after 1 minute (Bautitz and Nogueira, 2007). The rate of degradation is dependent on the initial concentration and the pH of the water. It has also been reported that in natural water samples the rate of photo -degradation is higher than in pure waters due to aquatic matrix effects (López-Peñalver et al., 2010). Homem and Santos (2011) report that with tetracylines over 80% reduction can be rapidly achieved by photo-degradation using advanced oxidation processes (1 -300 minutes depending on whether a catalyst was used and the pH of the reaction).

As described in **OX513A Technical Dossier Part B Section 2.4 Tetracycline in the environment in Saba**, the absence of large scale commercial agriculture and the associated use of tetracyclines either in the context of human therapeutic use, veterinary use or as a plant protection product, makes the likelihood of exposure of OX513A to environmental tetracycline's through this route negligible. Similarly, the environmental sources of tetracycline's such as rivers downstream from sewage treatment plant effluent as described in Curtis et al. (2015) are not present in Saba due to the absence of waste water treatment infrastructure.

The principle source of environmental tetracycline's in Saba is likely waste cesspits associated with the A.M. Edwards Medical Center hospital on Saba in The Bottom, as well as waste cess pits associated with private residences whereby individual inhabitants are receiving therapeutic doses of tetracyclines.

As noted above in this section, waste water is not the preferred breeding habitat of *Aedes aegypti,* as they prefer clean, still water such as rainwater filled vessels in the vicinity of human habitation. Additionally, the design of the cesspits or septic tanks (See ***Appendix 15-pg 36)*** makes them generally inaccessible to *Aedes aegypti* due to fact that they are generally sealed and covered to contain odours. Some reports have however suggested that *Aedes aegypti* can breed in septic tanks, usually where they are cracked or broken (Mackay et al., 2009; Barrera et al., 2008) but this tends to be in the clear water at the top of the tank, whereas tetracyclines tend to bind to the sediment which collects at the bottom Watkinson

et al., 2009; Brown et al., 2006) (thereby making any tetracycline's less accessible in the clear surface layer of water).

The survival of hemizygous OX513A progeny reared off tetracycline has been reported when reared on a specific brand of cat food (Massonnet-Bruneel et al., 2013) whereby 18% of a total 9847 larvae survived to adulthood where reared on commercial chicken based catfood, compared to 3.9% of a total 10413 reared on a commercial brand of fish food widely used in mosquito insectaries. 3.9% is consistent with the reported penetrance as described in **OX513A Technical Dossier Part A Section 5.2.6. Trait penetrance.** The presence of tetracycline in commercial cat food is described in the above noted study, which could suggest that the presence of commercial cat food in the receiving environment may represent a source of tetracycline sufficient to increase survival of OX513A. Potential sources of tetracycline via pet food in and around residences in the receiving environment would be highly unlikely to affect OX513A survival. Chicken based pet foods would need to be present, and further mixed with water to be dilute enough to present an attractive oviposition site, as *Aedes aegypti* prefer clean water. Further any dishes with the appropriate dilution and type of pet foods would have to remain in the environment long enough to allow development of eggs through to the pupal stage (~7-10 days). Additionally, animal-derived food products must have residue levels below established tolerance levels depending on their origin. In the U.S. for example of 2 ppm in muscle, 6 ppm in liver, and 12 ppm in fat and kidney (21 CFR 556.720), and plant-derived food products must have residue levels below 0.35 ppm on apples, peaches, and pears (40 CFR 180.337) which is not sufficiently high to affect OX513A survival as levels of tetracycline would likely have to be close to 1 µg/ml or higher in water to have an effect on eclosion and adult OX513A survival. To achieve sufficient sustained tetracycline levels in water from a tetracycline residue at the tolerance levels noted above, all the tetracycline in the animal-derived food would have to leach out into the water so that 50%, 16.7%, and 8.33% of the drinking water was muscle, liver, and fat/kidney respectively. An even higher percentage of the drinking water would need to comprise plant-based food to reach the concentration of tetracycline necessary to affect OX513A survival.

Any tetracycline or tetracycline derivative in pet food exposed to the environment would also be subject to photodegradation by exposure to light resulting in lower effective concentrations in any potential mosquito habitat. Additionally, such food would have to be left out continuously for 5-7 days and the container holding such food would need to contain sufficient fresh water throughout this time for the aquatic phase of the mosquito life cycle to be completed, allowing adults to eclose. The combined probability of all these events occurring at once is very low and, therefore, the risk of OX513A surviving, mating, producing eggs that survive, develop, and eclose, and resulting in the trait persisting in the environment is negligible.

Taken together, considering the levels of tetracycline(s) required for rescue of the OX513A phenotype, the relative low environmental concentrations of tetracyclines, the dynamics of degradation of tetracyclines, and the typical habitat for Aedes aegypti, the results suggest that OX513A larvae would not encounter concentrations of tetracyclines in the environment high enough to increase survival of OX513A.

Tetracycline loaded blood

There is a potential for small numbers of female OX513A to exist in the environment during a regulated environmental release under the following scenarios:

a) a small number of homozygous female mosquitoes to be released as a result of the mechanical sorting process (see **OX513A Technical Dossier Part B Section 1.5 Rearing of OX513A from egg to adult mosquitoes**). As detailed in Standard Operating Procedure ***TD-SOP-00293 OX513A Sex Sorting of Pupae for Release***, samples of 1000 pupae are taken subsequent to mechanical sorting, and if the threshold of more than 2 pupae are detected in the sample of 1000, the batch is re-sorted prior to advancing to the eclosion phase. Data from previous OX513A regulated environmental releases demonstrates a sorting accuracy of >99.9% is routinely achieved in operational scale projects (Carvalho et al., 2015; Gorman et al., 2015; Harris et al., 2012).

b) hemizygous females which hatch in the environment as a result of incomplete penetrance of the self-limiting trait (See **OX513A Technical Dossier Part A Section 5.2.6 Trait Penetrance**)

Tetracycline is an antibiotic used as a therapeutic and/or prophylactic agent in human and veterinary medicine and it may be present in the bloodstream of humans, companion animals or livestock following a recent dose of tetracycline. In vertebrates, the concentration of tetracycline in the blood usually reaches a peak 2-6 hours following an oral or injected dose, and then gradually declines due to the body's metabolic activity (Agwuh and MacGowan, 2006). In both humans and livestock, the peak concentration of tetracycline in blood (plasma) following standard therapeutic doses normally remains below 10 µg/ml (Bimazubute et al., 2011; Agwuh and MacGowan, 2006). The highest apparent concentration of tetracycline recorded in vertebrate blood is ~20 µg/ml (a level observed in pigs that received unusually high intra-muscular doses as part of experimental treatments) (Bimazubute et al., 2011).

Although there is no evidence suggesting that oral ingestion of tetracycline by a female mosquito results in deposition of active tetracycline in eggs, a study was conducted to test the hypothesis that providing high doses of dietary tetracycline to adult female *Aedes aegypti* has no effect on the penetrance of the OX513A phenotype in their hemizygous offspring (i.e. progeny either from homozygous OX513A mosquito females mated to wild-type males, or wild-type females mated to homozygous OX513A mosquito males). Study details are provided in **OX513A Technical Dossier Part A Section 5.2.5 Tetracycline loaded blood study.**

Crosses using females which had access to either tetracycline-free meals or meals containing high doses of tetracycline were made, and the penetrance of the OX513A phenotype in the hemizygous offspring was evaluated. The study used concentrations of tetracycline approximately ten times higher than the highest dose found in humans, and five times higher than the highest dose found in the blood of animals treated with tetracycline

No significant differences were observed between the control group and the experimental groups in any of the parameters examined, supporting the hypothesis that penetrance of the OX513A phenotype in the hemizygous OX513A offspring of females which have ingested high doses of tetracycline is no different from that observed in the offspring of females that did not ingest any tetracycline with their diet.

The results suggest that trait penetrance would not be significantly altered in OX513A offspring from a female which took a blood meal from an individual (human or animal) that had recently received a human or veterinary therapeutic dose of tetracycline, in comparison to a female not receiving a blood meal containing tetracycline.

Site specific features

Landscape or geophysical barriers to movement of *Aedes aegypti* include saltwater, rivers, roads, areas of vegetation without human habitation, (Hemme et al., 2010; Maciel-de-Freitas et al., 2010; Navarro et al., 2010). *Aedes aegypti* are reported not to survive in sea water at salinity levels around 35 g/l, although they have been found to survive to a limited extent brackish waters (Ramasamy et al., 2011) with lower saline levels. Other factors affecting distribution/dispersal of *Aedes aegypti* include the presence and type of water storage, as the mosquito is rare in deserts and desert-like conditions without human habitation, but conversely in parts of these regions where there are human habitations, there is also likely to be stored water and this can substantially increase the presence of the mosquito (Hayden et al., 2010; Sharma et al., 2008).

The geography of Saba is described in **OX513A Technical Dossier Part B Section 2.1.1 Geography** and the predicted habitat map presented in **Section 1.2 Figure 1 Location of releases** accounts for larger geographical barriers within the island. As Saba is an island surrounded by ocean, the release site is effectively isolated from any other landmass within the dispersal range of *Aedes aegypti.*

The species can also be dispersed by human activities such as passive transport on boats, trains, automobiles etc. (Gubler, 2006; Lounibos, 2002). Damal et al. (2013) reported that human aided activity, namely the availability of containers that serve as breeding sites, the presence of human hosts, and human mediated passive transport is the predominant means of dispersal of *Aedes aegypti*.

International Sanitary Regulations (WHO, 2005) require ports and airports to establish programs to control *Aedes aegypti* and other insect disease vectors for at least 400 m (1300 ft) from point of entry facilities.

Conclusion to step 1

Key considerations in the area of risk *Persistence and invasiveness, including vertical gene transfer* have been addressed for OX513A in the receiving environment with the following conclusions:

1. OX513A does not have the potential to persist or invade in the receiving environment:

2. OX513A is not able to reproduce successfully with insects of a different species in the receiving environment.

3. OX513A homozygous adults and OX513A hemizygous offspring do not have increased fitness that could allow it to persist or invade more than wild *Aedes aegypti*;

4. OX513A does not have introduced traits likely to alter the habitat and/or geographic range of the OX513A mosquito, or hybrid populations.

Through Step 1, potential hazards and pathways to exposure have been assessed through measurement endpoints. There is adequate information to formulate a conclusion that there is no plausible pathway to harm under the protection goals identified in Section 1.1, and that further hazard and exposure characterisation is not required.

Step 2-4: Hazard, Exposure and Risk characterisation

The potential for harm, under the protection goals identified in **Section 1.1,** through persistence or invasiveness as a result of the rDNA insert in OX513A has been assessed in step 1. The conclusion is that the potential for harm was negligible in all cases therefore no further Hazard characterisation (Step 2), Exposure characterisation (Step 3), or Risk Characterisation (Step 4) was necessary for this area of risk.

Step 5: Risk management strategies

No further risk management strategies are required for this risk area as no plausible pathway to harm under the protection goals identified in **Section 1.1** has been identified. It should be noted that the reduced fertility of OX513A is considered as a mitigation measure (EFSA, 2013) and that measures to avoid escape into unintended environments during production stages are implemented as a standard practice consistent with arthropod containment principles as described in **OX513A Technical Dossier Part B Section 1.4 Containment measures prior to release.**

Step 6: Conclusions

Using the information and evidence presented, it has been concluded in the problem formulation step through the identification of hazard and exposure pathways that the likelihood the deliberate environmental release of OX513A in Saba would represent a plausible pathway to harm through persistence and invasiveness is negligible, thus further hazard and exposure characterisation was not required.

2.2 Horizontal gene transfer

Step 1: Problem formulation (including identification of hazard and exposure pathways)

The horizontal gene transfer (HGT) is defined in EFSA (2013) as "any process in which an organism incorporates genetic material from another organism into its genome without being the offspring of that organism", or stated otherwise, the heritable transfer of a functional genetic element from one organism to another without mating.

HGT between certain bacteria and other single-celled (prokaryotic) organisms can occur at a detectable frequency and bacteria have obtained a significant proportion of their genetic diversity from distantly related organisms (Ochman et al., 2000). HGT from multicellular (eukaryotic) organisms, such as plants or insects, to other organisms is remarkably rare, occasionally being detected under optimized laboratory conditions (Crisp et al., 2015; Keese, 2008). Current scientific knowledge supports the idea that non-sexual gene transfer of non-mobile DNA fragments between unrelated organisms (such as insects to microorganisms) is extremely unlikely to occur under natural conditions and if it does happen it occurs on an evolutionary time span (Kuraku et al., 2012; Bartolome et al., 2009; Silva et al., 2004).

Under this area of risk, the assessment endpoint is the potential for HGT from OX513A, within semi-natural or natural habitats in the receiving environment.

It is important to note that HGT is not an adverse effect as such, but an event that may or may not lead to potential harm. The hypothesis that HGT between OX513A and other organisms in the receiving environment does not represent a plausible pathway to harm under the protection goals identified in **Section 1.1** has been tested.

The key considerations for this risk area which have been taken in evaluating measurement endpoints to test this hypothesis are (based on EFSA 2013):

1. The probability and frequency of HGT, and the heritability of insect DNA in the potential recipient organism, which considers:

- The amount and size of insect DNA exposed to various recipient organisms
- The presence of germline cells in multicellular organisms, or single celled organisms that are susceptible to direct DNA or DNA vector exposure
- The presence of mechanisms enabling such cells to take up recombinant insect DNA
- The existence of genetic recombination/integration processes by which translocated DNA could be incorporated and heritably stabilised in the germline cells, or replicating units

2. The biological relevance of HGT events which may occur at low frequencies, which is directly dependent on the likelihood of further vertical transmission, which considers:

- The presence of conditions leading to positive selection of the recipient of an HGT event such that the trait will propagate in the population.
- The presence of gene drive systems in the recombinant DNA leading to the possibility that an HGT event will increase in frequency during subsequent vertical transmission

Literature reviews have been conducted across potential routes of exposure by HGT in the receiving environment and are discussed below, namely for: *HGT between Insects; HGT to microorganisms: Acquisition of genes through oral ingestion; Exposure to the gut micro-flora of predators of the mosquito; Exposure to micro-organisms associated with parasitoids at the release site;* and, *Remobilisation of the transposon.* Measurement endpoints are evaluated in most cases through a qualitative overview of current opinion rather than specific experimental measurements. In specific cases, such as transposon remobilisation, and in demonstrating transgene stability, specific measurements endpoints have been experimentally evaluated (e.g. through sequence analysis of OX513A).

HGT between insects

Whilst direct mechanisms which enable gene transfer between bacterial species exist, such as bacterial competence to take up prokaryotic DNA, these are not present in eukaryotic organisms. Homologous recombination requires the presence of identical stretches of DNA sequence between the recombination points; therefore the lack of DNA sequence similarity represents a major barrier to inter-domain transfer between bacteria and insects. Insects exchange gametes internally and have complex mating behaviours and structures to prevent or limit interspecies gene transfer; exchange of genetic material between insects through any other mechanism is not observed. In the event that that elements of, or the entire OX513A construct was taken up into the cells of another insect species, in order to be heritable in a population it would require integration into germ-line cells of the insect. In order to persist in the recipient organism and propagate through the population it would additionally need to confer a benefit or selective advantage. The tTAV and DsRed2 traits as described in **Section 1.2.2** confer no known selective advantage to insects, and tTAV is in fact intended to confer a selective disadvantage by design (i.e. a self-limiting trait)

HGT to microorganisms

Compared to multicellular organisms with a low relative proportion of germline cells, all bacteria could, in principle, act as a recipient cell for HGT (EFSA, 2013; Dunning Hotopp, 2011; Keeling, 2009). It appears to be possible for some bacteria to acquire genetic material from multicellular organisms (Anderson and Seifert, 2011), however, the absence of frequent horizontal transfer events from multicellular organism sources into bacteria suggests a discriminate mechanistic, functional and/or selective barriers (EFSA, 2009; Keese, 2008; Andersson, 2005). Bacteria have a number of physical, biochemical, and genetic barriers to restrict non-sexual horizontal gene transfer (Thomas and Nielsen, 2005). Given the competence of most bacteria to take up foreign DNA, the major barrier to such inter-domain transfer appears to be the lack of sufficient DNA sequence similarities for homologous recombination to occur in bacteria. EFSA (2013) identifies several barriers for HGT from insects to micro-organisms, which include the lack of efficient mechanisms of integration of unrelated chromosomal DNA, the presence of introns and the limited potential for positive selection of the acquired recombinant traits. Every organism has a number of physical, biochemical, and genetic barriers to restrict non-sexual horizontal gene transfer

Additionally, for plants as another eukaryotic comparator, (EFSA, 2009) suggests that the main barriers for horizontal gene transfer from plants to bacteria are the lack of efficient mechanisms of integration of unrelated chromosomal DNA and the limited potential for

positive directional selection of the acquired recombinant gene-encoded traits. Unlike other uptake systems regulated by substrate availability, bacteria are not known to use DNA availability as a signal to induce the competence machinery, apparently because environmental DNA is present in most bacterial environments, especially biofilms (Mell and Redfield, 2014). In species for which competence triggering has been described, the environment and biochemical processes are involved (Seitz and Blokesch, 2013).

OX513A has been transformed using the non-autonomous transposon, *piggyBac* which enabled stable integration of the inserted genes into the *Aedes aegypti* genome. Non-autonomous transposons require the use of an exogenous transposase source for excision and integration into a genome during the transformation process. This enzyme was supplied on a separate plasmid during the transformation and was not integrated into the *Aedes aegypti* genome alongside with the required genetic elements. The inserted genetic elements have remained stable during rearing of the insects since 2002 (over 115 generational equivalents), the stability of the trait has been assessed through evaluation of the Mendelian inheritance ratios of the offspring as well as molecular techniques (see **OX513A Technical Dossier Part A Section 4 rDNA Insert and characteristics of modification**) These techniques have confirmed that the insert is a single copy that is stably integrated and behaves in a Mendelian fashion during colony breeding.

Note that antibiotic resistant marker genes were not integrated into the final OX513A strain thus there is no potential for HGT of antibiotic resistance genes

(see **OX513A Technical Dossier Part A Section 4.1 Detecting the absence of plasmid backbone in transgenic lines,** and ***Appendix 1*** referenced therein).

Acquisition of genes through oral ingestion

Nucleic acids are present in the cells of plants and animals used for food by humans and animals and are generally recognized as safe (GRAS) by the United States Food and Drug Administration (US-FDA) for food consumption (US-FDA Federal Register Volume 57, May 29, 1992; 22984[8]) as animals do not incorporate DNA into heritable germline cells through ingestion. Accordingly, there is no direct food consumption risk associated with exposure to the endogenous *Aedes aegypti* DNA or the #OX513 construct itself. Further, several studies have addressed the fate of ingested DNA in mammals and birds, including attempts to detect recombinant DNA in chicken (Khumnirdpetch et al., 2001) and in pork (Weber and Richert, 2001), pigs (Klotz et al., 2002), dairy cows, beef steers, and broiler chicken (Einspanier et al., 2001; Flachowsky et al., 2000), all fed with recombinant *Bacillus thuringiensis* corn. In none of those studies was recombinant DNA detectable by PCR in various samples. In reviews on the detection and fate of both recombinant DNA and protein in animals fed genetically engineered crops, Alexander et al. (2007) and Flachowsky et al., (2012) concluded that there were no safety concerns for livestock being fed feedstuffs derived from GM crops. Rizzi et al., (2012) conducted a comprehensive review of the stability and degradation of dietary DNA in the gastrointestinal tract of mammals with a view for implications for horizontal gene transfer and the biosafety of GMOs.

[8] http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Biotechnology/ucm096095.htm (accessed 08/09/2016)

In this review, over 150 relevant papers were cited, and the conclusion was that no study conclusively demonstrated the transfer of recombinant DNA from GM plants to naturally occurring bacterial or host cells in the gastro-intestinal tract of various mammals. Studies to date have focussed almost exclusively on the fate of GM plant material, as GM plants have become so widely integrated into agricultural systems internationally. In a review of risks from GMOs due to HGT by Keese, (2008), the conclusion was that HGT from GM plants poses negligible risks to human health or the environment. Notably, in characterising the pathways for HGT, Keese (2008) differentiated recipient and donor organisms as eukaryotes (i.e. including insects), prokaryotes, or viruses; HGT was examined in the context of multicellular eukaryotes as a whole throughout the review. The pathways, mechanisms, risks, and general conclusions of Keese (2008) may thus be inferred for insects based on the broad characterisation of eukaryotes as a whole in examining risks from GMOs of HGT.

As a supplement to a study principally aimed at assessing the potential for OX513A toxicity through oral exposure (see **OX513A Technical Dossier Part A - Section 5.4 Oral exposure studies)**, *Toxorhynchites spp.* fed on OX513A larvae were additionally subject to molecular analysis to assay for presence of OX513A rDNA in adult *Toxorhynchites spp.* (Nordin et al., 2013). The assay looked for unexpected persistence of OX513A rDNA in adult *Toxorhynchites spp*. as in indication of horizontal gene transfer (HGT). A total of 121 adults gave DNA of sufficient quality to test, as judged by amplification of a control DNA fragment; none were positive for OX513A rDNA. On average each Toxorhynchites larvae consumed 431 OX513A larvae, thus there were over 52,000 events that had the potential for HGT. Nordin et al., (2013) aknowledged that HGT rates are expected to be many orders of magnitude below the limits of sensitivity of this experiment, but the results suggest that no unrecognised high-efficiency mechanism for DNA persistence or transfer was observed through ingestion of OX513A by Toxorhynchites larvae.

Exposure to the gut micro-flora of predators of the mosquito

The gut flora of certain predators could be exposed to low levels of fragmented products of ingested OX513A DNA. Genomic DNA is a component of the diet of all organisms and becomes substantially degraded during digestion in the animal (or human) gastrointestinal tract and has been demonstrated whereby no full-length genes have been detected in the large intestine or in faeces of mammals (Rizzi et al., 2012; EFSA, 2009; Rizzi et al., 2008). Dietary assessments of predator/prey organisms consuming insectivore diets have shown that they are largely generalist organisms and only a small fraction of their diets is a single insect species e.g. (Clare et al., 2009; Durst et al., 2008). It is expected that the concentration and quality of transgenic DNA accessible to gut bacteria will be very low. DNA would be present in the environment mainly as double-stranded DNA and during transport across the bacterial cell wall and cytoplasmic membrane, it is converted to single-stranded DNA. In the bacterial cell, it is assumed to be degraded by exonucleases and restriction endonucleases (EFSA, 2009). It has been suggested that most bacteria living in the intestines of animals, or *Enterobacteriaceae*, are not naturally transformable (Sinha and Redfield, 2012; Johnsborg et al., 2007). The most immediate consequence of DNA uptake is nutritional as it is a source of deoxyribonucleotides (Mell and Redfield, 2014; Redfield, 1993; Barouki and Smith, 1985). These nucleotides are needed for replication of the bacteria's own genome, and require less energy than de novo nucleotide synthesis.

Exposure to micro-organisms associated with parasitoids at the release site

No specific parasitoids are known to be associated with *Aedes aegypti*. The Nematodes *Romanomermis culicivorax* and *Strelkovimermis spiculatus* from the family Mermithidae are generalist parasitoids infecting a number of mosquito species and, whilst these species are known to infect *Aedes aegypti* in the laboratory, they have not been found infecting natural populations (Wise de Valdez, 2007).

Remobilisation of the transposon

Key considerations in order to evaluate the potential for transposon mediated horizontal gene transfer to occur include:

- The ability of the transposon to move within the genome of the host
- The ability of the transposon to recombine in another organism
- Assessment of any benefit which could be conferred by the HGT of the transposon to another organism should the trait successfully integrate.

Insect heritable integration is routinely mediated through the use of Class II transposable elements (transposons) that are short inverted terminal repeat (ITR) transposons that transpose via a DNA-mediated intermediate in a cut-and-paste fashion (Handler and James, 1998). These elements have a transcriptional unit within the ITR sequences that encodes a transposase molecule that acts at or near the termini to effect the stable integration of DNA which is non-autonomous because the gene encoding the transposase enzyme, which is necessary for self-mobilisation, is removed. Therefore, transposon integration requires an external source of transposase for re-mobilisation. The non-autonomous transposon vector used in generating OX513A was the *piggyBac* vector isolated from the cabbage looper moth *Trichoplusia ni*, a vector which has been well studied and used to transform a wide range of insect taxa: Diptera, Lepidopteran, Coleoptera (Labbe et al., 2010; Koukidou et al., 2006; Kuwayama et al., 2006; Handler, 2002; Tamura et al., 2000; Jasinskiene et al., 1998).

Upon introduction into the embryo's germ cells through micro-injection, a transposase-encoding 'helper' plasmid or transposase mRNA, was used to mediate transposition of the *piggyBac* #OX513A construct into the receiving *Aedes aegypti* genome.

The integrated non-autonomous *piggyBac* vector is highly stable in the *Aedes* genome when exposed to exogenous transposase under a wide variety of conditions. Numerous studies indicate that inserted *piggyBac* elements are completely stable and unable to remobilize (Palavesam et al., 2013; Sethuraman et al., 2007; O'Brochta, 2003). Arensburger et al., (2011) has proposed that the stability of the transposons in *Aedes aegypti* is the result of a low proportion of transposon-specific piRNAs. Therefore, transposon mediated remobilization is not expected in OX513A, nor has any instability in the transformed line, OX513A been observed to date in over 115 generation equivalents.

As the transposase coding sequence does not integrate, there is no transposase source available in the genome to facilitate remobilisation of the transgene in subsequent generations.

Conclusion to step 1

The theoretical assumption of risk by horizontal gene transfer is largely based on that observed in prokaryotic bacteria. These mechanisms are not present in eukaryotic

organisms to the same extent and experimental attempts to establish microbial transformation with transgenic DNA under non-laboratory conditions have been uniformly negative or inconclusive (Demaneche et al., 2008; Keese, 2008; Bertolla and Simonet, 1999). This is consistent with inference from genome sequencing – HGT at any significant rate, even on a thousand- or million-year timescale, would so scramble the phylogenetic record that sequence-based phylogenies would be highly locus-dependent, and non-congruent either with each other or with classical taxonomy. Townsend et al., (2012) assessed the probability of detection of horizontal gene transfer events in bacterial populations. They found in relation to monitoring studies of genetically modified plants that only transgenes conferring strong selection co-efficients are likely to be detected, and even if the sample size is massively increased the chance of detecting HGT is not. The detection of HGT is therefore only feasible if there is strong selective pressure which not the case for the introduced transgenes in OX513A. The tTAV self-limiting trait confers conditional cell death at the larval stage, a strong selective disadvantage by design, as OX513A will not survive in the absence of the dietary antidote, tetracycline or its analogues, and the DsRed2 fluorescent marker protein has no known selective advantage to insects. Thus the #OX513 construct would not be expected to confer a selective advantage, but rather have a strong selective disadvantage in the unlikely event that they are transferred to another organism.

The potential for horizontal gene transfer from genetically engineered fruit flies was additionally reviewed in a United States Department of Agriculture (USDA) Environmental Impact Statement (EIS) in 2008 where they concluded that;

"*Exchange of genetic material between insects of different species, and between insects and other organisms, is biological improbable. Insects exchange gametes internally and have complex mating behaviours and structures. Although some higher organisms release genetic material into the surrounding environment, such as pollen or spores, insects are much more conservative in this respect and do not release their gametes freely into the environment*"

(https://www.aphis.usda.gov/plant_health/ea/downloads/eis-gen-pbw-ff.pdf) (Accessed 20/09/2016)

A review of current knowledge with respect to HGT, the nature of the *piggyBac* transformation vector, and the molecular characterisation data on OX513A have been considered in assessing the probability, potential frequency, and the heritability of a HGT event in potential recipient organisms. Additionally, the likelihood that expression of the #OX513 construct would have any biological relevance by providing a selective advantage to a potential recipient organism has been considered.

The key themes in the area of risk for horizontal gene transfer (HGT) have been addressed for OX513A in the receiving environment with the following conclusions:

1. The probability and frequency of HGT, and the potential for heritability of insect DNA in potential recipient organisms in the receiving environment is negligible.

2. The biological relevance of HGT events which may occur at low frequencies, which is directly dependent on the likelihood of further vertical transmission is negligible.

Through Step 1, potential hazards and pathways to exposure have been examined through the evaluation of assessment endpoints by means of literature review. There is adequate information to formulate a conclusion that there is no plausible pathway to harm under

the protection goals identified in Section 1.1, and that further hazard and exposure characterisation is not required.

Step 2-4: Hazard, Exposure and Risk characterisation

The potential for harm, under the protection goals identified in **Section 1.1,** through horizontal gene transfer as a result of the rDNA insert in OX513A has been assessed in step 1. The conclusion is that the potential for harm was negligible in all cases therefore no further Hazard characterisation (Step 2), Exposure characterisation (Step 3), or Risk Characterisation (Step 4) was necessary for this area of risk.

Step 5: Risk management strategies

No further risk management strategies are required for this risk area as no plausible pathway to harm under the protection goals identified in ***Section 1.1*** has been identified. It should be noted that the reduced fertility of OX513A is considered as a mitigation measure (EFSA, 2013) and that measures to avoid escape into unintended environments during production stages are implemented as a standard practice consistent with arthropod containment principles as described in **OX513A Technical Dossier Part B Section 1.4 Containment measures prior to release.**

Step 6: Conclusions

Using the information and evidence presented, it has been concluded in the problem formulation step through the identification of hazard and exposure pathways that the likelihood the deliberate environmental release of OX513A in Saba would represent a plausible pathway to harm through horizontal gene transfer is negligible, thus further hazard and exposure characterisation was not required.

2.3 Pathogens, Infections and diseases

Step 1: Problem formulation (including identification of hazard and exposure pathways)

Under this area of risk, the assessment endpoint is the efficacy of disease transmission of OX513A compared to its non-GM comparator within semi-natural or natural habitats in the receiving environment.

The hypothesis that OX513A or its progeny, will be no more efficacious for disease transmission than a non-GM comparator has been tested.

The key considerations for this risk area which have been taken in evaluating measurement endpoints to test this hypothesis are (based on EFSA 2013):

1. Whether the rearing and release of OX513A could lead to an altered transmission range or frequency of pathogen transmission.
2. Whether the rearing and release of OX513A could lead to the introduction/emergence/selection of new pathogens or pathogen strains with increased virulence.
3. The potential for OX513A to release metabolites that alter the pathogen population (addressed in ***Section 2.7 STEP 3- Exposure through OX513A saliva- the bite of an adult female***).
4. The potential for hazards related to disease transmission to derive from a potential failure of the OX513A rearing and release program.
5. The possibility for OX513A to introduce pathogens to environments were wild *Aedes aegypti* is not present and become a new source of disease.
6. Changes in the interactions with pathogens which result in an altered phenotype in OX513A that lead to increased transmission of pathogens.

Specific areas examined in addressing these considerations are; *Vertical Transmission, Vectorial capacity, Potential Release of Homozygous Females, Emergence of a non-target vector species, Susceptibility to chemical insecticides,* and, *Behavioural responses of OX513A to insecticides.* The potential for expansion of geographic range due to Temperature response may have implications in this area of risk and has been covered in **Section 2.1**.

Vertical Transmission

The passage of a disease causing agent or pathogen from an infected female to its offspring is known as vertical transmission. The vertical transmission of virus from adult female *Aedes aegypti* to offspring has been demonstrated for certain Dengue virus serotypes, and while its potential role in viral maintenance has been recognised, there is no consensus on its significance[9]. The importance of vertical transmission of dengue viruses by mosquitoes has been extensively reviewed (Grunnill and Boots, 2016) and the experimental evidence, mechanistic basis, and mathematical modelling taken collectively led to the conclusion that it is unlikely that vertical transmission is important for the epidemiological persistence of

[9] http://ecdc.europa.eu/en/healthtopics/vectors/mosquitoes/Pages/aedes-aegypti.aspx (accessed 02/09/2016)

dengue viruses. Vertical transmission of DENV 1, 3 and 4 has been reported in the environment (Martins et al., 2012; Mulyatno et al., 2012; Arunachalam, 2008; Thongrungkiat et al., 2003; Fouque and Carinci, 1996; Joshi et al., 1996) and in the laboratory for dengue 3 for *Aedes aegypti* (Joshi et al., 2002) and for dengue 1 for *Aedes albopictus* (Shroyer, 1990).

The capacity for the vertical transmission of DENV 1,2,3,4 and CHIKV was examined for OX513A and WT comparator *Aedes aegypti* females. Artificially infected females were allowed to lay eggs and their progeny reared and tested for live virus at larval stage. For both OX513A and the WT, positive vertical transmission was observed for dengue 1, 3 and 4 but none for DENV 2 and CHIKV; no significant differences were observed however between OX513A and the WT. Additional details are provided in **OX513A Technical Dossier Part A Section 5.7 Vertical transmission of Dengue and Chikungunya viruses in OX513A** and published in Mansor et al. (2016)

Additionally, it was previously reported that OX513A and WT females did not lay significantly different numbers of eggs when fed with non-infected blood (Lee et al., 2009b). In Mansor et al. (2016) females of both OX513A and WT were provided with an infected blood meal and it was concluded that there was no significant difference in vertical transmission rates between the strains, as well as in the fecundity (number of eggs laid per female). This result is similar to previous studies (Maciel- de- Freitas et al., 2011) and results obtained on other parasite/host systems (Hurd, 2001).

No other changes in OX513A phenotype have been observed that would suggest an enhanced ability to transmit diseases when compared to a WT counterpart.

It can be concluded that OX513A does not have an increased capacity for vertical transmission of the viruses tested in the adult females to the progeny compared to its wild type counterpart.

Vectorial capacity

In addition to vertical transmission the ability to transmit disease (vector capacity) has other entomological components, including the vector biting rate, vector density, vector survival and the duration of the virus extrinsic incubation period (EIP) in the mosquito, hence the longevity of the mosquito is an important component of vectorial capacity, as the shorter the lifespan the less likely to transmit disease. In order to transmit disease, the mosquito must live long enough to pick up the virus via a blood meal, survive the extrinsic incubation period and then pass on the virus during a subsequent blood meal. Consequently, the longer-lived the females are the more likely they are to transmit pathogens (Styer et al., 2007; Dye, 1992). The average EIP depends on temperature but is 15 days at 25^{o}C and 6.5 days at 30^{o}C (Chan and Johansson, 2012)

A potential exists for hemizygous OX513A female progeny to survive due to the reported incomplete penetrance of the self-limiting trait (Phuc et al., 2007). However as detailed in **OX513A Technical Dossier Part A Section 5.2.7 Non-penetrant OX513A progeny- Longevity and Fecundity** in fact median survival of non-penetrant females is significantly reduced (2 days vs 68 days). Approximately 20% do survive long enough to take two blood meals and some produced two clutches of eggs, however in no instance did non-penetrant OX513A hemizygous females survive longer that the wild-type *Aedes aegypti* counterpart. Thus, the disease transmission ability is even expected to be reduced compared to wild type mosquito.

The longevity of female heterozygotes surviving in the environment due to incomplete penetrance of the tTAV trait is not increased relative to their wild-type counterpart.

Potential Release of Homozygous Females

Mechanical size separation will be used to separate sexes as female pupae are larger than males (Ansari et al., 1977). As detailed in Standard Operating Procedure ***TD-SOP-00293 OX513A Sex Sorting of Pupae for Release***, samples of 1000 pupae are taken subsequent to mechanical sorting, and if the threshold of more than 2 pupae are detected in the sample of 1000, the batch is re-sorted prior to advancing to the eclosion phase. Data from previous OX513A regulated environmental releases demonstrates a sorting accuracy of >99.9% is routinely achieved in operational scale projects (Carvalho et al., 2015; Gorman et al., 2015; Harris et al., 2012).

Focks et al., (2000) attempted to estimate transmission thresholds for dengue based on the standing crop of *Aedes aegypt*i pupae per person in the environment. Recording the number of pupae per person in an area was considered the best way of estimating the actual adult mosquito population in a given area due to the low mortality of pupae compared to larvae. The thresholds calculated by Focks et al. (2000) can be used in the assessment of risk of transmission, and to inform the actual degree of suppression required to prevent or eliminate transmission. The assumption is that the introduction of a few infectious individuals into a community of susceptible individuals will not give rise to an outbreak unless the density of vectors exceeds a certain critical level, and is dependent on temperature (a key virus and mosquito development driver), and sero-prevelance in the local human population. The magnitude of new viral introductions into an area also would clearly impact the likelihood for an epidemic to occur and must be considered in modelling transmission thresholds. An example is given in **Table 4** (reproduced from Focks et al., 2000) as to how this model can be used to generate values for use in informing suppression programs. In this example the number of pupae/person required to result in a 10% or greater rise in sero-prevelance of dengue antibody over a one-year period resulting from 12 monthly viral introductions of a single viremic individual is estimated.

Table 4. Threshold for dengue transmission in pupae/person above which dengue transmission is more likely to occur than not occur. It describes the dependence of this threshold on temperature and host sero-prevalence. Reproduced from Focks et al. (2000)

Temperature (°C)	Transmission threshold by initial seroprevalence of antibody		
	0%	33%	67%
22	7.13	10.70	23.32
24	2.20	3.47	7.11
26	1.05	1.55	3.41
28	0.42	0.61	1.27
30	0.10	0.15	0.30
32	0.06	0.09	0.16

* In a series of simulations in the dengue simulation model, these values resulted in a 10% or greater increase in prevalence approximately 50% of the time.

The data on sorting efficiency of the mechanical sorting process can provide an estimate of the number of adult homozygous OX513A females potentially released during a sustained

release program. The corresponding number of pupae can then be estimated based on assumptions and calculation described in Focks et al. (2000) allowing an estimation as to whether the additional females released could be expected to contribute to the adult female standing crop with any significance to disease transmission.

Potential rates of OX513A females co-released in the environment have been calculated and transformed to pupae/person equivalent for a range of potential release rates (30-300 males/person/week) based on current operational guidance. Adaptive release rates based on continual population monitoring however allow current programs to minimize the release of males in excess of that needed for population suppression.

Table 5 summarizes calculations made for a daily survival probability (DSP_{ad}) range of 0.50 to 0.89 (Focks et al., 2000) with the following additional assumptions:

- OX513A Adult female standing crop; Calculated based on a mechanical sorting efficiency of 0.066 % taken from the highest published value from the Cayman Islands releases reported in Harris et al. (2012), and based on the average estimated over 42 days following 25 days of successive releases once the released female population can be assumed to be stabilized.
- Pupae development time = 2 (days)
- Sex Ratio of pupae = 0.5
- Rate of successful emergence: 0.83 (based on Focks et al., 2000)

Table 5. Potential rates of OX513A females co-released in the environment (transformed in pupae/person equivalent) depending on different male release rates and survival (DSP_{ad}).

DSP_{ad}	30 males /person/week	150 males /person/week	300 males /person/week
0.89	0.014	0.068	0.135
0.80	0.007	0.037	0.075
0.70	0.005	0.025	0.050
0.60	0.004	0.019	0.037
0.50	0.003	0.015	0.030

The daily survival probability (DSP_{ad}) of the very few potential co-released OX513A females is expected to be much lower than the 0.89 estimate of Focks et al (2000) for wild females due to the reduced longevity of multi-generation laboratory colonised strains. During a suppression programme, release rates as high as ~300/OX513A males/person/week are foreseen. Once suppression is observed in the treated areas, the releases of males will be reduced towards a maintenance phase level of release (e.g. ~30 males/person/week) aiming to prevent re-infestation, consequently, the number of potential females released will be reduced by as much as 10 fold (from an initial release rate of 300 males/person/week).

As suppression of the local *Aedes aegypti* population progresses in the release area, the number of total OX513A released would decrease and in the context of elimination of the

local *Aedes aegypti* population, the ratio of released OX513A : local wild *Aedes aegypti* is expected to increase as the population tends towards elimination. The presence of female OX513A *Aedes aegypti* representing a greater proportion of the total *Aedes aegypti* in this context does not represent an increase in the potential hazard due to pathogens, infections and diseases, as parameters examined in OX513A females do not indicate a greater vectorial capacity including longevity and temperature range, nor capacity for vertical transmission of virus than a relevant comparator.

The inadvertent co-release of OX513A mosquito females in the environment at the highest observed rates of female co-release, across a range of operational release rates would not represent a significant contribution to the local female mosquito population sufficient to increase virus transmission under the conditions encountered in the receiving environment.

Emergence of a non-target vector species

Aedes albopictus, has not been reported in Saba, but has been identified as a potential public health threat to Saba and placed on the Preliminary Alert List for the Leeward and Windward Dutch Islands (See ***Appendix 14*** *Key Elements Towards a Joint Invasive Alien Species Strategy for the Dutch Caribbean*).

If *Aedes albopictus* where to arrive in Saba, the potential for *Aedes albopictus* to displace existing *Aedes aegypti* in the receiving environment during the OX513A program, or become established after elimination targets have been achieved, and result in an increased hazard of arbovirus transmission in Saba compared to existing control measures has been examined by evaluating:

- If *Aedes albopictus* is more likely to occupy the *Aedes aegypti* ecological niche if *Aedes aegypti* numbers are reduced through the OX513A program compared to existing control measures; and,
- if *Aedes albopictus* represents a greater threat for arbovirus transmission that *Aedes aegypti*

The intended effect of the OX513A program is to target exclusively the *Aedes aegypti* population in the receiving environment thereby reducing the wild population. The species specific nature should thus have the potential to tend towards local elimination of the *Aedes aegypti* population, in the absence of immigration or re-introduction. As *Aedes albopictus* is one of the most closely related *Aedes* species to *Aedes aegypti* (Shepard et al., 2006) it has been suggested that *Aedes albopictus* ecology should be considered alongside *Aedes aegypti* in developing arbovirus control programs as this approach could increase the chance that *Aedes albopictus* displaces *Aedes aegypti*, (e.g. Miller and Loaiza, 2015).

It is often cited that that the ecological niche of *Aedes aegypti* is distinct from *Aedes albopictus*, whereby *Aedes aegypti* persists predominantly as an urban vector, breeding in artificial containers and feeding almost exclusively on humans (Powell and Tabachnick, 2013) and *Aedes albopictus* is described as predominantly associated with peri-urban and rural environments (Hawley, 1988) and in addition to humans, feeds on a variety of mammalian and avian species (Delatte et al., 2010). The global distribution of both species are known to be influenced by environmental factors such as temperature and demographic factors such as urbanisation. Kraemer et al., (2015a) examined documented occurrences of both

arbovirus vectors between 1960 and 2014 in compiling a global compendium of their occurrence, and while their global distribution is overlapping, the distinct nature of their ecological niches remains widely recognised across their distribution (Kraemer et al., 2015b).

As both *Aedes aegypti and Aedes albopictus* are container breeding species, capable of feeding on human hosts, a significant degree of habitat overlap could intuitively be expected in proximity to human habitation, and larval cohabitation of the two species is frequently observed (e.g. Camara et al., 2016; Serpa et al., 2013; Piovezan et al., 2012; Braks et al., 2003). It has been widely documented that the introduction and establishment of *Aedes albopictus* in an area has been associated with a concurrent decline in the population of *Aedes aegypti* (e.g. Reiskind and Lounibos, 2013; Raharimalala et al., 2012; Honório et al., 2009) and studies have been undertaken to evaluate both the impact of climactic and demographic factors. For example, Miller and Loaiza (2015) mapped the temporal expansion of *Aedes albopictus* in Panama to determine the ecological and non-ecological factors associated with its expansion, and came to a strong conclusion that road networks alone best explained the geographic expansion of *Aedes albopictus* across Panama. Leisnham and Juliano (2012) examined evidence for how climate, land use, and biological invasions may have direct abiotic and indirect community level impacts on immature developmental stages of *Aedes* mosquitoes, and suggested that climate and land use changes may be altering ecological interactions among Aedes species at the finer scale of individual water-holding containers. *Aedes aegypti* and *Aedes albopictus* populations have been the subject of long term monitoring in Florida (Britch et al., 2008) who identified that *Aedes albopictus* might be exploiting unoccupied or more marginal habitat than the habitat containing *Aedes aegypti* and this is particularly correlated with wet and dry periods, with *Aedes aegypti* being favoured in dry periods and both species increasing in the wet periods; this conclusion is supported in a further study conducted in urban Florida (Leisnham and Juliano, 2009). Lounibos et al. (2010) also identify that hotter and drier conditions are thought to favour *Aedes aegypti* as the eggs are more tolerant to desiccation. In the context of Saba, in considering the potential for *Aedes albopictus* to occupy the *Aedes aegypti* ecological niche if *Aedes aegypti* numbers are reduced, the assumption is thus made that the climactic, land use, and non-ecological factors would support the expansion of *Aedes albopictus* on Saba, and thus the focus of the analysis is on the potential role of inter-specific competition in the documented *Aedes aegypti* micro-habitat on Saba.

Aedes aegypti is generally understood to be competitively inferior to *Aedes albopictus*. Fader, (2016) reviewed the interspecific interactions between *Aedes albopictus* and various co-occurring species in North America. While the persistence of inferior competitors in predator rare habitats was acknowledged by Fader (2016), and the context dependence of biotic interactions in driving asymmetrical competition has been reviewed to better understand these interactions (Juliano, 2009), *Aedes albopictus* is consistently a superior competitor to essentially all native and resident species in the United States. Interspecific larval competition is thought to be the main factor that determines the abundance of mosquito species in sites with limited availability of food resource (Piovezan et al., 2012 and references therein) and multiple investigations have been undertaken which support the notion that resource availability is a consistent factor in driving competitive displacements of *Aedes aegypti* by *Aedes albopictus.* This is further supported by Leisnham and Juliano (2012) (and references therein) who note that that populations of Aedes in containers are

often resource limited and propose that competition for microbial food is likely the strongest ecological process structuring communities.

Braks *et al* (2003) examined the effects of species composition, larval density, and resource, on larval competition between *Aedes aegypti* and *Aedes albopictus* in field populations in Brazil, effectively replicating earlier studies in Florida (Juliano, 1998) but with differences principally in environmental conditions and nutrient source. While the populations in Brazil and North America were noted to have evolved from independent introductions, the conclusion was that *Aedes albopictus* was clearly the superior competitor in the Brazilian experiments, which aligned with the results observed in Florida. The effects of larval competition have further been examined with regards to the longevity of adult *Aedes aegypti* and *Aedes albopictus* (Reiskind and Lounibos, 2009), and significant intraspecific density dependant effects have been observed for *Aedes aegypti*, but not for *Aedes albopictus,* supporting the notion that indirect competition, such as through larval density or resource availability, is a principle driver of population displacements observed. Additionally, Yee (2016) undertook a review of documented responses of *Aedes albopictus* to different larval environments in order to better understand the larval ecology of *Aedes albopictus* in the United States as related to the use of habitats under different nutrient environments. *Aedes albopictus* was concluded to have a feeding niche wider than most resident species, and that this may be a major factor in the establishment of *Aedes albopictus* in the United States.

Based on observations of *Aedes albopictus,* Juliano and Lounibos (2005) modelled the effects of resource competition and predation as barriers to invasion, evaluating which community and ecosystem characteristics favour invasion. As superiority in interspecific competition is often listed as a characteristic of non-native species that enhances the likelihood of becoming invasive, resource competition, chemical or physical interference, and mating interference where identified as key mechanisms in producing negative effects on the focal population (e.g. *Aedes aegypti*). While mating interference was noted as unlikely to explain displacements of *Aedes aegypti* by *Aedes albopictus*, and the role of chemical interference as warranting further investigation, the effects of resource competition, as a principle driver in interspecific competitive displacement was well documented.

Taken together, the predominant weight of evidence suggests that the displacement of *Aedes aegypti* by *Aedes albopictus* is driven by the capacity of *Aedes albopictus* to outcompete *Aedes aegypti* for resource in the larval micro-habitat. The relatively low reported densities of *Aedes aegypti* on Saba (see **OX513A Technical Dossier Part B Section 2.2.1 Habitat of Aedes aegypti in Saba**), suggest a resource unconstrained environment in at least part of the available micro-habitat, which is not unexpected as the majority of inhabitants are reported to use rainwater collection and cisterns for water storage (see ***Appendix 15*** *"Towards a design for an improved drinking water supply system on Bonaire, St Eustatius and Saba*2). The establishment of *Aedes albopictus* in this environment could be expected to lead to co-habitation, as has been described by Rey et al. (2006) who in examining defined habitat variables and their influences on interspecific associations between the two Aedes species reported a significant container effect where-by *Aedes aegypti and Aedes albopictus* co-occurred more often in tubs than in cups. The larger relative volume of water in the tub container compared to the cup was attributed to a greater co-occurrence, implying less larval competition. If *Aedes aegypti* numbers were to increase and tend towards a habitat which was resource constrained, the introduction of Aedes albopictus in the area could

lead to its establishment through resource dependant larval competitive displacement. The capacity of *Aedes albopictus* to competitively displace *Aedes aegypti* in the larval environment, suggests that the habitat is already favourable to *Aedes albopictus* establishment, assuming other habitat and climatic variables were favourable.

On-going vector control methods in Saba through the use of larvivorous fish in breeding sites, as well as the application of products based on *Bacillus thuringiensis* are intended to reduce the *Aedes aegypti* population in a targeted manner, and will equally serve to target *Aedes albopictus* were it to arrive in Saba. The more precise species specific approach of the OX513A control program would not alter the larval microhabitat in such a way as to preferentially favour the establishment of *Aedes albopictus*.

Specific to the OX513A program, Gorman et al. (2015) demonstrated *Aedes aegypti* population suppression in releases of OX513A in Panama as summarised in **OX513A Technical Dossier Part A Section 7.1.5 Panama - Nuevo Chorillo.** Experimental goals also included an assessment of the effects on a co-existing population of *Aedes albopictus. Aedes albopictus* abundance, measured over an 84 day period commencing when significant *Aedes aegypti* population control began, was unaffected by a sustained reduction of *Aedes aegypti* by up to 93 % during the study period.

Potential for increased virus transmission by Aedes albopictus were it to arrive in Saba.

Juliano and Lounibos (2005) described an invasive mosquito that replaces a resident species via competition or apparent competition, as having the capacity to alter disease transmission if it is either a more or less efficient disease vector. Both *Aedes aegypti and Aedes albopictus* can transmit several arboviruses as has been comprehensively reviewed by Conway et al. (2014) where *Aedes aegypti* is described as the primary vector of dengue (DENV), chikungunya virus (CHIKV), and yellow fever virus (YFV), and *Aedes albopictus* as a competent vector for DENV and CHIKV but which typically leads to milder epidemics than *Aedes aegypti*. More recently, the principle vector associated with the 2015 Zika virus epidemic is identified as *Aedes aegypti,* with *Aedes albopictus* also becoming established as a competent vector (Vorou, 2016), and is described by the World Health Organisation (WHO) as a *"a proven or potential vector in some settings"* (WHO, 2016). Moore and Mitchell, (1997) in examining the public health implications of the ten-year presence of *Aedes albopictus* in the U.S., concluded that there was no evidence that *Aedes albopictus* is the vector of human disease in the United States. A comprehensive review by Gratz (2004) of the vector status of *Aedes albopictus* more precisely describes *Aedes albopictus* as a competent vector for DENV and other arboviruses of significance to human health, but that there was no evidence that *Aedes albopictus* is an important urban vector of dengue except in a limited number of countries where *Aedes aegypti* is absent. Evidence supported the possibility that *Aedes albopictus* may serve principally as a maintenance vector of arboviruses in rural areas. The notion that the replacement of *Aedes aegypti* by *Aedes albopictus* would not have a significant effect on dengue virus transmission is further supported by Lambrechts *et al*., (2010), who performed a meta-analysis of laboratory studies, as part of a broader review of the past role of *Aedes albopictus* in dengue epidemics.

The key parameters used to assess the potential for transmission of arboviruses by insect vectors are:

Vector competence which is a vector's biological capability to transmit a virus, and;

Vector capacity which is the efficiency with which the mosquito transmits a disease, based on things like preferred host, the number of feedings per cycle of egg production, its longevity, the density of the mosquito population, among other factors.

The WHO describes the vector competence of *Aedes aegypti* and *Aedes albopictus* is similar in general[10], although *Aedes albopictus* is considered to have lower vector capacity than *Aedes aegypti* for transmitting arboviruses, including Zika. Vector Competence of *Aedes aegypti* and *Aedes albopictus* for DENV was examined for colonies derived from populations from Florida Keys for example (Richards et al., 2012) where no significant differences were observed in rates of infection or dissemination between *Aedes aegypti* or *Aedes albopictus*. Similarly, Alto et al. (2014) examined the susceptibility of Florida *Aedes aegypti* and *Aedes albopictus* to dengue viruses from Puerto Rico and results did not demonstrate any increased risk of transmission by *Aedes albopictus* of DENV isolates from Puerto Rico.

The vector competence of 35 American *Aedes aegypti* and *Aedes albopictus* mosquito populations for three CHIKV genotypes has been assessed (Vega-Rua et al., 2014) and it was found that while both mosquito species were capable of transmitting all three CHIKV genotypes, *Aedes albopictus* was a better transmitter of only the epidemic mutant strain CHIKV_0621 of the East-Central-South African genotype. Chouin-Carneiro et al. (2016) evaluated the vector competence of *Aedes aegypti* and *Aedes albopictus* through oral exposure to an Asian genotype of ZIKV, the study suggested that although susceptible to infection, *Aedes aegypti* and *Aedes albopictus* were unexpectedly low competent vectors for ZIKV. Given the conclusion of the study that *Aedes aegypti* and *Aedes albopictus* exhibit similar transmission potential for ZIKV, the presence of *Aedes albopictus* would not represent an increased threat for Zika virus transmission.

Although *Aedes albopictus* is not currently reported as present on Saba, were it to appear there is no evidence that *Aedes albopictus* is a more competent vector for arboviruses of significance to human health, than is *Aedes aegypti.* The introduction and rapid establishment and spread of *Aedes albopictus* has not been correlated to explosive epidemics of DENV, CHIKV, or ZIKV as has been reported for *Aedes aegypti.*

Taken together, the weight of evidence suggests that an OX513A release program would be unlikely to alter the transmission of pathogens and diseases in the context of the emergence of a non-target vector species, compared to current control methods.

Susceptibility to chemical insecticides

Chemical insecticides are one of the management practices used to prevent the spread of mosquito vectors of disease. Susceptibility to chemical insecticides is an important feature for OX513A, as chemical insecticides can be used as part of a risk management strategy for rapid elimination of the OX513A strain from the environment if so desired.

[10] http://www.euro.who.int/__data/assets/pdf_file/0007/304459/WEB-news_competence-of-Aedes-aegypti-and-albopictus-vector-species.pdf (accessed 02/09/2016)

A study commissioned in 2011 by Oxitec, performed by the Liverpool School of Tropical Medicine, LSTM (**OX513A Technical Dossier Part A Section 5.2.3 Susceptibility to chemical insecticides**), tested the susceptibility of the OX513A strain relative to a wild-type comparator, to five commonly used insecticides using standard WHO procedures and discriminating doses[11] of: temephos, permethrin, deltamethrin, bendiocarb , and malathion. Additionally, OX513A was screened using molecular techniques for the presence of knock-down (kdr) mutations 1016 and 1534, which are associated with resistance to pyrethroids and DDT.

OX513A strain was found to be susceptible to discriminating doses of temephos, permethrin, deltamethrin and malathion, and it showed some resistance to bendiocarb. The level of resistance to bendiocarb in OX513A was comparable to that seen in the NEWORLEANS (control) strain. The NEWORLEANS strain is a long-standing laboratory strain that is considered susceptible to all known insecticides and was originally colonized by the Centre for Disease Control and Prevention (CDC) Atlanta, USA. This NEWORLEANS strain is an accepted standard in susceptibility assessments and continues to be widely used throughout the world. For the NEWORLEANS strain, none of the observed test results other than those for bendiocarb deviated from the values expected when assessing a fully-susceptible strain using the World Health Organization's recommended discriminating concentrations (i.e. 100% mortality). Therefore, there was no reasonable justification for suspecting that the integrity of the NEWORLEANS strain had been compromised (as results would likely have been skewed for more than just a single compound). In addition, the fact that the bendiocarb results observed for both OX513A and NEWORLEANS strains remained equal, thus OX513A was no more susceptible to bendocarb that the wild type comparitor. Results thus indicate that the WHO-recommended bendiocarb discriminating dose (0.1%) may not be not appropriate for *Aedes aegypti.* OX513A was also genotyped for two kdr mutations that are associated with pyrethroid and DDT resistance, in the same study. Results showed that these mutations were not observed in OX513A.

A separate study was conducted in Malaysia by Nazni et al., (2009b) comparing the susceptibility of the *Aedes aegypti* strains MyRIDL-513A[12] and MyWT results are summarised in **OX513A Technical Dossier Part A Section 5.2.3 Susceptibility to chemical insecticides**. Seven insecticides (DDT, fenitrothion, malathion, propoxur, permethrin, lambdacyhalothrin, cyfluthrin) were tested on adult mosquitoes following standard WHO methods. There were slight differences in the susceptibility of insecticides between the two strains, as MyWT was tolerant to propoxur and fenitrothion, whereas the MyRIDL513A strain was fully susceptible to both chemicals. Additionally, some level of resistance to DDT was detected in both strains, which the authors of the study attributed to the Malaysian genetic background shared by both strains (since use of DDT in the past in Malaysia caused the dissemination of resistance alleles in *Aedes aegypti* populations).

OX513A susceptibility to insecticides is thus consistent with that of wild type comparator, and known mutations associated with insecticide resistance could not be detected in OX513A.

[11] http://whqlibdoc.who.int/hq/2006/WHO_CDS_NTD_WHOPES_GCDPP_2006.3_eng.pdf (accessed 02/09/2016)

[12] The MyRIDL-513A strain was generated by out-crossing the original OX513A strain to the Malaysian MyWT strain. The resulting offspring (strain MyRIDL-513A) contains the genetic modifications associated with OX513A in a Malaysian genetic background.

Behavioural responses of OX513A to insecticides

The use of chemical pesticides is a widespread practice in disease-endemic countries, aimed at controlling the populations of arthropod vectors such as mosquitoes. Therefore, field releases of OX513A are likely to occur in areas where strategies such as indoor-residual spraying (IRS), insecticide treated bed nets (ITN), and/or ultra-low volume spraying are being used.

Because the success of an OX513A based strategy depends on the capacity of OX513A males to find and mate wild females in the release areas, it is important to understand how they will react to the presence of chemical irritants in the environment. To address this, a study was conducted to determine whether the behavioural responses of OX513A mosquitoes (strain OX513A-My, a derivate of the OX513A strain in a Malaysian genetic background) would be comparable to that of wild type strains (Kongmee et al., 2010). See **OX513A Technical Dossier Part A Section 5.2.4 Behavioural responses of OX513A to insecticides** for a summary.

Adult mosquitoes of two age groups (4-5 days and 8-10 days) were subject to contact and spatial irritancy assays and similar behavioural responses were observed between OX513A and a wild type comparator and no differences were observed.

Altered behavioural responses to certain chemical pesticides in OX513A relative to a wild type comparator were not observed

Rearing practices

Rearing management practices and release procedures are described and are highly unlikely to contribute to the introduction of pathogens into the receiving environment given that quality management procedures are in place to ensure OX513A quality for use in suppression programmes, and routine quality control testing is done to ensure conformance with product specifications. (See **OX513A Technical Dossier Part A Section 5.8.1 OX513A quality control**). More information on management practices can be found in **OX513A Technical Dossier Part B Section 1 Details of the proposed release on Saba.**

Blood feeding females for egg production in the UK facility

In the UK rearing facility, prior to the shipping step described in **OX513A Technical Dossier Part B Section 1.4 Containment measures prior to release.** OX513A eggs are produced under Containment Level 1 (CL1) conditions as regulated by the UK Health and Safety Executive (HSE) under *The Genetically Modified Organisms (Contained Use) Regulations 2014*. The facility is inspected at periodic intervals by the HSE for compliance with these regulations. HSE conducted the last inspection in 2013 and, from a verbal close out meeting, no deficiencies were noted.

Blood feeding of females is required in mass egg production. Animal blood (defibrinated horse blood, TCS Biosciences Ltd) is used in a heated membrane feeding system as the source of blood meals for the female mosquitoes. An aluminum plate is sealed on one side with a thin membrane such as Parafilm and blood is added between the membrane and the aluminum plate. The plate is then placed membrane side down on top of the cage and a heat source provided to heat the blood to approximately 37°C. Female mosquitoes readily feed through the mesh of the cage and engorge on blood. The animal blood is supplied through an authorized supplier and is tested for quality control including sterility and haemolysis.

Defibrinated blood is collected using sterile apparatus and processed aseptically from a closed herd of healthy horses permanently housed in the UK, under regular veterinarian supervision, that are screened for equine infectious anemia (EIA) and equine viral arteritis (EVA) among other pathogens, to minimize the potential for contamination of the blood by virus, bacteria, or other pathogenic agents. In the future, mosquito breeding requirements could require testing of blood for arboviruses, but at this time the host range of *Aedes aegypti* and *Aedes albopictus* does not extend to the UK (Kraemer et al., 2015b) so the risk of transmission of arbovirus such as dengue and chikungunya to these horses is negligible. As a result, the blood collected from the horses would be free of such arboviruses.

Conclusion to step 1

The key considerations for this risk area have been addressed with the following conclusions:

1. The likelihood that the rearing and release of OX513A could lead to an altered transmission range or frequency of pathogen transmission is negligible.
2. The likelihood that the rearing and release of OX513A could lead to the introduction/emergence/selection of new pathogens or pathogen strains with increased virulence is negligible.
3. The potential for OX513A to release metabolites that alter the pathogen population (addressed in ***Section 2.7*** *Impact on human and animal health Step 3 Exposure characterisation*) is negligible.
4. The potential for hazards related to disease transmission to derive from the possible malfunctioning of the OX513A rearing and release programme is negligible.
5. The possibility for OX513A to introduce pathogens to environments were wild *Aedes aegypti* is not present and become a new source of disease is negligible.
6. The likelihood that changes in the interactions with pathogens could result in an altered phenotype in OX513A that leads to increased transmission of pathogens is negligible.

Through Step 1, potential hazards and pathways to exposure have been assessed through measurement endpoints. There is adequate information to formulate a conclusion that there is no plausible pathway to harm under the protection goals identified in Section 1.1, and that further hazard and exposure characterisation is not required.

Step 2-4: Hazard, Exposure and Risk characterisation

The potential for harm, under the protection goals identified in **Section 1.1** through pathogens infections and diseases as a result of the rDNA insert in OX513A has been assessed in step 1. The conclusion is that the potential for harm was negligible in all cases therefore no further Hazard characterisation (Step 2), Exposure characterisation (Step 3), or Risk Characterisation (Step 4) was necessary for this area of risk.

Step 5: Risk management strategies

No further risk management strategies are required for this risk area as no plausible pathway to harm under the protection goals identified in ***Section 1.1*** has been identified. It should be noted that the reduced fertility of OX513A is considered as a mitigation measure (EFSA, 2013) and that measures to avoid escape into unintended environments during production

stages are implemented as a standard practice consistent with arthropod containment principles as described in **OX513A Technical Dossier Part B Section 1.4 Containment measures prior to release.**

Step 6: Conclusions

Using the information and evidence presented, it has been concluded in the problem formulation step through the identification of hazard and exposure pathways that the likelihood the deliberate environmental release of OX513A in Saba would represent a plausible pathway to harm through pathogens infections and diseases is negligible, thus further hazard and exposure characterisation was not required.

2.4 Interaction with target organisms

Step 1: Problem formulation (including identification of hazard and exposure pathways)

Under this area of risk, the assessment endpoint is the intended interaction between OX513A and the target organisms, wild *Aedes aegypti,* in the receiving environment. The interaction of OX513A with the target organism (TO) is the intention of the deliberate release. It is the intended impact of the mating that the hybrid offspring will fail to develop to maturity.

The hypothesis that OX513A will interact with target organisms (the *Aedes aegypti* mosquito) at the release site as intended and that the interaction with target organisms does not represent a plausible pathway to harm under the protection goals identified in ***Section 1.1*** has been tested.

The key considerations for this risk area which have been taken in evaluating assessment endpoints to test this hypothesis based on EFSA (2013) guidance regarding *Effects of suppression releases and preventative releases* are:

1. Has resistance to the conditional insect control trait been observed before?

2. Reduction in efficacy or resistance development in the target organisms against the GM insect mediated effect

3. Changes in interactions with target organisms arising from an altered genetic diversity of a reared GM insect population that may result in adverse effects.

4. Effects on target organisms due to the release of low-quality GM insects or non-GM insects that may result in adverse effects

Existing resistance to the OX513A trait

To date, resistance to the OX513A self-limiting trait has not been observed in over 115 generational equivalents reared since 2002. Quality assurance testing is undertaken at regular intervals on key parameters as described in **OX513A Technical Dossier Part A - Section 5.8.1 OX513A quality control.** Vector control organisations in areas where OX513A releases have taken place (referenced below), continue to monitor and control wild *Aedes aegypti* populations using existing interventions, and as part of Integrated Vector Management (IVM) programmes and resistance to the self-limiting trait has not been reported.

Development of resistance to the OX513A trait

The development of resistance of wild *Aedes aegypti* to the OX513A insect control trait is considered here as an effect on the target organism. Deliberate releases of OX513A have been conducted since late 2009 in collaboration with partners in both vector control programs and academia (see ***OX513A Technical Dossier Part A Section 7.1 Previous Aedes aegypti vector control projects using OX513A***) and significant (>90% based on egg counts) population suppression has been consistently observed in vector control projects.

IVM is the preferred approach to improving vector control (WHO, 2012). An IVM approach requires complimentary tools that in combination improve the efficacy, cost effectiveness,

ecological profile and sustainability of vector control interventions. Resistance management is a key outcome of IVM programmes, and vector control programs have engaged in evaluating OX513A as a tool for use in IVM approaches. The establishment of resistance in general, such as for insecticides, is well understood to be linked to direct selective pressures such as the continued application of an insecticide, and the period of the release is unlikely to be of sufficient duration to effect a change in the target population. The management of resistance to the OX513A trait is ultimately anticipated to be managed in conjunction with existing control measures for *Aedes aegypti* in the context of a broader IVM program.

As OX513A control programs are ongoing, population control data will continue to contribute to establishing a baseline standard for population control. The potential impact, depending on the prevalence of resistance should it occur, could ultimately result in failure of the program. Deviations in levels of population control may be indicative of control failure and warrant further investigation to establish if control failure was indeed due to resistance to the OX513A trait.

Change in the target organism population

Existing control programs attempt to control *Aedes aegypti,* thus population suppression is an accepted and intended consequence of any control program. *Aedes aegypti* is not native to the release area (See **OX513A Technical Dossier Part B - Section 2.2.2 Functions of *Aedes aegypti* in the ecosystem of Saba**) thus a reduction in population size could help to restore the environment to the state prior to the establishment of the non-native pest (EFSA, 2013).

Variability between the laboratory reared OX513A and the wild type mosquito strain

Quality control procedures are in place (**OX513A Technical Dossier Part A - Section 5.8.1 OX513A quality control)** to assess OX513A mating competitiveness against a baseline to ensure OX513A performs as specified for use in population control programmes. As the effect on target organisms is intended cell death at the larval stage of the offspring, any effect of variability in the OX513A laboratory reared population on the wild population would not be propagated. A key variable which would have an immediate effect on the wild population is the capacity of OX513A to mate with local *Aedes aegypti* and produce offspring.

Mating competitiveness studies for OX513A against wild-type strains from around the world have been carried out in a wide variety of laboratory settings with collaborative partners internationally ***(OX513A Technical Dossier Part A Section 5.1.3.1 Mating competitiveness in the laboratory)***. Additionally, various reproductive parameters, including insemination capacity and cost of mating have been examined in (***OX513A Technical Dossier Part A - Section 5.1.2 Insemination capacity and cost of mating***).

No variability was observed which could be expected to have an unintended impact on the target *Aedes aegypti* population. A reduced mating competitiveness, or variability in reproductive parameters, which could manifest as a reduction in overall control program efficacy for example has not been observed.

Quality of laboratory reared strain and potential consequences of the release of female mosquitoes

EFSA (2013) suggests that impacts on target organism could occur from unintended releases of untransformed fertile reared individuals, i.e. significant proportions of females when male-only releases are intended, or from insects contaminated with parasites or pathogens.

OX513A colonies are reared in a highly controlled environment under a quality management program to ensure that males released are of optimal health and quality to compete for mating wild females. This is integral to OX513A program delivery and key to success. Colonies are continually monitored and routinely subject to quality control testing for multiple parameters (See ***OX513A Technical Dossier Part A Section 5.8.1 OX513A quality control***).

In an OX513A release program, there is the potential for small quantities of two types of female mosquito to be present in the environment; the first is inadvertent co-release of homozygous (OX513A) females with homozygous males, and the second is hemizygous OX513A progeny of released males that have mated wild females and survive as a consequence of the incomplete penetrance of the self-limiting trait. The measurement endpoints for this potential area of risk are:

a) Sorting efficiency of males and female *Aedes aegypti* OX513A in large scale production; and

b) Survival of female heterozygotes.

These two items are discussed briefly below:

a) Sorting efficiency of males and female Aedes aegypti OX513A in large scale production;

The rearing of *Aedes aegypti* OX513A to adults involves the physical separation of male and female pupae as the female pupae are larger than the male pupae (sexual dimorphism). This is achieved using mechanical sorting as detailed in Standard Operating Procedure ***TD-SOP-00293 OX513A Sex Sorting of Pupae for Release***. Subsequent to mechanical sorting samples of 1000 pupae are taken, and if the threshold of more than 2 pupae are detected in the sample of 1000, the batch is re-sorted prior to advancing to the eclosion phase. Sorting procedures have been proven to be very efficient >99.9% (Carvalho et al., 2015; Gorman et al., 2015; Harris et al., 2012).

The release of homozygous OX513A females could impact the target *Aedes aegypti* population by potentially resulting in the generation of hemizygous OX513A progeny from an OX513A female and wild male *Aedes aegypti*. This scenario represents the intended the effect on the population, which would be the same as an OX513A male mating with a wild female *Aedes aegypti*. The consequence of a potential release of homozygous females is also examined in the risk area ***Section 2.3 Pathogens, Infections, and Diseases.***

b) Survival of female hemizygous OX513A

There is a potential for hemizygous females surviving in the field as a result of incomplete penetrance of the self-limiting trait (described in ***OX513A Technical Dossier Part A Section 5.2.6 Trait Penetrance***) to interact with the target population. The longevity and fecundity of OX513A hemizygous progeny has been investigated in the laboratory and is also discussed the risk area ***Section 2.3 Pathogens, Infections, and Diseases.*** The assessment parameters and details are described in ***OX513A Technical Dossier Part A Section 5.2.7 Non-penetrant OX513A progeny- Longevity and Fecundity***.

The longevity of both OX513A males and females was found to be significantly lower than that of wild type. Fecundity of OX513A hemizygous females reared in the absence of tetracycline was found to be slightly higher than that of wild-type females in this study. This may be a consequence of selection under mass-rearing conditions for early egg production, as this would be desirable in mass production. The apparent increase in fecundity, even if it were maintained throughout the female's lifetime, should not represent a large enough effect to outweigh the observed reduction in lifespan, and thus result in an overall impact on the wild population significantly different that the intended effect of the OX513A male release on the target population.

Conclusion to step 1

Key considerations in the area of risk *Interaction with Target Organisms* have been addressed for OX513A in the receiving environment with the following conclusions:

1. Resistance to the conditional insect control trait has not been observed before.

2. A reduction in efficacy, or resistance development in the target organisms against the GM insect mediated effect is unlikely to occur in the period of the proposed environmental release, or in the longer term in the context of an IVM program.

3. No changes in interactions with target organisms arising from an altered genetic diversity of a reared GM insect population that may result in adverse effects have been observed or are expected.

4. No effects on target organisms due to the release of low-quality GM insects or non-GM insects that may result in adverse effects are expected.

The interaction between OX513A and the target organism, wild Aedes aegypti, is the intended effect of an environmental release. Through Step 1, potential hazards and pathways to exposure have been assessed through measurement endpoints. There is adequate information to formulate a conclusion that there is no plausible pathway to harm under the protection goals identified in Section 1.1, and that further hazard and exposure characterisation is not required.

Step 2-4: Hazard, Exposure and Risk characterisation

The potential for harm, under the protection goals identified in **Section 1.1** through interactions with target organisms as a result of the rDNA insert in OX513A has been assessed in step 1. The conclusion is that the potential for harm was negligible in all cases therefore no further Hazard characterisation (Step 2), Exposure characterisation (Step 3), or Risk Characterisation (Step 4) was necessary for this area of risk.

Step 5: Risk management strategies

No further risk management strategies are required for this risk area as no plausible pathway to harm under the protection goals identified in ***Section 1.1*** has been identified. It should be noted that the interaction with the target organism (wild *Aedes aegypti)* and transmission of the self-limiting trait conferred by expression of the tTAV trait is the intended consequence of the environmental release. Measures to avoid escape into unintended environments during production stages are described in ***OX513A Technical Dossier Part B Section 1.4 Containment measures prior to release.***

Step 6: Conclusions

Using the information and evidence presented, it has been concluded in the problem formulation step through the identification of hazard and exposure pathways that the likelihood the deliberate environmental release of OX513A in Saba would represent a plausible pathway to harm through the interaction with target organisms is negligible, thus further hazard and exposure characterisation was not required.

2.5 Interactions with non-target organisms

Step 1: Problem formulation (including identification of hazard and exposure pathways)

Aedes aegypti is a peri-domestic species closely associated with human habitations. Breeding is tied to artificial water containers, such as potted plant holders, water tanks, tires, discarded plastic and metal containers such as soda cans, drains and roof guttering as well as ephemeral containers, such as puddles (Powell and Tabachnick, 2013). Once eclosed the adult *Aedes aegypti* mosquitoes live in and around houses where females have easy access to the blood meal necessary for egg development.

Aedes aegypti is an invasive species in Saba, as described in **OX513A Technical Dossier Part B Section 2.2.2 Functions of *Aedes aegypti* in the ecosystem of Saba,** whereby reference is made to ***Appendix 9*** *Introduced agricultural pests, plant and animal diseases and vectors in the Dutch Caribbean, with an "Alert species" list. Aedes aegypti* in Saba has therefore not co-evolved with other organisms in the receiving environment and is unlikely to represent a keystone species on which other organisms rely on for food.

In a recent risk assessment conducted for the release of *Aedes aegypti* carrying the intracellular bacterium *Wolbachia*, it was concluded that *Aedes aegypti* was unlikely to have interactions with natural ecosystems, and that it was unlikely that the other species rely heavily or even moderately on *Aedes aegypti* as a food item or provider of ecosystem services (Murphy et al., 2010). A reduction in the *Aedes aegypti* population is already achieved as a result of current vector control activities as briefly described in **OX513A Technical Dossier Part B Section 2.2.2 Functions of *Aedes aegypti* in the ecosystem of Saba**. In this context, the risk that a reduction of the population of *Aedes aegypti* would affect population dynamics of other functional groups or ecosystem services has already been considered as an acceptable outcome of vector control activities more broadly.

Under this area of risk, the assessment endpoint is potential adverse effects as a result of the interaction between OX513A and non-target organisms within semi-natural or natural habitats in the receiving environment.

The hypothesis that the interaction of OX513A with non-target organisms in the receiving environment does not represent a plausible pathway to harm under the protection goals identified in ***Section 1.1*** has been tested.

The key considerations for this risk area which have been taken in evaluating the assessment endpoint to test this hypothesis are, based on EFSA (2013) guidance regarding *Effects of suppression releases and preventative releases*:

1. Effects on abundance or species composition of:

- natural enemies/predators and the pest regulation service they provide
- competitors of GM insects and the ecological functions they provide
- pollinators and the pollination services they provide

2. Effects on biodiversity, concerning species of conservation value (rare or threatened species), or of cultural value (asthetic value) and food chain effects

3. Effects on other ecosystem services including *Aedes aegypti* as a decomposer and as a resource for decomposers, nutrient cycling, water regulation and purification.

4. Effects on abundance or species composition of host plants or host animals and the ecosystem services they provide.

5. Effects of toxins or allergens associated with the GM insect on insectivorous vertebrates. (Toxicity and allergenicity is additionally covered in ***Section 2.7 Impact on human and animal health***)

Predators of Aedes aegypti

Non-target predator organisms may include invertebrate species such as *Toxorhynchites spp*., dragonflies, spiders, water–borne Crustaceans such as Mesocyclops, amphibians, such as frogs, lizards and geckos, fish, insect feeding birds, and bats. It should be noted, however, that the scientific literature frequently indicates that mosquito predators are regarded as generalized predators (Shaalan, 2009; USFWS, 2004; Blum et al., 1997). As *Aedes aegypti* is invasive, there is no keystone species that is obligate on a single mosquito species and even generalist insectivores consume very small quantities of all mosquitoes (Lounibos et al., 2002; Blum et al., 1997).

There are generalist insect predators that may be found out of doors in the urban setting of a release area, which may incidentally come into contact with OX513A adult mosquitoes or larvae. The likelihood of species interaction with adults is however very restricted outside of the immediate timing of release activities, as the *Aedes aegypti* mosquitoes are not typically found flying outside of houses since they are day biting and typically live inside houses resting on walls. In the aquatic environment, the larvae have a number of predators including other invertebrates, tadpoles, and fish. Aquatic invertebrate predators from the Coleoptera (beetles), Diptera (flies), Hemiptera (True bugs) and Odonata (dragonflies) orders are known to prey on all mosquito larvae in the same environment (Shaalan, 2009). Because *Aedes aegypti* usually uses man-made containers such as gutters, water containers, cans, and tires as breeding sites, there appears to be no specific predator that preys on *Aedes aegypti* but rather predators that are generally opportunistic and feed on larvae if and when they encounter them. Predators can significantly affect the survival, development and recruitment levels of mosquitoes in their aquatic breeding sites, as there is some evidence that the presence of predators affects oviposition by *Aedes aegypti* (Albeny-Simoes et al., 2014), where they are attracted to predator kairomones (similar to pheromones) and lay their eggs in these vessels. Mogi, (2007) reviewed mosquito invertebrate predators and concluded that they are usually absent or sparse in man-made containers in residential areas.

Additionally, the inserted traits are neither toxic or allergenic as they have no homology to known toxins or allergens as evaluated through bio-informatics analysis (see ***OX513A Technical Dossier Part A Section 4.5 Potential for toxicity and allergenicity of the introduced proteins***)

Predatory mammals, birds, amphibians, invertebrates, and fish are discussed individually below.

Predatory mammals

Since adult mosquitoes typically remain indoors they would not form a large proportion of the diet of any species typically found out of doors. Notably in the case of the bat species, the likelihood of encountering OX513A males is very low, as bats are nocturnal, and OX513A population density out of doors would be only be periodically high during the period immediately following daytime releases.

There are 5 species of bats reported on Saba as listed in **OX513A Technical Dossier Part B Section 2.3 Flora and Fauna.** Insectivorous bats are often anecdotally regarded to be a significant predator of mosquitoes and are thought to eat large quantities of mosquitoes. In the case of bats there is temporal separation between the diurnal (daily) habits of bats and *Aedes aegypti* mosquitoes. *Aedes aegypti* mosquitoes are active in the day whereas bats are principally active at dawn and dusk. Gonsalves et al. (2013) found that mosquitoes were not always available as diet to bats and therefore only a small fraction of their diet. This was due to their small size, poor detectability by low frequency echolocation, and variable field metabolic rates. The American Mosquito Control Association (AMCA) also reviewed the role of bats for mosquito control[13], indicating that although bats do eat mosquitoes, the consumption of mosquitoes by bats comprised of less than 1% of their gut contents of wild caught bats in the studies reviewed to date, and other insects, such as moths provide better nutritional value. An analysis of the diet through stomach content analysis or fecal pellet analysis shows that bats are opportunistic feeders; Whitaker and Lawhead (1992) analysed the brown bat fecal pellets and showed 71% small moths, 16.8% spiders and 1.8% mosquitoes while the diet of the big brown bat was dominated by beetles and caddisflies (reviewed in (Agosta, 2002).

In the receiving environment in Saba (described in OX513A Technical Dossier Part B Section 2), due to the temporal separation in activity periods, and the spatial separation afforded by habitat, Aedes aegypti is likely to form a negligible part of the bat diet. The likelihood of bats encountering OX513A males is very low, as bats are nocturnal, and OX513A population density out of doors would be only be periodically high during the period immediately following daytime releases.

Predatory birds

Insectivorous birds of the order Passeriformes, which may potentially feed on mosquitoes may be found out of doors in the release area in Saba, notably the Scaly-breasted Thrasher, the Pearly-eyed Thrasher, the Trembler, the Lesser Antillean Bullfinch, and the Blue-crowned Euphonia are reported (**OX513A Technical Dossier Part B Section 2.3 Flora and Fauna).** The consumption of insects by insectivorous birds can depend on the abundance of the insect population itself, where there are abundant insects then consumption is likely to increase (Glen, 2004). However, even if the consumption increases in times of abundant insect populations, the birds remove an extremely small proportion of the insects. Perhaps the most frequently anecdotally cited bird regarding the consumption of mosquitoes is the Purple Martin (*Progne subis*), the largest species of martin in North America; however both the American Mosquito Control Association and the Purple Martin Conservation Association (PMCA)[14] declare that this is erroneous and not supported by evidence. There is temporal

[13] http://www.mosquito.org/faq (accessed 05/09/2016)

[14] http://www.purplemartin.org/update/MosCont.html (accessed 05/09/2016)

isolation between the Purple Martin and the mosquito flight patterns, with the birds and mosquitoes not flying at the same times or altitudes, and that they form only a small part of the overall diet of the birds (Johnstone, 1967). An intensive 7-year diet study conducted at PMCA headquarters in Edinboro, PA, failed to find a single mosquito among the 500 diet samples collected from parent martins bringing beakfuls of insects to their young[15].

In the receiving environment in Saba (described in OX513A Technical Dossier Part B Section 2) due to the temporal separation in activity periods and that Aedes aegypti is likely to form only a negligible part of the diet of insectivorous birds, the likelihood that insectivorous birds will contact OX513A is very low.

Predatory amphibians

The only amphibian reported on Saba is the piping frog (*Eleutherodactylus Johnstonei*). Amphibians do have the capacity to consume mosquito larvae, for example a controlled laboratory study study showed that large numbers (200-400 3rd instar larvae of *Culex* species per day) could be consumed by salamander species, but this where mosquitoes were the only food source and there was no prey choice (DuRant and Hopkins, 2008). Blum et al., (1997) found that through the diet analysis of anurans (newts) that mosquitoes made up only 0.16% of the content. In the context of potential use as biological control agents for mosquitoes for example, amphibian predators such as frogs and reptiles are not recognised as consumers of mosquitoes in sufficient number for mosquito control[16]. There are unlikely to be significant habitat overlap with piping frogs in the same breeding sites as *Aedes aegypti, as Aedes aegypti* is more associated with human habitats, and piping frogs are reported on Saba to occur in primary forest due to the lack of other species of *Eleutherodactylus* (see **Appendix 11** pg 36 *Biological Inventory of Saba*).

In the receiving environment in Saba (described in OX513A Technical Dossier Part B Section 2) due primarily to spatial separation by means of habitat, Aedes aegypti is likely to form only a negligible part of the diet, the likelihood that predatory amphibians would contact OX513A is very low.

Predatory invertebrates

Insects from the orders Odonata, Coleoptera, Diptera and Hemiptera all may opportunistically feed on mosquito adults or larvae encountered incidentally in the environment. Ants (Lee et al., 1994), coleopterans (Yang, 2006), cockroaches (Russell et al., 2001), and pillbugs (Focks et al., 1993) have been reported to prey on eggs of *Aedes aegypti* or related species. These are generalist predators that are not reliant on a single species of mosquito for their food source. Indoors, household spiders may have the most likely opportunity to feed on *Aedes aegypti* adult mosquitos. Studies looking at the efficacy of *Aedes aegypti* predators for pest control have noted a number of household spiders which eat *Aedes aegypti*. In Malaysia spiders of the genera Araneus and Neoscona tested positive for gut content containing *Aedes aegypti*, indicating that they were natural predators of *Aedes aegypti* (Sulaiman et al., 1990). Further reports from Thailand and India show that the spider *Crossopriza lyoni* is also a predator of *Aedes aegypti*, (Strickman et al., 1997; Nandi and Raut, 1986). These spiders all have a broad distribution worldwide.

[15] http://www.purplemartin.org/update/MosCont.html (accessed 05/09/2016)
[16] http://www.michigan.gov/emergingdiseases/0,4579,7-186-25805_25824-75797--,00.html (accessed 05/09/2016)

Aedes aegypti larvae are known to form part of the diet of carnivorous mosquito species such as *Toxorhynchites* which have been evaluated for biological control of mosquito larvae (Amalraj et al., 2005). These mosquitoes are reported to inhabit most of the tropical regions of the world (Collins and Blackwell, 2000). *Toxorhynchites* are most commonly found in tree-holes, bromeliads and other ephemeral containers. The gut content of *Toxorhynchites rutilus* from wild treeholes were analysed and were found to contain 20 taxa of aquatic prey (Campos and Lounibos, 2000). Mosquito larvae accounted for only 6% of prey items from tree-holes and only 5% of prey items from car tires.

Reports on aquatic predators of mosquitoes such as Heteropteran species indicate that these species are capable of switching prey species depending on the different mosquito population density changes that are common in temporary breeding sites (Saha et al., 2009). Thus while Heteropteran are not exclusively dependant on any one species, it is conceivable that predatory invertebrates with similar feeding habits could consume proportionally larger amounts of OX513A during the release program if there was sufficient exposure. The oral exposure to OX513A in the predatory invertebrate *Toxorhynchites spp.* has been evaluated experimentally as described below.

Toxorhynchites are large species and are easily reared in the laboratory where they can be fed exclusively on mosquito larvae. To evaluate effects on predatory arthropods feeding exclusively on a diet of OX513A mosquito larvae, two different species of *Toxorhynchites* (*Tx. splendens* and *Tx. amboinensis*) were fed larvae of OX513A constituting 100% of their diet (Nordin et al., 2013). The *Toxorhynchites* species were also fed a diet of wild-type *Aedes aegypti* and OX513A mosquito reared on tetracycline. Further details are provided in ***OX513A Technical Dossier Part A Section 5.4.1 Toxorhynchites spp. (predatory Mosquito).*** Effects on life history parameters of all life stages were compared to *Toxorhynchites spp*. being fed on wild-type larvae of the same background strain, any significant differences found were attributed to differences between species and there was no evidence of an adverse impact.

There was no evidence that the development, fecundity, and longevity of the two Toxorhynchites species were adversely effected by consumption of OX513A larvae.

Predatory fish

Certain fish species are recognised as predators of mosquitoes when sharing mosquito breeding habitats, and have been evaluated as a potential mosquito control measure (Louca et al., 2009). *Poecilia reticulate* are used in Saba as part of the vector control program for *Aedes aegypti* (see **Appendix 8).**

An independent laboratory study of the toxicity of the OX513A over a prolonged period (max 28 days) has been conducted. OX513A larvae and pupae were fed to the guppy fish, *Poecilia reticulate* (Actinopterygii: Poeciliidae) according to the OECD No. 204 Guideline (1984) modified for oral route exposure. See ***OX513A Technical Dossier Part A Section 5.4.2 Poecilia reticulate (Guppy Fish)*** for additional details and reference to the full report.

No adverse acute or sub-lethal effect on any of the endpoints was observed in the fish group fed with OX513A mosquitoes between 7 and 14 days, whilst control mortality remained at an accepted level (10%).

Parasitoids of Aedes aegypti

No specific parasitoids are known to be associated with *Aedes aegypti*. The nematodes *Romanomermis culicivorax* and *Strelkovimermis spiculatus* from the family Mermithidae are generalist parasitoids infecting a number of mosquito species. Although these species are known to infect *Aedes aegypti* in the laboratory, they have not been found infecting natural populations (Wise de Valdez, 2007).

Aedes aegypti as a decomposer

Aedes aegypti larval development is in an aquatic environment, predominantly in artificial breeding sites which frequently contain detritus metabolised by the microbial communities. Although there is limited research in this area, it is thought that *Aedes aegypti* survive on the micro-organisms that break-down the detritus, and it is the nitrogen, phosphorus and carbon availabilities that influence relative abundance of *Aedes aegypti* in breeding sites (Otero et al., 2006). As the microorganisms break down the detritus there are number of metabolites and volatiles which act as attractants to gravid mosquitoes and stimulate egg laying in containers which are enriched with bacteria (Ponnusamy et al., 2008). So although *Aedes aegypti* occupy man-made or artificial containers where plant and animal detritus is broken down, it is unlikely to be the mosquito itself contributing to the direct decomposition of the material. It is likely that the mosquito mainly acts as a consumer of the elements from the breakdown of detritus by other organisms, rather than as a decomposer.

Aedes aegypti as a resource for decomposers

Fungi such as *Metarhizium anisopliae*, a well-known entomopathogenic fungus which is found in the soil is capable of infecting *Aedes aegypti* eggs (Leles et al., 2012). The fungus *Beauveria bassiana* has also recently been evaluated as a potential biological control agent for *Aedes aegypti* (Navarro et al., 2010; Sharma et al., 2008).

Aedes aegypti role in pollination

Although female *Aedes aegypti* mosquitoes take blood meals from humans in order to obtain protein for ovary development, mosquitoes of both sexes require plant juices as an energy source. Floral nectars are the best-known sources, but mosquitoes also are also known to obtain sugars from extra-floral nectaries, damaged fruits, damaged and intact vegetative tissues, and honeydew (Clements, 2000). Some responses of mosquitoes to flower features have been described. *Aedes aegypti*, for example, is known to react positively or negatively to different floral scents and to prefer green flowers (Argue, 2012). *Aedes aegypti* are adapted to domestic and urban environments that tend to be low in sugar sources but allow easy and unlimited access to blood meals, such as those around human habitations. It is likely that *Ae. aegypti* males are reliant on potted plants or plant species which are around houses for sugar sources due to the anthropophilic nature of the mosquito (Martinez-Ibarra et al., 1997). There is limited information on the pollination of plant species by mosquitoes. However, there are no reports that *Ae. aegypti* is a pollinator for any plant species. Despite feeding on plant nectar mosquitoes play little role in pollination (Foster, 1995) however there is one notable exception in the literature; *Ae. communis* and *Ae. Canadensis candensis* are known as pollinators of an orchid in Northern Canada; *Habenaria obtust* (Thien, 1969). This lack of pollination activity may be because, as an invasive species, the mosquito has not been present in the ecosystem for sufficient time to develop an essential ecosystem function. Dedicated pollinator species for particular flowers require

close evolution for many thousands of years. Additionally, previous mosquito control efforts in various territories (Brathwaite Dick et al., 2012; Wheeler and Petrie, 2007) have resulted in the complete eradication of the mosquito from large areas with no reports of any adverse effect on the reproductive capacity of the native or crop plant species documented during this period.

Possibility for adverse effects on charismatic or protected species

An analysis of protected, charismatic and valued species that may occur in the release areas on Saba has been conducted using detailed online searches from source such as the International Union for Conservation of Nature (IUCN) Red List as well as biological inventory reports from the Caribbean Research and Management of Biodiversity foundation, and information from the Dutch Caribbean Nature Alliance. Results are summarised in ***OX513A Technical Dossier Part B Section 2.3 Flora and Fauna.***

The Red-bellied Racer (*Alsophis rufiventris*) is listed as endangered, however there is negligible potential for exposure to OX513A due to lack of habitat overlap, and non-insectivorous feeding habits. For the Saban Anole[17] *(Anolis sabanus)* the potential for exposure due to habitat overlap may be considered moderate. Within inhabited areas it is described as usually resting on warm rocks and on walls of houses, while in forest areas it is typically found perched on trees and leaves. The Saban Anole is described generically as eating mostly small insects[17]. The dietary habits of *Anolis* lizards across island sites in the West Indies, and mainland Central and South America have been analysed (Andrews, 1979) and results provide some indication as to the dietary make-up and feeding habits of *Anolis sabanus.* From (Andrews, 1979) Formicidae (Ants) and Isoptera (Termites) were found to comprise the most significant proportion of prey in certain locations, with Lepidoptera (butterflies and Moths) comprising the most significant proportion in other sites. Various Diptera (Fly) and Coleoptera (Beetle), as well as spiders and mites were consistently recorded, along with a variety of miscellaneous arthropods. The food habits of *Anolis opalinus* (Jamaican Lizard) have also been well characterised for example (Floyd and Jenssen, 1983) and are consistent with Andrews (1979) in that ants were found to primarily comprise the diet by number, but with lepodopteran larvae, and orthopterans comprising the main food items by bulk.

The *Aedes aegypti* habitat is considered as urban/peri-domestic, with breeding sites in standing water surrounding human habitation and adults generally living within human dwellings. With the potential for habitat overlap the Saban Anole could encounter OX513A adults or larvae incidentally and thus have a moderate level of exposure, as the lizards of the Anole family are generalist insectivorous predators. The potential for harm is negligable as the hazard would be considered negligible as supported by the evidence detailed in ***OX513A Technical Dossier Part A Section 4.5 Potential for toxicity and allergenicity of the introduced proteins***

[17] http://www.dcnanature.org/saban-anole/ (accessed 14/09/2016)

Aedes aegypti is invasive on Saba and is already controlled with existing interventions. In comparison to the current control systems, OX513A is not likely to result in harm to charismatic or key species.

Conclusion to step 1

Whilst there are many non-target species which can opportunistically feed on OX513A larvae or adults, both out of doors and inside human habitation, there is no one species which relies completely on *Aedes aegypti* as a food source. There is limited chance of habitat overlap for most species, and additionally the charismatic and protected species in Saba have been characterised in this regard.

Predatory species found within the temporary mosquito breeding sites are not anticipated to be permanent or specialist *Aedes aegypti* feeders but rather generalist opportunistic feeders. *Aedes aegypti* is also a highly urbanised species which lives in and around human habitation and is unlikely to form a significant proportion of any one species diet. In the event that OX513A is consumed by a predatory species, laboratory feeding studies with selected non-target organisms have not demonstrated an observable effect due to oral exposure at high dose rates, and the inserted traits have no homology to known toxins or allergens based on bio-informatics analysis.

Relative to current control mechanisms the high mating competitiveness of OX513A and the penetrance of the self-limiting trait enable a species-specific population control with limited non-target impact in comparison to current control methods, and to the wild local *Aedes aegypti* population.

Through Step 1, potential hazards and pathways to exposure have been assessed through literature analysis and measurement endpoints. There is adequate information to formulate a conclusion that there is no plausible pathway to harm under the protection goals identified in Section 1.1 and that further hazard and exposure characterisation is not required.

Step 2-4: Hazard, Exposure and Risk characterisation

The potential for harm, under the protection goals identified in **Section 1.1** through interactions with non-target organisms as a result of the rDNA insert in OX513A has been assessed in step 1. The conclusion is that the potential for harm was negligible in all cases therefore no further Hazard characterisation (Step 2), Exposure characterisation (Step 3), or Risk Characterisation (Step 4) was necessary for this area of risk.

Step 5: Risk management strategies

No further risk management strategies are required for this risk area as no plausible pathway to harm under the protection goals identified in ***Section 1.1*** has been identified. It should be noted that the interaction with the target organism (wild *Aedes aegypti)* and transmission of the self-limiting trait conferred by expression of the tTAV trait is the intended consequence of the use of OX513A for vector control. Measures to avoid escape into unintended environments during production stages are implemented as a standard practice consistent with arthropod containment principles as described in ***OX513A Technical Dossier Part B Section 1.4 Containment measures prior to release.***

Step 6: Conclusions

Using the information and evidence presented, it has been concluded in the problem formulation step through the identification of hazard and exposure pathways that the likelihood the deliberate environmental release of OX513A in Saba would represent a plausible pathway to harm through the interaction with non-target organisms is negligible, thus further hazard and exposure characterisation was not required.

2.6 Environmental impacts of the specific techniques used for the management of OX513A

Step 1: Problem formulation (including identification of hazard and exposure pathways)

Aedes aegypti is a vector of human disease, thus certain elements of management practices of *Aedes aegypti* are covered in the ***Section 2.3 Pathogens, Infections and Diseases*** area of risk.

Under this area of risk, the assessment endpoint is the potential adverse environmental impacts of the specific techniques used for the management of OX513A in the receiving environment.

The hypothesis that the specific management practices of OX513A do not represent a plausible pathway to harm under the protection goals identified in ***Section 1.1*** has been tested.

The key considerations for this risk area which have been taken in evaluating the assessment endpoint to test this hypothesis based on EFSA (2013) guidance are:

1. Changes in vector management practices associated with the production and subsequent release of OX513A.

2. Potential adverse effects to the environment resulting from these changes in management practices.

3. The overall risks associated with changes in management of the production and release of OX513A and their environmental consequences.

Conventional control measures

The use of control measures at the release site are fully compatible with the OX513A program. A site visit to Saba occurred early in 2016, and discussion with vector control confirms that the principle means of control are with the biological control agents Larvivorous fish (Guppy) (*Poecilia reticulata)* and *Bacillus thuringiensis* Var. Israelensis SH 14 (Bti)

Information on these methods can be found in the ***Appendix 8*** *Final Report of the Application stage of the Biological Vector Control Project in Saba Island (Dutch Caribbean).*

If vector control methods were to change in Saba, the impact of OX513A releases in areas which are using pesticide applications for example is generally that application schedules are adjusted so as to avoid adulticide applications the same day as OX513A releases are taking place.

Risk management strategies for rearing and release of OX513A

Representative conditions for OX513A production and release have been previously described (Carvalho et al., 2014). OX513A production for deliberate release is a two-step process: A UK-based facility produces OX513A eggs which are then shipped to a local facility in Saba for rearing to the adult stage for release.

The mass rearing facility is described in ***OX513A Technical Dossier Part B Section 1.4.1 Mobile Rearing Unit Overview*** and gives specific references to SOPs for; Emergency Procedures; Cleaning and Waste Procedures; Biosafety; Unit Decontamination; and, Entry and Exit.

SOPs for emergency procedures cover circumstances which have the potential to cause inadvertent release through damage to the integrity of the rearing facility. These include, but are not limited to: natural events such as adverse weather, long term power outages, fire, or and vandalism among others. In the event of the above specific incidents these cases, the facility manager or the study director will undertake detailed step-wise procedures.

Additionally, SOPs including cleaning and waste disposal procedures and entry and exit are in place to prevent the inadvertent release of OX513A, based on principles established for standard arthropod containment measures (e.g. Arthropod Containment Level 2 - ACL2). SOPs will be in place at the local rearing facility to cover elements of operations related to quality assurance, and occupational health and safety as referenced throughout ***OX513A Technical Dossier Part B Section 1 Details of the proposed release on Saba***.

The local rearing facility will comply with any additional local regulations not related explicitly to biosafety approvals (e.g. building code requirements, health and safety) for facilities of this category in Saba. It is therefore not expected that the rearing facilities, which will have been authorized, would have an additional impact on the environment that is not acceptable to local authorities.

Egg production

In the UK egg production facility, eggs are continually produced from a cycling colony of homozygous OX513A. Oxitec Ltd. has dedicated UK production facilities for OX513A which are licensed by the UK Health and Safety Executive (HSE) for the holding of GM organisms in contained use, under the UK Genetically Modified Organisms (Contained Use) Regulations 2014. Maintaining compliance with these regulations ensures appropriate risk assessment and management activities are undertaken in the UK.

Eggs are shipped in multiple shipments throughout the course of the programme to a locally based rearing facility where they are reared to adults for use in the release programme. ***OX513A Technical Dossier Part B Section 1.4 Containment measures prior to release*** describes how OX513A will be contained from the receipt of eggs through the hatching and rearing process up to the release.

Egg transportation to the local facility

Prior to shipping to the local rearing facility, import permit conditions such as specific packaging and labelling requirements for Saba will be assessed to ensure compliance. ***OX513A Technical Dossier Part B Section 1.4 Containment measures prior to release*** refers to the SOPs used in preparing and packaging egg shipments for release. Labelling and record keeping requirements are established and referenced through-out ***OX513A Technical Dossier Part B Section 1,*** to ensure chain of custody is maintained and all regulated material is accounted for. As standard practice eggs from the UK production facility are packed in at least two levels of shatterproof containment (e.g., sealed plastic bags/polystyrene container/cardboard boxes) and shipments are labelled as to be kept above 10^{o}C and to only be opened by inspection officials, or the importer to prevent inadvertent release. Boxes are

shipped through a courier service that has a tracking facility to ensure the location of the shipment can be monitored. Shipping from the UK to the local facility will need to occur regularly (e.g. bi-weekly) prior to, and during the programme. In conformance with protocols at the local facility, upon receipt, shipments will only be opened by staff previously authorised through training activities; and, as eggs are a non-motile life stage of *Aedes aegypti* and under the correct conditions can remain viable for several months, shipping materials will be disposed of by freezing at ≤ -15°C for at least 12 hours to render non-viable any remaining eggs prior to disposal through local waste handling streams.

Adult mosquito production

OX513A Technical Dossier Part B Section 1.5 Rearing of OX513A from egg to adult mosquitoes provides details and reference to specific SOPs used in adult mosquito production described briefly below:

In the rearing facility eggs are hatched under prescribed conditions to the larval stage (approximately 1 day) and further aliquoted by volume to trays for feeding and pupal development (approximately 8 days). Pupae are then mechanically sex sorted based on the difference in size between male and female pupae (sexual dimorphism) using a proprietary mechanical device. Male pupae are placed into release devices to emerge and mature before release. Quality control procedures are undertaken on an ongoing basis throughout production to assess the sex sorting efficiency after each mechanical sorting activity.

Female pupae and undeveloped larvae are killed by freezing ((-15^{o}C) for more than 12 hours and disposed of through local waste handling streams.

Transport and release

OX513A Technical Dossier Part B Section 1.6 Transport and adult release provides details and reference to specific SOPs used in the transport and release of OX513A described briefly below:

Release devices for adult release are packed in the vehicle at the local rearing facility and double contained for transport to the release site. The release device itself serves as the primary level of containment, with secondary containment a suitable container, such as a polystyrene box, sealed bag, chest cooler etc. If temperatures are determined to be too high and may compromise OX513A quality, cooling devices such as ice packs may be used in the transport containers.

Chain of custody is maintained such that release devices are signed out of the rearing facility, and signed for upon receipt by authorized personnel at the release site if personnel change between steps. At the appropriate release coordinates, a release device is removed from double containment and the lid is opened to release OX513A. Release devices are placed back into the container or bag for transport of back to the local facility and frozen ((-15^{o}C) for more than 12 hours when returned to kill any remaining adults. All frozen life stages not required for analysis will be discarded by freezing ((-15^{o}C) for more than 12 hours and disposed of through local waste handling streams.

Ovitrap processing and analysis

Samples from traps will be returned to a designated space in the local facility for analysis. Samples will include both OX513A, and their progeny as well as wild *Aedes aegypti*. All wastes are frozen ((-15^{o}C) for over 12 hours prior to disposal in the local waste handling stream.

Samples required for further molecular analysis will be stored frozen in 70% ethanol or DNA/RNA Sheild™ prior to shipping to the UK or to collaborative partners.

Post release

In the context of island-wide elimination of *Aedes aegypti*, a re-introduction would be expected to follow the same pattern as initial establishment of *Aedes aegypti* as an invasive species in the area. Cessation of OX513A releases prior to an elimination endpoint would remove the consequent suppression pressure on the wild *Aedes aegypti* population. As released OX513A and the first generation offspring die as an intended effect, the wild *Aedes aegypti* population in the release area would be expected to equilibrate to the pre-release state, or no action alternative over time. The timing would be expected to depend on the degree of suppression of the *Aedes aegypti* population as a result of the OX513A program and on the conventional control measures in place.

Post release monitoring of the release site in Nuevo Chorillo Panama in 2014, as described in ***OX513A Technical Dossier Part A Section 7.1.5.1 Environmental persistence*** and Gorman et al. (2015), revealed no persistent OX513A genes in the environment 12 weeks following the final OX513A release. The absence of OX513A genes in over 20,000 larvae recovered from traps and screened could only be confirmed from 12 weeks post-release onward due to a disruption in data collection between weeks 4-12, however a significant drop in OX513A larvae detected at week 4 may be suggestive of a trend from that time point.

It is notable that the intended effect is *Aedes aegypti* population suppression to an end-point consistent with local vector control program objectives, which may include local eradication of *Aedes aegypti* in an area not subject to *Aedes aegypti* immigration pressures.

Potential release of tetracycline into the environment

The maximum amount of tetracycline likely to be generated on a weekly basis as a result of the rearing practices for OX513A males has been estimated as a maximum of 1.7 grams/week as calculated in ***OX513A Technical Dossier Part B Section 1.5.1 Tetracycline use.***

The amount of tetracycline generated by the rearing facility is negligible when taken in comparison to that used in medical applications for humans. The typical tetracycline dosage for humans use in treating Acne infections in adult humans for example is 7 grams/week (500 mg x 2 times /day) for a 2 week period[18]. The WHO International Programme on Chemical Safety, Who Food Additives Series 41[19] reports that in humans receiving oral therapeutic doses, 30% of the dose of chlortetracycline is absorbed, as compared with 60-80% of an oral dose of tetracycline, and that tetracycline is excreted mainly in the urine, and chlortetracycline is excreted in urine and faeces. It is thus clear that the weekly output of chlortetracycline from the mobile rearing unit would be less than that passed from a single individual in Saba receiving a therapeutic dose of tetracycline for adult acne. Disposal of waste water from the rearing unit in manner consistent with that of the residential waste disposal systems would thus represent a negligible contribution to tetracycline in the environment on Saba.

[18] Example from https://www.drugs.com/dosage/tetracycline.html (accessed 05/09/2016)

[19] http://www.inchem.org/documents/jecfa/jecmono/v041je07.htm (accessed 05/09/2016)

Potential for tetracycline resistant bacteria to be released into the environment.

The potential for insect gut bacteria acquiring antibiotic resistance genes during rearing of OX513A aquatic life stages in the presence of tetracyclines, then spreading those genes to other organisms in the environment upon their release has been considered. There is no causal pathway for this to occur as gut bacteria are expelled during mosquito metamorphosis from larvae to adults (Moll et al., 2001; Demaio, 1996). Larvae are treated with tetracycline, but as described above the gut bacteria are lost during the pupal stage (e.g., stay in the rearing water), pupae and adults are not subsequently treated with tetracycline during the rearing, and pupae are washed in fresh water several times during the sorting process. The possibility of superficial bacteria present on the body surface of eclosed adults acquiring antibiotic resistance genes due to OX513A eggs and larvae being raised in tetracycline containing water is very low. There is no causal pathway for this to occur because pupae would be washed several times in fresh water during sorting, the pupae would be raised in fresh water, and the adults eclosing from these pupae would not have extensive enough contact with the pupal case or the water surface for acquisition of bacteria that could be harbouring antibiotic resistance genes. Additionally, bacteria would need to be present in the rearing trays, acquire tetracycline resistance genes, and spread those acquired resistance traits across the general bacterial population which would have to persist in the fresh water used to maintain sorted pupae. The combined probability of all these events happening is negligible.

Conclusion to Step 1

Conventional vector control measures at the release site are not anticipated to be modified as a result of the OX513A release program such that the intended vector control outcomes are altered. In the event of local eradication of *Aedes aegypti,* the local vector control program in the longer term may adjust control measures. In this scenario the outcome of the OX513A releases would be consistent with vector control program objectives which look to reduce the *Aedes aegypti* population as much as possible.

Many of the measures described in this section regarding the rearing and handling of OX513A in deliberate release programmes, and in the referenced SOPs in **OX513A Technical Dossier Part B - Section 1 Details of the proposed release on Saba,** are generally implemented globally in projects to date as a matter of regulatory compliance in the various jurisdictions where OX513A release projects have been undertaken. The risk management strategies have thus not necessarily been put in place in response to specific items identified in the risk characterisation steps, but as best practice measures consistent with past project permit conditions.

In conclusion:

1. Negligible risk has been identified associated to changes in management practices associated with the production and subsequent release of OX513A.

2. Potential adverse effects to the environment have not been identified resulting from these changes in management practices.

3. The overall risks associated with changes in management of the production and release of OX513A and their environmental consequences are negligible.

Through Step 1, potential hazards and pathways to exposure have been evaluated. There is adequate information to formulate a conclusion that there is no plausible pathway to harm under the protection goals identified in Section 1.1, and that further hazard and exposure characterisation is not required.

Step 2-4: Hazard, Exposure and Risk characterisation

The potential for harm, under the protection goals identified in **Section 1.1,** through environmental impacts of the specific techniques used for the management of OX513A has been assessed in step 1. The conclusion is that the potential for harm was negligible in all cases therefore no further Hazard characterisation (Step 2), Exposure characterisation (Step 3), or Risk Characterisation (Step 4) was necessary for this area of risk.

Step 5: Risk management strategies

No further risk management strategies are required for this risk area as no plausible pathway to harm under the protection goals established in ***Section 1.1*** has been identified. Measures to avoid escape into unintended environments during production stages are implemented as a standard practice consistent with arthropod containment principles as described in ***OX513A Technical Dossier Part B Section 1.4 Containment measures prior to release.*** Additionally, it should be noted that the reduced fertility of OX513A is considered as a mitigation measure (EFSA, 2013).

Step 6: Conclusions

Using the information and evidence presented, it has been concluded in the problem formulation step through the identification of hazard and exposure pathways that the likelihood the deliberate environmental release of OX513A in Saba would represent a plausible pathway to harm through the environmental impacts of the specific techniques used for the management of OX513A is negligible, thus further hazard and exposure characterisation was not required.

2.7 Impact on human and animal health

Step 1: Problem formulation (including identification of hazard and exposure pathways)

An assessment of the potential impacts of OX513A on human and animal health is conducted to evaluate whether the GM insects present a hazard for human and animal health. Both immediate and delayed effects resulting from potential direct and indirect interactions with OX513A are assessed, such as the risks for workers and members of the public coming into contact with OX513A around the release area. OX513A is not intended for human or animal food or feed use. Therefore, effects considered are mainly from routes other than ingestion or intake such as dermal exposure or exposure through insect biting.

Under this area of risk, the assessment endpoint is the potential adverse impact on human and animal health through the release of OX513A in the receiving environment.

The hypothesis that the environmental release of OX513A will not have an adverse impact on human and animal health is tested.

The key considerations for this risk area which have been taken in evaluating the assessment endpoint to test this hypothesis based on EFSA (2013) guidance are:

1. Potential toxic effects of the new compound(s), their derived metabolic products and/or the GM insects to humans and animals, e.g. qualitative or quantitative change in the production of toxins by the GM insects when compared with their non-GM comparators.

2. Potential allergenic effects of the new compound(s), their derived metabolic by-products and /or the GM insects to humans.

3. Loss of immunity in the human population and reliance on continued long-term positive effects of vector suppression or replacement strategy.

OX513A has not been developed as a product for food or feed consumption, and oral exposure in relation to *animal* safety through feeding has been comprehensively assessed in ***Section 2.5*** *Interactions with non-target organisms* and the risk has been concluded to be negligible.

The interaction between human and the released male OX513A are expected to be limited to accidental dermal, ocular or inhalation, the likelihood of which can be considered to be negligible. The most plausible route of human exposure would be through biting of OX513A females. The allergenic and toxic potential of the introduced proteins tTAV and DsRed2 is examined in this section, while their presence in OX513A saliva is further explored in *Step 3 - Exposure characterisation*.

Notably, OX513A has been reared and used in contained use and in regulated environmental releases since 2002 (over 115 generations) with no reported adverse effects on the staff working with the strain.

Toxicity and allergenicity of the OX513A traits

OX513A Technical Dossier Part A Section 4.5 Potential for toxicity and allergenicity of the introduced proteins references specific studies and data to support the conclusions presented below.

Subcutaneous routes of exposure in general in the context of the safety of recombinant vaccines, have been widely researched, including that of the tetravalent dengue vaccine (Osorio et al., 2014; Dayan et al., 2013). Additionally, the World Allergy Organization regards recombinant proteins as promising new approaches to target allergy immunotherapy (Canonica et al., 2014). Three studies (Yukselen et al., 2012; Keles et al., 2011; Eifan et al., 2010) looked at both an oral route of exposure (under the tongue, known as sublingual) and a subcutaneous route of exposure for the efficacy of allergen immunotherapy, and both routes of exposure reduced the incidence of allergy in the patients exposed, with the subcutaneous route better in one study (Yukselen et al., 2012). Consequently, based on this limited evidence, either route of exposure to known protein allergens is likely to illicit a systemic immune response in humans. The International Codex Alimentarius, Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (FAO/WHO, 2001) referenced below focuses on the evaluation of amino acid sequence similarities of the protein with known toxins and allergens. This approach should thus be equally applicable to both oral and injection routes of exposure in the current context. This view is further supported by expert opinion (see ***OX513A Technical Dossier Part A - Appendix 3.1***)

To assess whether the tTAV or DsRed2 proteins contain sequences that are likely to represent potential hazards to animal or animal health as a result of toxic or allergenic properties, a comprehensive independent bioinformatics analysis was conducted by the Food Allergy Research and Resource Program at the University of Nebraska U.S.A. (***OX513A Technical Dossier Part A- Appendix 2***). The program develops and provides expert services relating to allergenic and novel foods and food ingredients including GM products. The study examined the homology between tTAV and DsRed2 and known allergens and known toxins in accordance with published guidelines of the International Codex Alimentarius, Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (FAO/WHO, 2001). The bioinformatic searches performed did not uncover any concerns of potential allergenicity, allergenic cross-reactivity or potential toxicity that would demonstrate a need for further testing regarding safety of the tTAV and DsRed2 proteins. Additionally, highly conservative comparisons did not identify sequence similarities that would suggest the proteins are allergens or are sufficiently similar to an allergen to cause cross-reactivity. The study also concluded that although the Codex Guidelines are primarily intended to evaluate potential of food safety concerns regarding potential risks from genetically engineered organisms, the same safety evaluation is scientifically sound as an approach for evaluating other potential routes of exposure, namely through insect bites and mosquito saliva.

As part of the analysis by the Food Allergy Research and Resource Program at the University of Nebraska U.S.A., scientific literature searches in the PubMed (NCBI) database maintained by the US National Library of Medicine were conducted to address the issue of whether the tTAV and DsRed2 proteins had reported toxic or allergenic properties. The literature search analysis did not uncover any concerns of potential allergenicity, allergenic cross-reactivity or potential toxicity that would demonstrate a need for further testing regarding safety. As the above noted search only represented tTAV and DsRed2, a supplemental literature search (***OX513A Technical Dossier Part A- Appendix 3***) using the same methodology, explored the potential toxicity, allergenicity and pathogenicity of the other genetic elements in the #OX513 construct. These analyses determined that there are no sequences in the construct

that are directly or indirectly likely to be toxic or allergenic or pathogenic or in other ways harmful to humans, animals or the environment.

Additionally, ***Appendix 3.1 Expert opinion 2015 Transgenic protein tTAV - assessment of allergenic risk*** presents an analysis of the allergenic potential of tTAV or DsRed2 in OX513A by Professor Ian Kimber, currently Professor of Toxicology in the Faculty of Life Sciences at the University of Manchester[20], which draws the following conclusions:

- The available evidence indicates that tTAV (and DsRed2) lacks the inherent potential to induce allergic sensitisation.
- In addition, neither tTAV, nor DsRed2, display a level of homology with known human allergens that would be required for the elicitation of cross-reactive allergic reactions.
- Levels of exposure to tTAV (and DsRed2) via mosquito bite will be extremely low, if present at all, and unlikely to initiate an immune response.
- The transgene proteins to not pose human health risks with regard to allergy or allergic sensitisation.

Human applications of the tet-system.

The tTAV system described in section ***OX513A Technical Dossier Part A Section 4.4.2 Self-limiting trait tTAV*** was derived from that originally described by Gossen and Bujard (1992). Sun et al. (2007) described Tet regulatory systems as the most widely used regulatory systems for conditional gene expression (at the time). Most recently, Das et al. (2016) describe the tetracycline-controlled Tet-Off and Tet-On gene expression systems as being used to regulate the activity of genes in eukaryotic cells in systems varying from basic biological research, biotechnology applications and gene therapy applications. The use of Tet systems for control of gene expression, particularly in early stage research on gene therapy applications, has thus been extensive since the initial description in 1992. A highlight of recent publications is available at:

http://www.tetsystems.com/science-technology/highlighted-publications/ (accessed 05/09/2016)

The body of scientific literature supports the fact that the tet-system is well tolerated and used widely in mammalian systems.

DsRed2

A New Protein Consultation (NPC0004) has been carried out by the U.S. Food and Drug Administration (FDA) -Center for Food Safety and Applied Nutrition (CFSAN)[21] on DsRed2 expressed in plants as part of an application for deregulation in the USA for DP 32138-1 Maize. Additionally, a Pink Bollworm expressing fluorescent genes similar to DsRed2 (Enhanced Green Fluorescent Protein) was assessed by the USDA-APHIS and it was concluded that it was unlikely to present an environmental risk [22]. (See ***OX513A Technical Dossier Part A -Section 4.5.3 Additional DsRed2 Toxicity and Allergenicity Assessment***).

A bioinformatic analysis was conducted in accordance with the international Codex Alimentarius; Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from

[20] https://www.liverpool.ac.uk/drug-safety/staff/professoriankimber/ (Accessed 27/06/2016)
[21] http://www.fda.gov/AboutFDA/CentersOffices/OfficeofFoods/CFSAN/ (Accessed 17/05/2016)
[22] https://www.aphis.usda.gov/brs/aphisdocs/05_09801r_ea.pdf (Accessed 17/05/2016)

Biotechnology (FAO/WHO, 2001) and revealed no identities between known or putative protein allergens or toxins and the DsRed22 protein sequence. Furthermore, no short (≥ eight amino acids) identical polypeptide matches were shared between the DsRed2 protein and proteins in the allergen database. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the DsRed2 protein and known or putative allergens or protein toxins.

The weight of evidence developed showed that DsRed2 was unlikely to be allergenic using bioinformatics comparisons of the amino acid sequence of the DsRed2 protein with known or putative protein allergen sequences; evaluation of the stability of the DsRed2 protein using an *in vitro* gastric digestion model, and assessment of the DsRed2 gene source and history of use and exposure. Further evaluations in the Early Food Safety Evaluation (EFSE) of DsRed2 used bioinformatics to show that the marker showed no homology to any known toxin protein, additional information supporting the lack of toxicity to the DsRed2 protein is implied by is homology to the GFP and the GFP's safety assessment which concluded that when ingested by rats it was non-toxic (Pavey and Fedorova, 2006). Direct analysis of the effect of GFP when fed to rats has also demonstrated no adverse effects of oral administration (Richards et al., 2003). The USDA-APHIS issued a Finding of No Significant Impact[23] stating that the maize variety expressing the DsRed2 marker would have *"no significant impact on human or animal health*".

The DsRed2 sequence in strain OX513A is identical to the sequence in the FDA-CFSAN NPC0004, apart from OX513A having a three-amino acid extension at the N-terminus, which probably originated from a linker sequence during cloning.

Additionally, there were no detrimental effects or signs of toxicity when DsRed variants were over-expressed in small mammals (Arao et al., 2009; Luckow et al., 2009; Turchin et al., 2006) or in the arachnid (*Ixodes scapularis*) (Kurtti et al., 2008). Direct analysis of the effect of fluorescent proteins fed to rats has demonstrated no adverse effects of oral administration in the following study:

- Richards et al., (2003) Safety Assessment of recombinant green fluorescent protein orally administered to weaned rats. J Nutr 133:1909-1912.

For insects expressing both the tTAV and DsRed proteins (See ***OX513A Technical Dossier Part A -Section 4.5.3 Additional DsRed2 Toxicity and Allergenicity Assessment***):

- An Environmental Assessment (EA) and Finding of No Significant Impact (FONSI) has been issued by the United States Department of Agriculture- Animal and Plant Health Inspection Service (USDA-APHIS) for the field Release of an Oxitec Diamondback moth expressing both the tTAV and DsRed2 genes[24].
- The United States Food and Drug Administration- Center for Veterinary Medicine (US FDA-CVM) have issued (August 5, 2016) an EA and FONSI which states "*FDA found that the probability that the release of OX513A male mosquitoes would result in toxic effects*

[23] https://www.aphis.usda.gov/brs/aphisdocs/08_33801p_fonsi_rtc.pdf (Accessed 17/05/2016)
[24] https://www.aphis.usda.gov/brs/aphisdocs/13_297102r_fonsi.pdf and https://www.aphis.usda.gov/brs/aphisdocs/13_297102r_dea.pdf (Accessed 31/08/2016)

in humans or non-target animals or allergenic effects in humans is extremely low and the risk is negligible." [25]

Consistent with the findings of both the USDA and US-FDA in independent reviews, the proteins expressed in OX513A have not been demonstrated to be sufficiently homologous to known toxins or allergens nor induce any toxic or allergenic effect in humans or animals.

Herd immunity

It has been suggested that, paradoxically, vector control could lead to increased dengue transmission, through reduced immunity in the human population as less people are exposed (Thammapalo et al., 2008; Goh, 1997). This is not a unique consideration for the use of OX513A in a control program, but an issue for all successful vector control methods, as demonstrated in Singapore (Ooi et al., 2006). Herd immunity provides a degree of protection for individuals who have not developed immunity (John and Samuel, 2000). This protection is because the chain of infection tends to break if a high proportion of individuals in the population are immune. Immunity can be through vaccination or simply by recovery to an immune state after exposure to the pathogen. Though normally considered for diseases that are transmitted directly from human to human, the same principle applies to mosquito-borne diseases.

A potential therefore exists where the prevention of transmission of a disease for a period of time might reduce the level of herd immunity and therefore the protective herd effect, leaving the population more vulnerable if the pathogen were reintroduced. Thammapalo et al. (2008) suggested that in countries with very high transmission rates, reduction in transmission could increase the frequency of dengue hemorrhagic fever even while decreasing the incidence of dengue fever. The proposed mechanism for this is a lower per-infection risk of dengue hemorrhagic fever if a person is infected with a second virus serotype while still cross-immune due to recent infection with a different serotype. This effect requires (a) several virus serotypes to be present and (b) very high transmission rates so that a significant proportion of second infections are within this cross-immune period. The authors assumed a cross-immune period of around 2 years.

If transmission were successfully suppressed over a multi-year period by any method, then over time, recruitment of immunologically naïve individuals (births and immigration of such individuals) and loss of those previously exposed to one or more serotypes (death and emigration) would gradually reduce the seroprevalence level. This successful control would significantly reduce human morbidity and mortality while in place, but in principle, the concomitant decline in herd immunity could allow resurgence of transmission if control were to be abandoned. As described by Ooi et al. (2006) vector control to an intermediate level could prevent transmission for a period, but then allow transmission to resume, albeit at a lower level, if there were sufficient vectors to maintain transmission at herd immunity levels above zero.

The level of control of wild populations of *Aedes aegypti* demonstrated through the use of OX513A are substantial for vector control operations ***OX513A Technical Dossier Part A Section 7.1 Previous Aedes aegypti vector control projects using OX513A.*** Vector control projects conducted to date have however demonstrated exclusively entomological

[25] http://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/GeneticEngineering/GeneticallyEngineeredAnimals/ucm446529.htm (Accessed 23/08/2016)

endpoints. The impact of a reduction in vector population density on the transmission threshold of dengue has however been estimated by Focks et al. (2000) in relation to pupae per person^{-1} (as a proxy for adult mosquito population), ambient temperature and herd immunity[26]. For a mean temperature of 28°C Focks *et al.* (2000) calculated an epidemic transmission threshold of 0.42, 0.61 or 1.27 pupae per person for initial sero-prevalence[27] of 0%, 33% and 67%, respectively. Using calculations and assumptions given in Focks et al., (2000), we estimate that average pupae person^{-1} decreased in our treated area from 0.7 pre-treatment to 0.04 post-treatment, which in their model would be sufficient to prevent epidemic transmission under these conditions, or indeed under the most adverse conditions modelled for a naive human population with 0% sero-prevalence. **Figure 5** represents an example of how the wild *Ae. aegypti* population reduction achieved in Itaberaba Brazil (described in ***OX513A Technical Dossier Part A Section 7.1.2 Brazil- Itaberaba***) relates to transmission thresholds at 3 levels of sero-prevelance.

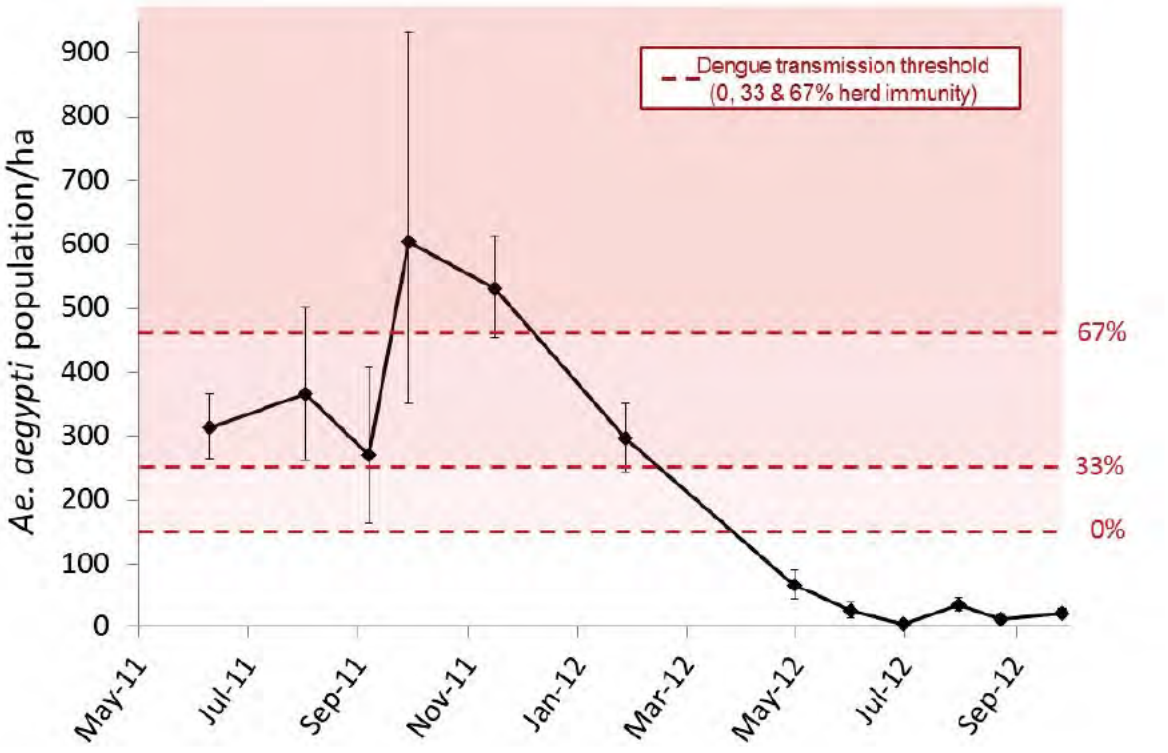

Figure 5. Change in adult *Ae. aegypti* population density throughout the vector control project conducted in Itaberaba, Brazil (Carvalho et al., 2015). Dengue transmission threshold for three sero-prevelance values are indicated by red lines. The average temperature during the peak dengue transmission season (January – July was 27.7°C.) *Ae. aegypti* population density calculated based on methods of (Focks et al., 2000).

[26] Herd immunity describes immunity that occurs when the vaccination of a portion of the population (the "herd") provides protection to unprotected individuals. Herd immunity theory proposes that, in diseases passed from individual to individual, it is difficult to maintain a chain of infection when large numbers of the population are immune.

[27] Seroprevalence is the number of persons in a population who test positive for a specific disease based on serology (blood serum) specimens; often presented as a percent of the total specimens tested or as a proportion per 100,000 persons tested.

Potential for exposure of humans and animals to tetracycline resistance genes.

No tetracycline resistance genes have been used in the construction of OX513A. The potential source of tetracycline resistance discussed in this section relates to the potential development of tetracycline resistant bacteria within the controlled conditions of the rearing process within the MRU. The development of tetracycline resistance under these conditions is also discussed in **Section 2.6** *Potential for tetracycline resistant bacteria to be released into the environment.*

The possibility of DNA transfer from OX513A to microorganisms (e.g., bacteria in the gut of OX513A mosquitoes, humans, or other animals; bacteria present in soil and involved in decomposition of organic matter) has been considered in ***Section 2.2*** *Horizontal Gene Transfer* in the context of potential rDNA transfer from OX513A***.*** It was concluded that such transfer is highly unlikely due to a number of physical, biochemical, and genetic barriers that restrict horizontal gene transfer (Thomas and Nielsen, 2005). Despite the fact that prokaryotes are exposed to an abundance of genetic material from eukaryotic organisms, the presence of eukaryotic genes in the genome of prokaryotes is extremely limited and suggests the existence of functional and selective barriers that limit the acquisition of eukaryotic genes by bacteria (Andersson, 2005).

Tetracycline resistant bacteria, even if present in the larval or pupal stages, would be highly unlikely to be present in adult OX513A mosquitoes due to the fact that gut bacteria are expelled during mosquito metamorphosis from larvae to adults (see **Section 2.6**). Antimicrobial resistance arising in bacteria in the rearing water and the subsequent transfer of this trait to other bacteria that could cause food or water-borne diseases would also be highly unlikely due to the short duration of the mosquito life cycle. Waste water from the MRU will be disposed of in a manner consistent with wastewater disposal on Saba (see ***OX513A Technical Dossier Part B Section 1.5.1 Tetracycline use***) further precluding the potential for exposure of humans and animals to bacteria in the waste water. Also, process controls that would be implemented at the MRU (e.g., use of personal protective equipment) would eliminate the potential for transfer of antibiotic resistant bacteria to personnel involved in the production of OX513A. The likelihood of adverse effects in the receiving environment, associated with development of anti-microbial resistance due to the production and release of OX513A is negligible.

Conclusions to Step 1

No evidence was found to suggest the tTAV or DsRed2 proteins expressed in OX513A represent risks of allergy or toxicity to humans through both bioinformatics analysis, as well as literature review. The potential for tTAV or DsRed2 to be present in OX513A female mosquito saliva has however additionally been examined in *Step 3 Exposure Characterisation*, to further support the overall risk characterisation.

A population suppression approach using OX513A, could be very well placed to reduce the vector population below a disease transmission threshold even with zero herd immunity. Situations in which reduced transmission could result in a loss of immunity in the local human population should consider a control method which provides the potential to reduce the vector population below key transmission thresholds, and has the potential to lead to vector elimination. SIT analogous programmes are very effective against low vector populations in this regard. If a vaccine were to become available, even modest coverage of the population, or an incompletely effective vaccine, could eliminate the issue of herd

immunity. Conversely, for vector-borne diseases such as dengue, effective vector control is likely to be required in conjunction with any vaccine, in all but the most of optimistic of scenarios (Alphey et al., 2010).

1. Potential toxic effects of the new compound(s), their derived metabolic products and/or the GM insects to humans and animals, e.g. qualitative or quantitative change in the production of toxins by the GM insects when compared with their non-GM comparators have not been identified.

2. Potential allergenic effects of the new compound(s), their derived metabolic by-products and /or the GM insects to humans have not been identified.

3. Loss of immunity in the human population and reliance on continued long-term positive effects of vector suppression or replacement strategy does not pose any additional risk, relative to existing or intended interventions.

Through Step 1, potential hazards and pathways to exposure have been assessed through literature analysis and measurement endpoints. There is adequate information to formulate a conclusion that there is no plausible pathway to harm under the protection goals identified in Section 1.1, and that further hazard and exposure characterisation is not required.

Exposure characterization has however been carried out in Step 3 to further support the overall risk characterisation in the area of Human Health.

Step 2: Hazard characterisation

The potential for harm, under the protection goals identified in **Section 1.1,** through the impact on human and animal health has been assessed in step 1. The conclusion is that the potential for harm was negligible in all cases therefore no further Hazard characterisation (Step 2) has been undertaken. Although no hazard has been identified, Exposure Characterisation (Step 3) has been undertaken to further support the Risk Characterisation (Step 4) and contribute to the weight of evidence in formulating the Conclusion (Step 6).

Step 3: Exposure characterisation

Adult female *Aedes aegypti* live in the terrestrial environment in and around houses in dark shaded areas where there are opportunities to blood feed on their human hosts. Adult males live in close proximity to the females and survive on plant nectars and juices and do not require human blood. Males are incapable of biting as they do not have penetrating mouthparts (Christophers, 1960).

Exposure through OX513A saliva- the bite of an adult female

Saliva from *Aedes* species mosquitoes contains secreted proteins which play a role in sugar and blood feeding (Dhar and Kumar, 2003). These have been characterized by proteomic studies of saliva itself (Chisenhall et al., 2014), as well as by studies of the sialome (the set of messages and proteins expressed in saliva glands) (Valenzuela et al., 2002; Racioppi and Spielman, 1987). There is an amino acid signal sequence typically associated with proteins that are secreted into saliva which is cleaved during the process of protein secretion into saliva in mosquitoes (e.g. James et al., (1991). In addition, (de Lara Capurro et al., 2000) confirmed that in order to secrete engineered short chained variable fragment (scFV)

antibodies into the saliva, a mosquito secretory signal sequence, fused to the upstream region of the coding sequence is required for functional expression. The signal peptide serves to direct the protein into the golgi and is cleaved during the process (Stark and James, 1998; James et al., 1991). tTAV contains no such signal sequence for secretion; or homology to such sequences and therefore it is not anticipated to find tTAV in the saliva of OX513A. However to confirm this, a study was conducted to provide data on whether the synthetic proteins tTAV and DsRed2 were present in the female OX513A saliva (see ***OX513A Technical Dossier Part A Section 5.6 Analysis of expression of the introduced proteins in female mosquito saliva***) In order to present a potential risk to human health, tTAV protein would have to (a) be expressed in salivary glands, (b) be secreted into the saliva, and (c) be toxic or otherwise hazardous to humans if injected in relevant quantities. Of these, (a) and (b) relate to potential exposure, while (c) relates to potential hazard (addressed in step 1).

Saliva was collected from OX513A and WT females and pooled separately. 5 µl of OX513A saliva equates to the quantity of saliva collected from approximately 5.5 female adult mosquitoes. Samples of 5 µl were analysed by Western blot using chemiluminescent detection. A limit of detection (LOD) was determined for each of tTAV and DsRed2 (using recombinant versions of the protein due to the limiting factor of saliva collection), and determined to be 0.8 ng for TAV and 5-2.5 ng for DsRed2. tTAV and DsRed 2 were not detected in the saliva of OX513A at the limit of detection (LOD) of the assay.

tTAV and DsRed2 are not expected to comprise a significant fraction of protein in OX513A saliva, and the potential for exposure of the human population to the tTAV and DsRed2 proteins via the bite of OX513A females is considered negligible.

Step 4: Risk characterisation

The likelihood that the deliberate release of OX513A will result in harm to human and animal health has been assessed in Step 1 through the problem formulation step, and additionally exposure was characterised in step 3. The risk is considered negligible and therefore no further risk characterisation is necessary.

Step 5: Risk management strategies

No further risk management strategies are required for this risk area as no plausible pathway to harm under the protection goals identified in ***Section 1.1*** has been identified. Measures to avoid escape into unintended environments during production stages are implemented as a standard practice consistent with arthropod containment principles as described in ***OX513A Technical Dossier Part B Section 1.4 Containment measures prior to release.***

Step 6: Conclusions

Using the information and evidence presented, it has been concluded in the problem formulation step through the identification of hazard and exposure pathways that the likelihood the deliberate environmental release of OX513A in Saba would represent a plausible pathway to harm through the impact on human and animal health is negligible, thus further hazard and exposure characterisation was not required.

Further exposure characterisation was however carried out in Step 3 to further support the overall risk characterisation in the area of human health and the potential for exposure was considered to be negligible.

3. Overall risk evaluation and conclusions

3.1 Uncertainty in the Environmental Risk Assessment (ERA)

Uncertainty in the ERA may arise from various sources such as variability in parameters measured, assumptions and extrapolations made, and the limitations of the current scientific literature and viewpoints. The ERA for the deliberate environmental release of OX513A on Saba is qualitative in nature, while often reliant on quantitative measurement endpoints to formulate conclusions. In qualitative risk assessments, expert judgment in the field is the basis of informing the degree of uncertainty. The key areas of uncertainty which have been identified through this ERA are addressed below:

- What is the likelihood of dispersal of OX513A and their progeny beyond Saba?

There is a high degree of certainty that OX513A released males would have limited dispersal due to the geographic isolation of Saba, and the known dispersal range of *Aedes aegypti,* and that of OX513A as determined experimentally. This is based on results from previous trials of OX513A in other countries and information from the published literature and the location and features of the Saba.

- What is the likelihood for establishment of OX513A in Saba?

Sufficient information from previous field releases of OX513A, where the lifespan of the released insects was approximately 1-3 days and the fact that more than 95% of progeny die before reaching adulthood as well as evidence from the scientific literature on potential sources of tetracycline provide a high degree of certainty that the OX513A would be unlikely to establish in the environment, as addressed in **Section 2.1** *Persistence and invasiveness, including vertical gene transfer*.

Ongoing monitoring of the local mosquito population is integral to the OX513A program and is fundamental to vector control activities generally. Post suppression- phase monitoring will allow an ongoing assessment of the lack of establishment of OX513A in Saba.

- What is the likelihood of inadvertent release of OX513A?

There is a high degree of confidence in the containment measures at the MRU through the risk management strategies in place, as referenced throughout the ERA. Rearing would be conducted consistent with ACL2 containment levels. Staff working at the MRU would be under the supervision of an Oxitec site manager with a high degree of experience in handling OX513A and other GM insects in contained conditions. Locally recruited staff working in the MRU would be trained in the Standard Operating Procedures (SOPs) for the OX513A program as referenced throughout the ERA.

Some uncertainty exists for the occurrence of adverse weather conditions being encountered and preventing rearing or release. This is minimized by a Hurricane Preparedness Policy being in place, where adult and larval insect life stages would be killed within an appropriate time frame in advance of an adverse weather warning of a defined magnitude. Even if some OX513A were to escape containment, the intended effect of the self-limiting trait would prevent establishment in the environment.

Other uncertainties include the nature of incursion of wild Aedes *aegypti* during and post release intervention phase and the quantity of OX513A mosquitoes required in the release

and maintenance phase required for the control of the wild *Aedes aegypti* population. These would be assessed and adjusted dynamically in the preparation phase as well as during the course of the phased control approach.

3.2 Conclusions

This Environmental Risk Assessment (ERA) has been conducted following the methodology and guidelines described in the European Food Safety Authority (EFSA) –*Scientific Opinion – Guidance on the environmental risk assessment of genetically modified animals* (2013). Seven specific areas of risk have been evaluated according to the six steps recommended by (EFSA, 2013) as an interpretation of Directive 2001/18 EC.

The risk assessment was carried out considering one of the following comparators:

a) Wild-type *Aedes aegypti* (unmodified laboratory strains)

b) Wild *Aedes aegypti* (native wild local populations)

c) Existing control measures for *Aedes aegypti*

A comparative safety assessment has been conducted using a weight-of-evidence approach which considers the molecular characterisation of OX513A together with its phenotypic and behavioural characteristics. This assessment has been used to establish whether the intended or any unintended changes in OX513A, as a result of the genetic modification, present a plausible pathway to harm under protection goals defined in the areas of human and animal health, and biodiversity and ecosystem services.

The results of this comparative safety assessment demonstrated that the only differences of biological relevance are the expression of the two introduced traits; the self-limiting trait (tTAV) and the fluorescent marker trait (DsRed2). A significant focus of the environmental risk assessment was thus to characterise tTAV and DsRed2 with respect to the potential adverse effects on environmental or human health. In this regard, although the potential for tTAV and DsRed2 to elicit an immune response or have toxic effects was determined to be negligible, further exposure characterisation under the risk area *Impact on human and animal health* was also carried out to further support the risk characterisation, and the potential for exposure was determined to be negligible.

For all other areas of risk, through *Step 1- Identification of potential hazards and pathways to exposure* there was adequate information to formulate a conclusion that there is no plausible pathway to harm under the protection goals identified and that further hazard and exposure characterisation was not required.

Measures have been described regarding physical containment and procedural controls which are intended to prevent the unintended environmental release of OX513A as a matter of good practice and consistent with common conditions for regulatory compliance. While the measures described may be characterised as risk management measures, they are not in response to any identified risk in this ERA for OX513A in Saba.

In all regulated environmental releases to date OX513A has performed consistently with respect to parameters identified in risk assessment submissions, and no un-anticipated results nor unintended effects have been observed, nor required regulatory reporting.

4. References

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OX513A Technical Dossier

Part D – Advanced Informed Agreement Notification

Submission to the GMO Office of the National Institute of Public Health and the Environment of the Netherlands (RIVM) for the technical evaluation of the release of *Aedes aegypti* OX513A in Saba.

September 2016 v.1

Oxitec Limited

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The information provided in Part D constitutes the information requirements outlined in *Annex I* to the *Cartagena Protocol on Biosafety to the Convention on Biodiversity,* and is to be notified to the Competent Authority of Saba prior to the importation of OX513A eggs into Saba.

A brief summary of the information or source of information to be provided under the headings below is given. Full details will be provided in the format below once required permits and agreements are in place to proceed with OX513A program deployment.

(a) Name, address and contact details of the exporter.

- *Eggs to be shipped from Oxitec UK*

(b) Name, address and contact details of the importer.

- *Department of Vector Control Saba (or appropriate local entity)*

(c) Name and identity of the living modified organism, as well as the domestic classification, if any, of the biosafety level of the living modified organism in the State of export.

- *Reference to relevant sections of OX513A Technical Dossier Part A*
- *Aedes aegypti is not subject to a domestic biosafety classification level in the UK.*
- *The use of GM animals is regulated under The Genetically Modified Organisms (Contained Use) Regulations 2014.*

(d) Intended date or dates of the transboundary movement, if known.

- *To be determined pending project approvals*

(e) Taxonomic status, common name, point of collection or acquisition, and characteristics of recipient organism or parental organisms related to biosafety.

- *Reference to relevant sections of OX513A Technical Dossier Part A*

(f) Centres of origin and centres of genetic diversity, if known, of the recipient organism and/or the parental organisms and a description of the habitats where the organisms may persist or proliferate.

- *Reference to relevant sections of OX513A Technical Dossier Part A and Part B (Saba specific habitat)*

(g) Taxonomic status, common name, point of collection or acquisition, and characteristics of the donor organism or organisms related to biosafety.

- *Reference to relevant sections of OX513A Technical Dossier Part A*

(h) Description of the nucleic acid or the modification introduced, the technique used, and the resulting characteristics of the living modified organism.

- *Reference to relevant sections of OX513A Technical Dossier Part A*

(i) Intended use of the living modified organism or products thereof, namely, processed materials that are of living modified organism origin, containing detectable novel combinations of replicable genetic material obtained through the use of modern biotechnology.

- *Reference to relevant sections of OX513A Technical Dossier Part A and Part B*

(j) Quantity or volume of the living modified organism to be transferred.

- *The amount of OX513A eggs needed during the course of the project will be indicated in grams to be shipped in approximate monthly shipments. 1 gram off eggs is roughly equivalent to 95 000 eggs; approximately 1 OX513A adult male is produced for release for each 7 eggs imported.*

(k) A previous and existing risk assessment report consistent with Annex III [of the Cartagena protocol].

- *OX513A Technical Dossier Part C- Environmental Risk Assessment consistent with 2001/18 EC Annex II*

(l) Suggested methods for the safe handling, storage, transport and use, including packaging, labelling, documentation, disposal and contingency procedures, where appropriate.

- *Reference to relevant sections of OX513A Technical Dossier Part B Section 1*

(m) Regulatory status of the living modified organism within the State of export (for example, whether it is prohibited in the State of export, whether there are other restrictions, or whether it has been approved for general release) and, if the living modified organism is banned in the State of export, the reason or reasons for the ban.

- *OX513A is regulated under The Genetically Modified Organisms (Contained Use) Regulations 2014 in the UK. Oxitec operates a containment level 1 (CL1) (low risk) facility under licence from the UK Health and Safety Executive for the use of genetically modified organisms.*
- *Aedes aegypti is not subject to a domestic biosafety classification level in the UK.*
- *Aedes aegypti is also regarded as a potential carrier species for animal pathogens, imports of which are regulated under The Importation of Animal Pathogens Order 1980 by the Department of Environment Food and Rural Affairs (DEFRA).*

(n) Result and purpose of any notification by the exporter to other States regarding the living modified organism to be transferred.

- *To be provided for projects to date at the time of notification to Saba.*

(o) A declaration that the above-mentioned information is factually correct.

- *To be provided at the time of notification to Saba.*

Abbreviations

Master list to accompany the submission to the GMO Office of the National Institute of Public Health and the Environment of the Netherlands (RIVM) for the technical evaluation of the release of *Aedes aegypti* OX513A in Saba.

Companion to *OX513A Technical Dossier- Part A, B, C*

#OX513	The genetic construct inserted into the OX513A
µg	microgram
ACL2	Arthropod Containment Level 2
AMCA	The American Mosquito Control Association
BLAST	Basic Local Alignment Search Tools
CAC	Codex Alimentarius Commission
CDC	Centre for Disease Control and Prevention
CFR	Code of Federal Regulations (U.S.)
CFSAN	Center for Food Safety and Applied Nutrition
CHIKV	Chikungunya virus
CL1	Containment Level 1
CSC	Container Safety Convention
DDT	dichlorodiphenyltrichloroethane
DENV	Dengue virus
DNA	deoxyribonucleic acid
DSP	daily survival probability
DsRed2	fluorescent marker gene from Discosoma species
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
ECL	Enhanced Chemiluminescence
EFSA	European Food Safety Authority
EFSE	Early Food Safety Evaluation
EIA	equine infectious anemia
EIP	extrinsic incubation period

EIS	Environmental Impact Statement
ERA	Environmental Risk Assessment
EU	European Union
EVA	equine viral arteritis
FAO	Food and Agriculture Organisation of the United Nations
ft	feet
GIS	Geographical Information System
GM	Genetically Modified
GMO	Genetically Modified Organism
GRAS	generally recognized as safe
HGT	Horizontal Gene Transfer
HSE	UK Health and Safety Executive
ICGES	Instituto Conmemorativo Gorgas de Estudios de la Salud
IPPC	International Plant Protection Convention
IRS	indoor-residual spraying
IRR	Initial Release Rate
ISO	International Organization for Standardization
ITN	treated bed nets
ITR	inverted terminal repeat
IUCN	International Union for Conservation of Nature
IVM	Integrated Vector Management
kdr	knock-down [mutations]
km	kilometre
L	litre
L1	first instar Larva
LOD	Limit of Detection
LSTM	Liverpool School of Tropical Medicine
mi	miles
ml	millilitre
MRCU	Cayman Mosquito Research and Control Unit

MRU	Mobile Rearing Unit
MyRIDL513A	a derivate of OX513A in a Malaysian genetic background (see literature
MyWT	Malaysian background wild-type *Aedes aegypti*
NCBI	National Center for Biotechnology Information
NOEL	No Observable Effect Level
NTO	Non Target Organism
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator - Australian Government Department of Health
OIE	Office International des Epizooties
ORF	Open Reading Frame
piRNA	Piwi-interacting RNA
PMCA	Purple Martin Conservation Association
ppm	parts per million
QMS	Quality Management System
rDNA	recombinant Deoxyribonucleic Acid
RNA	Ribonucleic Acid
S.D.	Standard Deviation
scFV	single-chain variable fragment
SIT	Sterile Insect Technique
SOP	Standard Operating Procedure
TO	target organism
tRE	tetracycline response element
tTAV	insect-optimized tetracycline repressible transactivator protein inserted in the OX513A mosquito
U.S.	United States of America
UK	United Kingdom
USDA	United States Department of Agriculture
USDA-APHIS	United States Department of Agriculture- Animal and Plant Health Inspection Service

US-FDA	United States Food and Drug Administration
USFWS	United States Fish and Wildlife Service
VCAG	Vector Control Advisory Group- WHO
WHO	World Health Organisation
WT	Wild-type
YFV	Yellow fever virus

Oxitec Internal Research Reports

Submission to the GMO Office of the National Institute of Public Health and the Environment of the Netherlands (RIVM) for the technical evaluation of the release of *Aedes aegypti* OX513A in Saba

OXITEC

INTERNAL RESEARCH REPORT

1. **Reference Number**: PH-2013-2-V2a

2. **Issuing Date**: 21 April 2015

3. **Title**: Ingestion of tetracycline by adult female *Aedes aegypti* does not affect penetrance of the OX513A transgenic phenotype

4. **Statement Of Data Confidentiality Claims:**

   Confidential Business Information (CBI) has been deleted from this report

5. **Statement Concerning Good Laboratory Practices:**
   This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

6. **Authors:**

Study Coordinator (Signature):	**Study Supervisor (Signature):**
Study Coordinator (Name And Position):	**Study Supervisor (Name And Position):**
Senior Scientist	Chief Scientific Officer
Date Signed: 21 APRIL 2015	**Date Signed:** 21 April 2015

7. **Associated Personnel:**

Name	Tasks
	Study coordination, experimental design, data analysis, report writing
	Experimental design, report writing and approval
	Insectary work, data collection, report writing
	Insectary work, data collection, statistical analysis
	Statistical analysis
	Insectary work
	Study Sponsor

8. **Test Facility:**

This research was performed at Oxitec's research facility located at:
46a Western Avenue,
Abingdon,
Oxfordshire,
OX14 4RU
United Kingdom

9. **Objectives:**

The objective of this study was to test the hypothesis that providing high doses of dietary tetracycline to adult female *Aedes aegypti* (either homozygous OX513A transgenic females mated to wild-type males, or wild-type females mated to homozygous OX513A transgenic males) has no effect in the penetrance of the OX513A lethal phenotype observed in their heterozygous offspring.

10. **Summary:**

Oxitec's transgenic *Aedes aegypti* strain OX513A has been recently used in open releases of transgenic mosquitoes in the environment and successful suppression of field mosquito populations by the release of genetically sterile males. Because the lethal phenotype displayed by this strain is repressible by the addition of tetracycline to the larval rearing medium, we wanted to establish whether the oral administration of high doses of tetracycline to parental female mosquitoes (either homozygous OX513A females mated to wild-type males, or wild-type females mated to homozygous OX513A males) has any measurable effect on the penetrance of the transgenic phenotype observed in the offspring of said females.

Our data indicate that the penetrance of the OX513A phenotype in heterozygous offspring of female mosquitoes which have ingested high doses of tetracycline is not significantly different from that observed in the offspring of females that were not provided with tetracycline in their diet.

11. **Introduction:**

First developed in 2002 at Oxford University, the *Aedes aegypti* RIDL strain OX513A was originally produced by transforming the Rockefeller strain using a *piggyBac*-based construct. This strategy allowed the integration of a repressible dominant lethal system in the *Ae.*

aegypti genome whereby, in the absence of tetracycline, >95% of the heterozygous progeny of mating between RIDL males with wild type females (and of RIDL females with wild-type males) die before becoming functional adults due to the accumulation of high intracellular levels of tTAV protein produced by a positive feedback loop. However, if tetracycline is added to the larval rearing medium in sufficient quantities (e.g. to 30 µg/ml), tTAV expression is repressed, allowing for normal development to adulthood (Phuc *et al.*, 2007).

Recently, strain OX513A has been used in the first open release of transgenic mosquitoes in the environment (Harris *et al.*, 2011) and the first successful suppression of field mosquito populations by the release of genetically sterile males (Harris *et al.*, 2012). Due to the high penetrance of the lethal phenotype expressed by mosquitoes inheriting the OX513A construct, the overwhelming majority of the offspring of released transgenic males will die before reaching adulthood. However, under laboratory conditions, a small percentage of these offspring (<5%) might survive to produce flying adults, of which approximately half will be female (Phuc *et al.*, 2007). Although preliminary data suggest that this survival rate is probably much reduced in heterozygous individuals produced in the field (due to the many environmental challenges that mosquitoes face in the wild relative to benign laboratory conditions), it is nonetheless possible that a few female individuals carrying the OX513A construct could make it to adulthood following a field release. Furthermore, although the sex-separation techniques used to eliminate females from the released cohorts are highly efficient (well above 99% female elimination) (Harris *et al.*, 2012), they are not perfect, and therefore the potential exists for a small proportion of OX513A adult females to remain in the male populations released during a field trial.

Tetracycline is an antibiotic used extensively as a therapeutic and/or prophylactic agent in human and veterinary medicine. Therefore, it is possible that a female mosquito could feed on a person or animal that had recently received a dose of tetracycline and carries some level of this antibiotic in the bloodstream. In vertebrates, the concentration of tetracycline in the blood usually reaches a peak 2-6 hours following an oral or injected dose, and then gradually declines due to the body's metabolic activity (Agwuh and MacGowan, 2006). In both humans and livestock, the peak concentration of tetracycline in blood (plasma) following standard therapeutic doses normally remains below 10 µg/ml (Agwuh and MacGowan, 2006; Bimazubute *et al.*, 2011). To the best of our knowledge, the highest concentration of tetracycline recorded in vertebrate blood is ~20 µg/ml (a level observed in pigs that received unusually high intra-muscular doses as part of experimental treatments) (Bimazubute *et al.*, 2011).

Although we are not aware of any evidence suggesting that oral ingestion of tetracycline by a female mosquito results in deposition of active tetracycline in her eggs, we wanted to investigate whether providing an adult female mosquito with tetracycline-containing meals would have any measurable effect on the penetrance of the phenotype of her heterozygous offspring. Therefore, we hypothesized that providing high doses (50-100 µg/ml) of dietary tetracycline to adult female *Ae. aegypti* (either homozygous OX513A females mated to wild-type males, or wild-type females mated to homozygous OX513A males) should have no effect in the penetrance of the OX513A phenotype observed in their heterozygous offspring. To test this hypothesis, we set up crosses using females which had access to either tetracycline-free meals or meals containing high doses of tetracycline, and we evaluated the penetrance of the lethal phenotype on their heterozygous offspring.

Our results suggest that the ingestion of high concentrations of tetracycline by a female mosquito does not affect the penetrance of the lethal OX513A phenotype in her offspring.

12. **Methods:**

Strains

This study was performed using the following *Ae. aegypti* strains:

- **Latin wild-type:** This non-transgenic strain was originally collected in the region of Chiapas (Mexico) and was transferred to Oxitec from Mexico's Institute of Public Health in 2006 (D. Nimmo, personal communication). This strain will be henceforth referred to as WT.

- **OX513A (bi-sex lethal RIDL strain):** In the absence of tetracycline, this transgenic strain expresses high levels of the tTAV protein during immature stages through a positive feedback loop, resulting in high mortality. However, when reared in the presence of tetracycline, expression of tTAV in this strain is repressed, allowing for high survival levels (Phuc *et al.*, 2007). Individuals of this strain can be identified during immature stages by the expression of the fluorescent protein DsRed2 in their bodies in a characteristic punctate pattern driven by the Actin5C promoter. The particular strain used for this study was generated by introgressing the original OX513A strain into the aforementioned Latin WT genetic background.

Insect Rearing

All specimens were reared under standard insectary conditions: 27°C [+/- 1°C], 70% [+/- 10%] relative humidity, 12h: 12h light: darkness cycle. Larvae were fed finely ground Tetramin© fish flakes (Tetra GmbH, Germany) and adults were provided with 10% sucrose solution. To obtain eggs, mated females were provided with defibrinated horse blood (TCS Biosciences Ltd., UK) and given access to wet filter paper (Whatman, UK) as oviposition substrate.

In the case of groups requiring rearing on-tetracycline ('ON-tet'), 30 µg/ml chlortetracycline hydrochloride (Sigma-Aldrich, USA) was added to the larval rearing water.

Crosses

Adult virgin homozygous OX513A individuals (reared ON-tet) were crossed to adult virgin WT individuals. To emulate all potential scenarios in the field following a mass-release of transgenic mosquitoes, both reciprocal crosses (OX513A♀ vs. WT♂, and OX513A♂ vs. WT♀) were performed in tetracycline-loaded cohorts.

Tetracycline-loading

To evaluate the effects of the ingestion of high tetracycline concentrations, selected groups of female mosquitoes (henceforth referred to as 'tet-loaded' groups) received both blood and sugar meals containing a pre-determined dose (either 50 µg/ml or 100 µg/ml) of chlortetracycline hydrochloride.

Experimental design

Table 1 provides a description of the different control and experimental groups set up for this experiment. With the exception of group F, all groups consisted of six repeats containing 200 L1 larvae each. Group F consisted of six repeats containing 85-200 L1 larvae each (see table 1 and annex 1 for details).

Table 1. **Control and experimental groups.**

GROUP	DESCRIPTION	PARENTAL CROSS	TET-LOADING DOSE*	LARVAL REARING MEDIUM
A	Non tet-loaded control	OX513A ♂ vs. WT ♀	None‡	OFF-TET
B	Experimental	OX513A ♂ vs. WT ♀	50 µg/ml	OFF-TET
C	Experimental	OX513A ♀ vs. WT ♂	50 µg/ml	OFF-TET
D	Experimental	OX513A ♂ vs. WT ♀	100 µg/ml	OFF-TET
E	Experimental	OX513A ♀ vs. WT ♂	100 µg/ml	OFF-TET
F†	Rearing control	OX513A ♂ vs. WT ♀	None‡	ON-TET
G†	Rearing control	OX513A ♂ vs. WT ♀	50 µg/ml	ON-TET

*Refers to concentration of tetracycline offered to parental females in both blood and sugar meals.

‡The parental females of groups A and F received only tetracycline-free diets.

†Groups F and G were set-up to control for mortality caused by factors independent from the penetrance of the phenotype (i.e. environmental conditions, manipulation, etc.) and were therefore excluded from statistical analysis.

Within each group, we evaluated the following parameters:

a) Pupation: Survival from first larval instar to pupation.
b) Adult emergence: Survival from first larval instar to the appearance of adults, regardless of the fitness or longevity displayed by adult mosquitoes.
c) Number of flying adults: Number of adults which were able to fly ≥48 hours after emergence. This category was created to differentiate fully functional adults from those that die soon upon emergence from the puparium (often without being able to leave the rearing water).

Statistical analysis

Data were analysed using the RStudio software package version 0.97.237 (RStudio, USA). Normality was tested using the Shapiro-Wilk method. For normally-distributed data (survival to pupation, adult emergence), parametric significance tests were carried out using ANOVA and, when required, Tukey's honestly-significant-difference (HSD) tests for post-hoc analysis. For non-normally distributed data (number of flying adults), non-parametric testing was performed using the Kruskal-Wallis test, followed by post-hoc analysis using the Nemenyi test (Zar, 1999).

13. RESULTS

For all parameters analysed, numeric data (raw and average) are presented in Annex 1.

Survival to pupation: ANOVA revealed significant differences between groups for this parameter ($F(4,25)=4.57$, $p=0.007$). Post-hoc analysis using Tukey's HSD test indicated a

statistically significant difference ($p<0.01$) in the average survival to pupation between groups B and E (fig. 1). No other significant differences were observed during pair-wise post-hoc testing.

Adult emergence: Statistical testing (ANOVA) revealed no significant differences between groups for this parameter ($F(4,25)=1.616$, $p=0.201$) (fig. 1).

Flying adults: Kruskal-Wallis test indicated significant differences between groups for this parameter ($H(4)=9.929$, $p=0.04164$). Nemenyi post-hoc tests revealed a significant difference ($p<0.05$) in the average number of flying adults between groups C and E (fig. 1). No other significant differences were observed during pair-wise post-hoc testing.

14. Discussion and Conclusions:

No significant differences were observed between the non tet-loaded controls and the experimental groups in any of the parameters examined in this study, supporting the hypothesis that penetrance of the OX513A phenotype in the heterozygous offspring of female mosquitoes which have ingested high doses of tetracycline (50-100ug/mL) is not different from that observed in the offspring of females that did not ingest any tetracycline with their diet.

Post-hoc testing of our results identified significant differences between various tet-loaded groups in two parameters (survival to pupation between groups B and E, and number of flying adults between groups C and E). The fact that no significance was observed when comparing either of those groups to their corresponding non tet-loaded controls suggests that the observed differences are caused by factors unrelated to the ingestion of tetracycline. Although the exact nature of these factors remains to be described, we believe they are probably related to environmental conditions during rearing, and therefore not relevant to the specific objectives of this study.

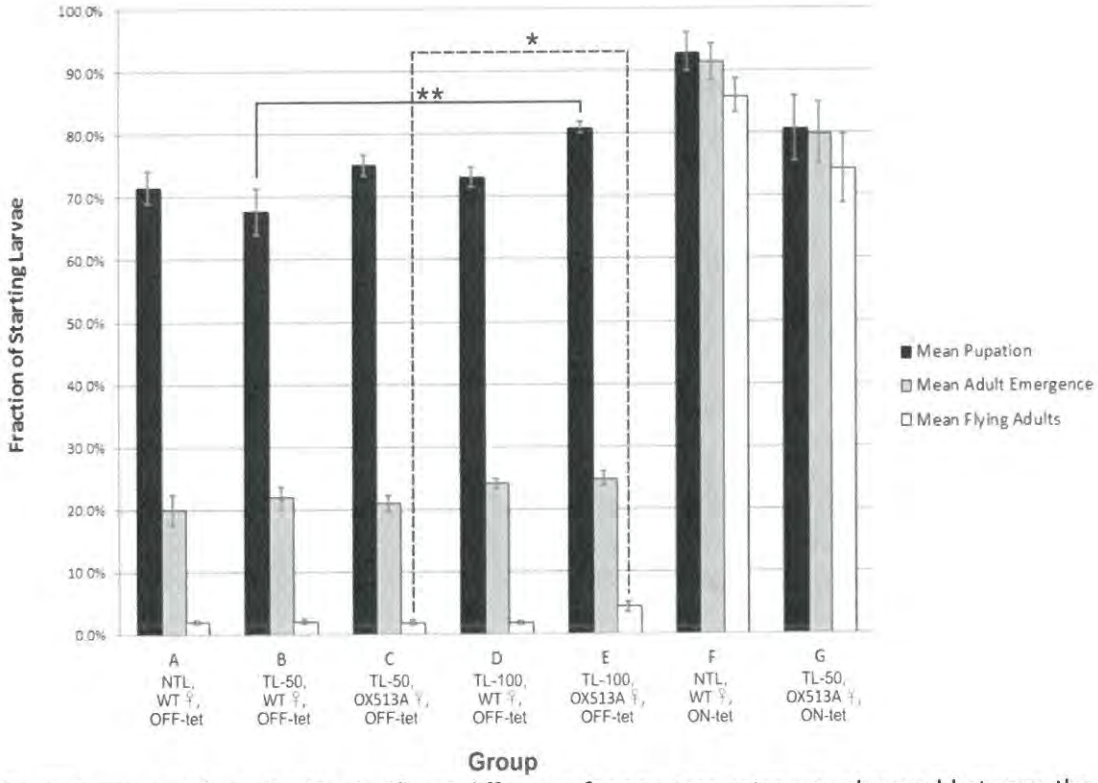

Fig. 1. **Summary of results**. No significant difference for any parameter was observed between the non tet-loaded control group (A) and any of the treatment groups (B-E). Significant differences were only observed in pupation between groups B and E ($p<0.01$), and in the number of flying adults between groups C and E ($0.01<p<0.05$). Values for the ON-tet control groups (F,G) are shown for reference. NTL: Non tet-loaded. TL-50: Tetracycline loaded, 50µg/ml. TL-100: Tetracycline loaded, 100µg/ml. WT ♀: Female of parental cross was wild-type. OX513A ♀: Female of parental cross was transgenic. OFF-tet: Larvae reared without tetracycline. ON-tet: Larvae reared with tetracycline added to the rearing water.

It is important to highlight that the highest dose of tetracycline used in this study is 10-fold higher than the normal concentration found in the blood of humans or animals receiving usual therapeutic doses of tetracycline, and 5-fold higher than the highest dose reported (to the best of our knowledge) from any animal blood. This suggests that during a field release of transgenic mosquitoes, the OX513A phenotype should not be compromised by the presence of individuals (human or animal) receiving tetracycline treatments in the target area, whether on an individual basis in terms of survival of heterozygous progeny from released specimens, or on a population basis in terms of the suppressing effect of systematic mass releases of OX513A mosquitoes.

15. Literature:

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Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J., Pape, G., Fu, G., Condon, K.C., Scaife, S., Donnelly, C.A., *et al.* (2007). Late-acting dominant lethal genetic systems and mosquito control. BMC biology 5, 11.

Zar, J.H. (1999). Biostatistical Analysis. Prentice-Hall. New Jersey, USA. 663 pp.

OXITEC

1. **Reference Number**: PH-2013-3-V3a

2. **Issuing Date**: 25 February 2016

3. **Title**: Assessment of heterozygous OX513A individuals surviving without provision of tetracycline in the diet: Longevity and Fecundity

4. **Statement Of Data Confidentiality Claims:**

   Confidential Business Information (CBI) has been deleted from this report

5. **Statement Concerning Good Laboratory Practices:**
   This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

6. **Authors:**

Study Coordinator (Signature):	**Study Supervisor (Signature):**
Study Coordinator (Name And Position):	**Study Supervisor (Name And Position):**
Senior Scientist	Chief Scientific Officer
Date Signed: 25 Feb 2016	**Date Signed:** 25 FEB 2016

7. **Associated Personnel:**

Name	Tasks
	Study coordination, experimental design, data collection and analysis, report writing
	Experimental design, approval
	Experimental design, report editing, study supervisor and approval
	Data collection
	Study Sponsor

8. **Test Facility:**

This research was performed at Oxitec's research facility located at:
46a Western Avenue
Abingdon, Oxfordshire,
OX14 4RU
United Kingdom

9. **Objectives:**

The objective of this study was to assess the fitness of the small fraction of heterozygous OX513A individuals that reach adulthood following rearing in the absence of tetracycline.

10. **Summary:**

This study assessed the longevity of adult male and female OX513A *Aedes aegypti*. The homozygous OX513A strain, used for field trials in Brazil, was outcrossed to wild-type of Latin background to generate heterozygous eggs. These eggs were hatched and reared in the absence of the antibiotic tetracycline that is required for survival of most individuals. Emerged, flying adults were collected and housed in single-sex groups. The longevity of these individuals was assessed over a period of over 12 weeks alongside that of non-transformed insects of the same background reared with tetracycline (1 µg/mL) in the rearing water, and wild-type individuals. Rearing in the absence of tetracycline mimics the conditions heterozygous offspring of OX513A males will encounter in the wild. The 1 µg/mL dose was selected because it is the minimum dose needed to provide good survival of OX513A heterozygotes (See PH-2013-4-v1), yet well over the amounts animals might encounter in the field (Le-Minh et al., 2010; Locatelli et al., 2011). Longevity of homozygous OX513A individuals reared on the standard tetracycline dose of 30 µg/mL was also assessed. These experiments therefore examine the longevity of the two types of OX513A female most plausibly present in the field – homozygous females inadvertently co-released with homozygous males, and heterozygous progeny of released males that have mated with wild females and survive as a consequence of incomplete penetrance of the lethal trait. The lifespan of OX513A homozygotes and heterozygotes was found to be significantly reduced relative to wild type comparators. Since longevity is an important component of vectorial capacity, shorter lifespan implies reduced vectorial capacity, especially for heterozygous females reared without tetracycline (median lifespan 2 days relative to wild type 68 days). This reduction in longevity also implies that the mean fitness of heterozygous OX513A males and females reared without tetracycline is even lower than one would estimate simply by considering survival to adulthood.

11. Introduction:

Aedes aegypti RIDL strain OX513A was developed at Oxford University in 2002 by injection of the OX513A *piggyBac*-based construct into the Rockefeller strain (Phuc et al., 2007). The dominant-lethal phenotype of this strain, when reared without tetracycline, results in death of immature heterozygous progeny at a rate of approximately 95%. The basis of this phenotype is the accumulation of the tTAV protein via a positive feedback loop that can be broken by the binding of tetracycline to tTAV itself. When homozygous males bearing this trait are released into the environment to mate with wild females, matings are unproductive as the progeny have no access to tetracycline, and the population is suppressed.

The use of OX513A has recently been shown to be effective as a genetic SIT strategy in an open field trial (Harris et al., 2012). The high penetrance of heterozygous lethality means that very few heterozygous progeny emerge as functional adults[1] – less than 5% under laboratory conditions. Models suggest that RIDL-based control of *Aedes aegypti* should be effective so long as the average fitness of heterozygous progeny is less than 10% relative to wild type (Phuc et al., 2007). The average fitness of OX513A heterozygous progeny in the field is predicted to be lower than indicated by laboratory based studies based on the presumed rigours of life in natural habitats, as compared with the protected environment of the laboratory, but risk assessments should assume that a non-zero fraction of heterozygous females will reach adulthood in the wild. Additionally, sex-separation methods designed to allow only the release of males are very good (>99% accurate), but not perfect, and a small number of homozygous females are expected to be released over the course of a control programme.

Of particular interest for risk assessment is the ability of OX513A females to serve as disease vectors which is heavily dependent on their ability to live long enough to take at least two bloodmeals, separated by the extrinsic incubation period (EIP) of flaviviruses such as dengue, estimated to range from 7-15 days, depending on environmental temperature (Chan and Johansson, 2012). Evaluation of both the longevity of both relevant types of adult female was therefore conducted in this study: heterozygous OX513A reaching adulthood without the provision of tetracycline, and homozygous OX513A reared with tetracycline. The null hypotheses being tested is that OX513A-bearing individuals are not more long-lived than their wild type comparators. The assessment of homozygous OX513A females will help establish the risks associated with accidental release of females as part of the control programme. The fitness of the heterozygous offspring also relates to the potential efficacy of such a programme – modelling indicates that the predicted efficiency and effectiveness of the method would be substantially reduced it the net fitness of offspring were >10% that of wild type. Lifespan is a major component of fitness. Additionally, an assessment of the fecundity of surviving OX513A heterozygous females that are reared without tetracycline is presented; fecundity is another element of biological fitness.

[1] Progeny types include dead larvae, dead pupae, non-viable/non-functional adults (dead adults on the water (of the weigh boat), dead adults on the floor of the cage and non-flying adults) and functional adults (adults capable of flight); all these classes except the last are considered to have zero fitness as *Aedes aegypti* court and initiate mating on the wing, and flight ability is required in the field to avoid predators and to find mates, hosts and oviposition sites.

12. **Methods:**

Strains

The following strains were used for this experiment:

Latin Wild-type (LWT): This non-transgenic strain was originally collected in the region of Chiapas (Mexico) and was transferred to Oxitec from Mexico's Institute of Public Health in 2006 (D. Nimmo, personal communication).

OX513A Latin1 (bi-sex lethal RIDL strain): In the absence of tetracycline, this transgenic strain expresses high levels of the tTAV protein during immature stages through a positive feedback loop, resulting in high mortality. However, when reared in the presence of tetracycline, expression of tTAV in this strain is repressed, allowing for high survival levels (Phuc *et al.*, 2007). Individuals of this strain can be identified during immature stages by the expression of the fluorescent protein DsRed2 in their bodies in a characteristic punctate pattern driven by the Actin5C promoter. The particular strain used for this study was generated by introgressing the original OX513A strain into the aforementioned Latin WT genetic background.

Insect Rearing

All strains were reared under standard insectary conditions: 27°C [+/- 1°C], 70% [+/- 10%] relative humidity, 12 h:12 h light:dark cycle. Larvae were fed finely ground Tetramin© fish flakes (Tetra GmbH, Germany) on the standard diet and reared at a density of 1 larva/mL. Adults were provided with 10% sucrose solution *ad libitum*. Strains were reared with the addition of chlortetracycline (tetracycline) to the rearing water at concentrations of 0, 1 or 30 µg/mL, as described.

Fecundity Study

All larvae for this study were reared in the absence of tetracycline. New larval rearing trays and food were used to avoid inadvertent introduction of tetracycline. Adult females were mated to LWT males 4 days post eclosion. Males were allowed to cohabitate with females for 2 days, after which they were removed. A blood meal was offered 7 days post eclosion. Two days post-bloodmeal, females were transferred to entomological tubes with wetted cotton wool in the bottom to promote individual egg laying. Females were discarded and eggs were counted 3 days post transfer of the females to entomological tubes. Eggs were vacuum hatched in pure water 5 days post laying, and L1 larvae counted.

Longevity Study

New larval rearing trays and food were used to avoid inadvertent introduction of tetracycline in tetracycline-free experiments. Pupae were picked and sexed daily. Each 15 cm^3 cage received 25 male or female pupae, all picked on the same day to allow near-synchronous eclosion unless otherwise noted. Females were mated for two days, and the males then removed. Two blood meals of defibrinated horse blood (TCS Biosciences Ltd., UK) were provided on days 7 and 17 with eggs collected on a wet filter paper (Whatman, UK) 4 days later (Figure 2). In addition to 10% sucrose solution, adults were also provisioned with pure water *ad libitum*. Dead adults were removed from cages daily and counted. Cages were rotated in the insectary daily to control for environmental factors based on position in the insectary; sugar and water feeders were replaced every two weeks.

Statistical Analyses

Data were analysed using the RStudio software package version 0.97.237 (RStudio, USA) running version 2.15.0 of the R statistical software. For longevity analysis, the Survival Analysis package, survival (2.37-2), was used to plot Kaplan-Meier curves and test for significance. Normality and homoscedasticity were tested using Shapiro-Wilk and Bartlett-box tests, respectively. Parametric significance tests were carried out using ANOVA, and post-hoc testing was performed using the Tukey HSD method. Average egg-laying numbers were compared using a student's t-test, and hatch rates were compared using the Mann-Whitney *U* test.

13. Results

Fecundity of OX513A

A total of 18 heterozygous OX513A females and 22 LWT females laid eggs. Each strain had one egg clutch that did not hatch, so 17 OX513A egg clutches and 21 LWT egg clutches were examined as part of this study. Only the first gonotrophic cycle was observed. As shown in Figure 1, the mean clutch size for OX513A was 69.9 eggs (S.D. 13.9) and for LWT it was 54.8 eggs (S.D. 12.4). Analysis by t-test revealed a significant difference between average values (p=0.001), indicating that the OX513A strain lays a larger egg clutch during the first gonotrophic cycle, compared to its wild-type background. The mean hatch rates were 92% (S.D. 14) and 82% (S.D. 18) for OX513A and LWT, respectively. Statistical testing did not reveal any significant difference between these values (p=0.089). It should be noted the OX513A strain has been intensively mass reared for over 100 generation equivalents while the LWT strain has not, which may explain the differences observed.

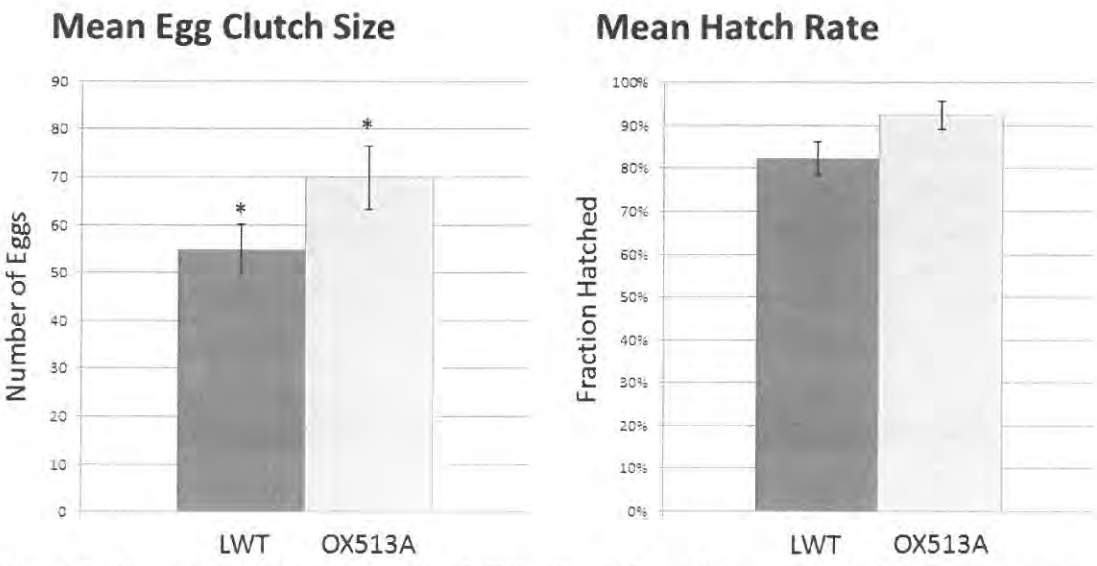

Figure 1: Fecundity study results. * p=0.001. The 95% confidence interval is shown for egg clutch size, and the standard error of the mean for hatch rate.

Longevity of OX513A

Three longevity comparisons were carried out: Heterozygous OX513A without tetracycline in the rearing water, heterozygous OX513A reared with 1 µg/mL tetracycline provided in the rearing water, and homozygous OX513A reared with 30 µg/mL tetracycline provided in the rearing water, each compared to a LWT cohort reared under the same conditions. For

both studies where tetracycline was provided, 25 pupae of each sex were placed into a cage on Day -2, and counting commenced on Day 1 (Figure 2).

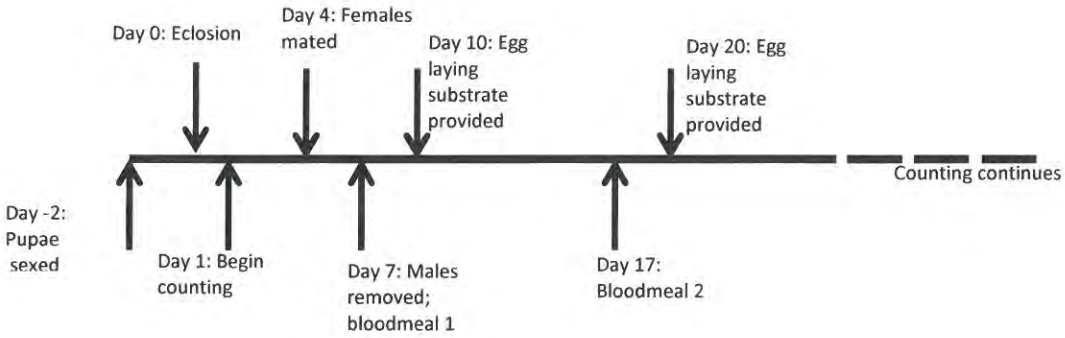

Figure 2: SFFigure 2: Schematic of female longevity experiments. Males were allowed to eclose and then counted each day, starting on Day 1.

Due to anticipated and observed death of the majority of OX513A heterozygous pupae, large, steep-sided eclosion containers (16 oz deli pots, Robertson Packaging, UK, SICC65) containing hundreds of pupae were put into cages for these trials so that the few flying adults could leave the surface of the water and thereby separate from the larger number of non-flying individuals unable to leave the container. In total, 87 females eclosed and were able to exit the eclosion container from a starting cohort of 4000 L1 individuals. The total number of flying adults, male and female, represents 4.4 % of starting L1 larvae. Not all motile females are included in this study as 6 eclosed too late to be used, and females unable to fly on the morning of Day 1 were also excluded. For this reason, the cohort sizes in these cages range from 17 to 30.

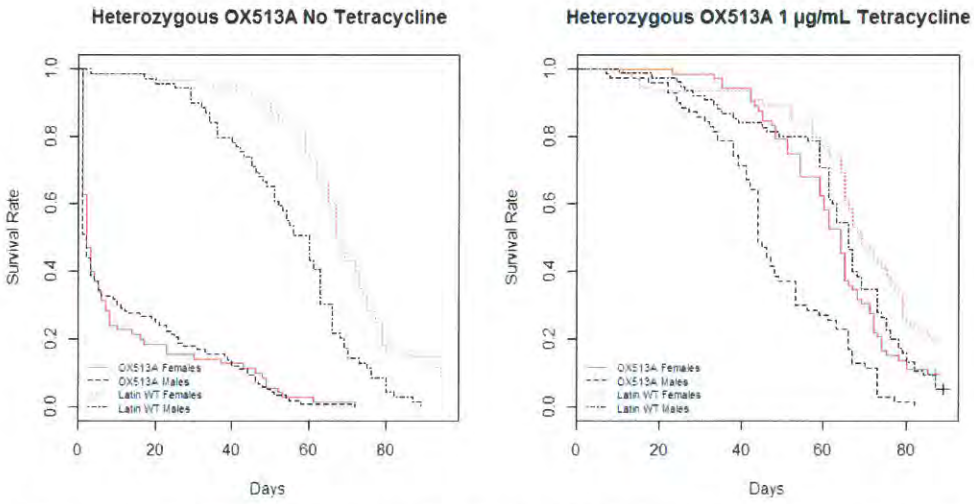

Figure 3: Survival curves for heterozygous OX513A reared without tetracycline or with 1 µg/mL in the larval rearing water.

Substantial mortality was observed within the first few days post-eclosion for the OX513A heterozygous males and females reared without tetracycline (Figure 3), although a small fraction (~20%) do survive long enough to take two blood meals and some produced two clutches of eggs. Very little mortality was observed in the week post-eclosion in the LWT strain for both males and females, which contrasts strongly with the OX513A strain (p-

value=0). Median survival of both OX513A males and females is 2 days compared to LWT males and females with median survival of 60 and 68 days, respectively.

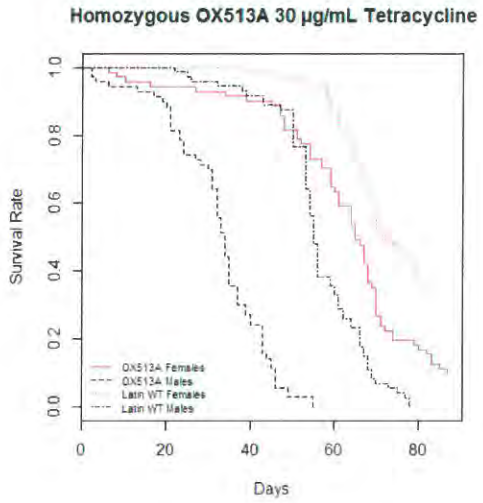

Figure 4: Survival of homozygous OX513A individuals that were reared with 30 µg/mL tetracycline compared to LWT reared under the same conditions.

Survival of heterozygous OX513A is imperfectly rescued by the provisioning of 1 µg/mL in the larval rearing water, as evidenced by comparing their survival curves to those of their LWT counterparts ($p < 0.001$ for males and $p = 0.005$ for females). Median survival times for wild-type and OX513A heterozygous males were 66 days and 44 days, respectively. Median survival of OX513A heterozygous females was 64 days, and median survival of LWT females is 69 days.

Survival of the homozygous line reared according to standard procedures was also assessed (Figure 4). Homozygous OX513A males have a reduced fitness compared to their LWT counterparts ($p = 0$). Their median survival is 34 days; median survival of LWT males reared on the same dose of tetracycline is 55 days. The median survival of the LWT females has has been reach at 72 days, but a significant number of females are still alive at the time of this writing. OX513A females have a median survival of 65 days, which is significantly shorter ($p=0.0006$).

14. Discussion and Conclusions:

Understanding the overall fitness of heterozygous OX513A individuals that may survive in the wild contributes to an assessment of the safety and efficacy of a RIDL-based SIT programme.

Longevity: Longevity of three types of OX513A females was assessed: homozygous females reared on 30 µg/ml tetracycline, heterozygous females reared in the absence of tetracycline, and heterozygous females reared on 1 µg/ml tetracycline. In no case was the lifespan of the transgenics longer than that of the wild type comparators, and so the null hypothesis is confirmed. Indeed, the median lifespan of the OX513A females was significantly shorter than that of the wild type comparator in all three cases.

Vectorial capacity: Since the females have to survive at least the extrinsic incubation period (EIP, typically 7-15 days for dengue (Chan and Johansson 2012)) to have any possibility of transmitting the virus, vectorial capacity is very sensitive to vector longevity. OX513A females were found to have a significantly reduced lifespan relative to wild type, especially when heterozygotes were reared in the absence of tetracycline (median survival 2 days vs wild type 68 days). This implies that the vectorial capacity of OX513A females is significantly less than that of wild type females. Environmental factors are thought to reduce daily survival and hence lifespan considerably in the wild relative to the laboratory environment, with possibly as few as 9% of wild females living over 15 days (Joy et al., 2012). Nonetheless, the shorter lifespan observed in these laboratory experiments, particularly for OX513A heterozygotes reared without tetracycline, likely indicates an underlying reduction in fitness that would lead to an equivalent or greater proportional reduction in lifespan under harsher conditions. In practice, these data indicate a very reduced probability that OX513A females will be able to survive long enough to bite humans, and an even further reduced ability to survive the extrinsic incubation period of the virus.

Fitness: Modelling indicates that for efficient use of a RIDL strain of *Aedes aegypti*, such as OX513A, the mean fitness of heterozygous offspring of the released homozygous RIDL males with wild females should be <10% that of wild type (Phuc et al., 2007). Based on survival to functional adults, the fitness of OX513A heterozygotes in this and similar studies is about 4% that of wild type. However, this is likely to be an overestimate as it assumes that these rare survivors are fully fit, i.e. equivalent to wild type. This study found that this is not the case, in particular the longevity of both males and females is significantly lower than that of wild type. Fecundity of OX513A heterozygous females reared in the absence of tetracycline was found to be slightly higher than that of LWT females. This may be a consequence of selection under mass-rearing conditions for early egg production, and does not imply increased per-lifetime female productivity. In any case, the apparent fecundity increase, even if it were maintained throughout the female's lifetime, was nowhere near a large enough effect to outweigh the observed reduction in lifespan; the mean fitness of heterozygous offspring of released homozygous RIDL males with wild females is therefore well below 4% that of wild type, and correspondingly well below the 10% threshold of Phuc et al. (2007). Therefore, the survival of a small proportion of heterozygous individuals is not expected to compromise the efficiency of a mass release program for population control based on systematic releases of OX513A .

As well as programme effectiveness, fitness of transgenic individuals also relates to the potential for the transgene to persist in the environment. Even a modest fitness penalty would be sufficient to ensure the eventual loss of the transgene from a large wild population by natural selection. Here we find that the transgenics have reduced fitness relative to wild type, irrespective of their exposure to tetracycline. The mean fitness of the key class, OX513A heterozygotes reared without tetracycline, is well below 4% relative to wild type; this would lead to extremely rapid elimination of the transgene from a large wild population were releases to stop.

15. **Literature:**

Chan, M., and Johansson, M.A. (2012). The incubation periods of dengue viruses. PloS ONE 7, e50972.

Harris, A.F., McKemey, A.R., Nimmo, D., Curtis, Z., Black, I., Morgan, S.A., Oviedo, M.N., Lacroix, R., Naish, N., Morrison, N.I., et al. (2012). Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. Nature Biotech 30, 828-830.

Le-Minh, N., Khan, S.J., Drewes, J.E., and Stuetz, R.M. (2010). Fate of anitbiotics during municipal water recycling treatment processes. . Water Research 44, 4295-4323.

Locatelli, M.A.F., Sodre, F.F., and Jardim, W.F. (2011) Determination of antibiotics in Brazilian surface waters using liquid chromatography-electrospray tandem mass spectrometry. *Arch Environ Contam Toxicol*, 60 (385-393).

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Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J., Pape, G., Fu, G., Condon, K.C., Scaife, S., Donnelly, C.A., et al. (2007). Late-acting dominant lethal genetic systems and mosquito control. BMC Biology 5, 11.

Suchman, E.L., Kononko A., Plake, E., Doehling, M., Kleker, B, Black IV, W.C., Buchatsky, L., Carlson, J. (2011). Effects of AeDNV infection on *Aedes aegypti* lifespan and reproduction, Biological Control, *120* (465-473).

OXITEC

INTERNAL RESEARCH REPORT

1. **Reference Number**: PH-2013-5-V3a

2. **Issuing Date**: 25 February 2016

3. **Title**: Investigating the effects of larval rearing temperature on the phenotype of *Aedes aegypti* OX513A.

4. **Statement Of Data Confidentiality Claims:**

   Confidential Business Information (CBI) has been deleted from this report

5. **Statement Concerning Good Laboratory Practices:**
   This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

6. **Authors:**

Study Coordinator (Signature):	**Study Supervisor (Signature):**
Study Coordinator (Name And Position):	**Study Supervisor (Name And Position):**
Research Scientist	Chief Scientific Officer
Date Signed: 25 Feb 2016	**Date Signed:** 25TH FEB 2016

7. Associated Personnel:

Name	Tasks
	Study coordination, experimental design, data collection and analysis, report writing
	Data collection
	Experimental design
	Experimental design, data analysis, report writing
	Experimental design, approval
	Study Sponsor

8. Test Facility:

This research was performed at Oxitec's research facilities located at:

43b Western Avenue,
Abingdon,
Oxfordshire,
OX14 4RU
United Kingdom

71 Innovation Drive,
Abingdon,
Oxfordshire,
OX14 4RQ
United Kingdom

9. Objectives:

The objectives of this study were to determine;

a) If the penetrance of the phenotype of OX513A heterozygotes varies when reared at temperatures different than the laboratory standard.
b) If OX513A has altered survival at temperatures outside of *Aedes aegypti's* natural range, compared to wild-type.

10. Summary:

Aedes aegypti larvae, heterozygous for the OX513A construct, were reared at five temperatures ranging between and including 9°C and 37°C. Larvae were reared in the absence of tetracycline, which as a dietary supplement allows survival of OX513A individuals. Latin wild-type (WT) larvae, the background strain of the OX513A strain, were reared under the same conditions as a control. Five repetitions were conducted for each temperature point. We found that OX513A larvae and Latin WT larvae died before pupation when reared at 9°C and 37°C. This demonstrates that the presence of the OX513A insertion does not extend the viable temperature conditions for *Ae. aegypti* such that they can develop to functional adults at these temperatures. No evidence was therefore found to indicate that OX513A might be able to spread beyond the current temperature-bounded range of wild *Ae. aegypti*. OX513A larvae reared at intermediate temperatures within this range did not show a higher than expected proportion (<5%) of individuals surviving from L1

to functional adult (range 0-2%). Together, these studies demonstrate the phenotype of OX513A is stable over the range of temperatures that larvae are likely to encounter in the field and that they will not be able to expand the habitable geographic range of *Ae. aegypti.*

11. Introduction:

First developed in 2002 at Oxford University, the *Ae. aegypti* RIDL strain OX513A was originally produced by transforming the Rockefeller strain using a *piggyBac*-based construct (Phuc *et al.*, 2007). This strategy allowed the integration of a repressible dominant lethal system in the *Ae. aegypti* genome whereby, in the absence of tetracycline, >95% of the heterozygous progeny of a mating between RIDL males with wild-type females die (and of RIDL females with wild-type males) due to the accumulation of high intracellular levels of tTAV protein produced by a positive feedback loop. However, if tetracycline is added to the larval rearing medium, tTAV expression is repressed, allowing for normal development to adulthood.

Recently, OX513A has been used in the first open field release of transgenic mosquitoes into the environment (Harris *et al.*, 2011). Due to the high penetrance of the lethal phenotype expressed by mosquitoes inheriting the OX513 construct, the overwhelming majority of the offspring of released transgenic males will die before reaching maturity. However, under laboratory conditions, a small percentage of these offspring (<5%) survive to produce functional adults.

Here we attempt to ascertain the effect that rearing larvae at different temperatures has on the OX513A phenotype, and ultimately the percentage of flying adults surviving without tetracycline. The temperatures used in this experiment were chosen based on the reported habitable temperature range of *Ae. aegypti* (e.g., Hemme *et al* 2009, Richardson *et al* 2011). Consequently, we tested temperatures of 9°C and 37°C which represent temperature points somewhat beyond the lower and upper bounds of the reported habitable temperature range. Within the reported habitable range we tested 18°C, 24°C and 30°C, temperatures larvae are likely to encounter in the field. The null hypotheses tested were that

1. there is no difference between OX513A individuals and the wild-type comparator in respect of survival outside the temperature range permissive for egg-to-adult development and
2. the penetrance of the lethal phenotype of OX513A is not temperature dependent (<5% at all temperatures)

12. Methods

Strains

OX513A (bi-sex lethal RIDL strain): In the absence of tetracycline, this strain expresses high levels of the tTAV protein during immature stages through a positive feedback loop, resulting in high mortality. However, when reared in the presence of tetracycline, expression of tTAV is repressed, allowing survival (Phuc et al., 2007).

Latin wild-type: the background strain of OX513A, collected from Chiapas, Mexico in 2007.

Insect Rearing

L1 larvae were counted into pots (16 oz deli pots, Robertson packaging, UK, SICC65), 200 larvae per pot, 200 ml deionised water. Five repeats of OX513A and Latin WT were set up for each of the five temperatures. Larvae were fed according to a standard feeding regime of finely ground Tetramin© fish flakes (Tetra GmbH, Germany). Due to the expected differential development rate of larvae between temperatures, pots were not fed if food remained from the previous day.

Temperatures were maintained using incubators for the 9°C, 18°C and 37°C experiments and heat mats (Habistat, UK) for the 24°C and 30°C experiments. Evaporation from the pots was compensated for by adding deionised water as required to maintain pots at starting levels (200mL). Water temperatures were monitored using Thermochron iButtons (Maxim, UK).

Live pupae were counted and placed into cages (15x 15 x 15 cm, Bugdorm-Megaview, Taiwan) which in turn were placed into the relevant incubator or onto the heat mat. Dead larvae and dead pupae in the pots were counted and discarded. Pots were discarded when there were fewer than six larvae (3% of starting L1s) remaining.

Adults were provided with 10% sucrose solution *ad libitum*. Adult cages were assessed[1] for emergence three days after the last pupa was added.

Statistical Analyses

Data were analysed using R (Version 2.15.0) in R Studio (Version 0.97.237). Parametric significance tests were carried out using ANOVA and post-hoc testing using the Tukey HSD method, using the multcomp package. Non-parametric data was tested for significance using Kruskal-Wallis rank sum test. Confidence intervals were bootstrapped.

13. RESULTS

OX513A heterozygous larvae and Latin WT larvae were reared at five different temperatures ranging from 9°C to 37°C. Each temperature had five repeats of each strain with 200 larvae per repeat, reared in the absence of tetracycline.

The results displayed in Table 1 show that all OX513A larvae and Latin WT larvae reared at 9°C and 37°C died before pupation.

Strain	Temperature	Dead larvae	Total eclosion	Flying adults
OX513A	9°C	100.0%	0.0%	0.0%
	37°C	100.0%	0.0%	0.0%
Latin	9°C	100.0%	0.0%	0.0%

[1] Assessment included counting the total number of dead pupae, non-viable adults (dead adults on the water, dead adults on the floor of the cage and non-flying adults) and functional adults (flying adults).

WT	37°C	100.0%	0.0%	0.0%

Table 1. Developmental fate of heterozygous OX513A and Latin WT *Ae. aegypti* larvae reared at temperatures outside the normal range for *Ae. aegypti*. All larvae reared in the absence of tetracycline.

Table 2 shows the results for OX513A larvae reared at the intermediate temperatures of 18°C, 24°C and 30°C. There was no deviation from the expected proportion of flying adults (<5%), in fact, the percentages were very low with 18°C having no flying adults.

A very high proportion (95.5%) of OX513A larvae reared at 30°C died prior to pupating; this was consistent between repeats but not seen in the Latin WT (Table 2).

OX513A also showed a variation in the total eclosion[2] between these intermediate temperatures. 18°C and 30°C had a significantly lower total eclosion compared to the standard laboratory rearing temperature of 24°C (p=0.000 for both temperatures). This indicates that OX513A larvae do less well, i.e. die earlier in development, if reared at either higher or lower temperatures than nominal (24°C). Latin WT's total eclosion did not vary significantly between 18°C, 24°C and 30°C (p=0.912).

Strain	Temperature	Dead larvae	Total eclosion	Flying adults
OX513A	18°C	27.6% (24.8%-30.3%)	0.8% (0.3%-1.4%)	0.0% (0.0%-0.0%)
	24°C	39.0% (36.3%-42.3%)	16.2% (13.9%-18.5%)	1.0% (0.4%-1.7%)
	30°C	95.5% (94.2%-96.7%)	2.0% (1.2%-2.9%)	2.0% (1.2%-2.9%)
Latin WT	18°C	18.7% (16.3%-21.1%)	71.2% (68.4%-74.0%)	59.6% (56.5%-62.6%)
	24°C	30.0% (27.2%-32.9%)	69.4% (66.6%-72.2%)	68.3% (65.4%-71.2%)
	30°C	30.1% (27.4%-32.9%)	67.0% (64.2%-69.8%)	65.8% (62.9%-68.6%)

Table 2. Developmental fate of heterozygous OX513A and Latin WT *Ae. aegypti* larvae reared at temperatures within the normal habitable range for *Ae. aegypti*. All larvae reared in the absence of tetracycline. Confidence intervals in parentheses.

14. **Discussion and Conclusions:**

Determining the effect, if any, of larval rearing temperature on the penetrance of the OX513A phenotype is relevant to risk assessment as we expect insects in the wild to experience a variety of temperatures. There are two separate issues: first, does OX513A show differential penetrance at temperatures other than those typically used in laboratory culture, and second, does the presence of the OX513A gene preferentially allow the engineered insects to colonise areas that were previously uninhabitable to wild *Ae. aegypti*.

[2] Total eclosion is the percentage of L1 larvae which eclose to adults including flying function adults, dead adults on the surface of the water, dead adults on the surface of the cage and non-flying adults.

The data presented here show that at the larval rearing temperatures of 9°C and 37°C, somewhat outside the permissive range for wild type *Aedes aegypti* development, neither OX513A nor Latin WT were able to survive to pupation, demonstrating that field-released OX513A would not represent an establishment hazard to *Ae. aegypti*-free areas outside the current temperature-limited range of the species.

The data also show that at the range of intermediate temperatures tested there was no significant difference in the penetrance of OX513A (proportion of L1 larvae developing into functional adults). In other words, across the range of normal habitable temperatures, <5% functional adults were observed, showing that OX513A has a consistent penetrance of the lethality phenotype. This demonstrates that the penetrance of the OX513A lethal trait will not be adversely affected by the temperature of the larval habitats in the receiving environment.

15. **Literature:**

Harris, A.F., Nimmo, D., McKemey, A.R., Kelly, N., Scaife, S., Donnelly, C.A., Beech, C., Petrie, W.D., and Alphey, L. (2011). Field performance of engineered male mosquitoes. Nature Biotechnology 29, 1034-1037.

Hemme, R.R., Tank, J.L., Chadee D.D., and Severson, D.W. (2009). Environmental conditions in water storage drums and influences on *Aedes aegypti* in Trinidad, West Indies. Acta Tropica 112, 56-66.

Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J., Pape, G., Fu, G., Condon, K.C., Scaife, S., Donnelly, C.A., et al. (2007). Late-acting dominant lethal genetic systems and mosquito control. BMC biology 5, 11.

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OXITEC

INTERNAL RESEARCH REPORT

1.	**Reference Number**	PH-2013-6-v.1
2.	**Issuing Date**	May 1st 2013
3.	**Title**	Evaluation of Trait Penetrance in the Field by Polymerase Chain Reaction (PCR) Analysis of Field Collected Adults

4. Statement of Data Confidentiality Claims

This document contains no confidential business information

5. Statement Concerning Good Laboratory Practices

This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

6. Authors

Study Coordinator (Signature):	Study Supervisor (Signature):
Study Coordinator (Name And Position):	**Study Supervisor (Name And Position):**
Senior Scientist	Chief Scientific Officer
Date Signed: 10/5/13	**Date Signed:** 10 May 2013

7. Associated Personnel

Name	Tasks
	Study coordination, experimental design, data collection and analysis, report writing
	Experimental design, report writing and approval
	Experimental design, insectary work
	Experimental design, sample collection, statistical analysis, report writing
	Statistical analysis
	Experimental work, Data collection
	Experimental work, Data collection
	Experimental work, Data collection
	Study Sponsor

8. Test Facility

This research was performed at Oxitec's research facility located at:
71 and 46a Milton Park,
Abingdon, Oxfordshire,
OX14 4RX
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9. Objectives

The objectives of this study were to (i) genotype adult female field samples from Cayman Islands and Brazil for at the OX513A insertion locus and (ii) using this data estimate the effective penetrance of the OX513A lethal trait under field conditions.

10. Summary

Adult mosquitoes were collected in traps (BG Sentinel type) as part of multi-component programmes also involving the sustained release of OX513A males in the Cayman Islands (East End) and two release sites in Brazil (Itaberaba and Mandacaru).
Samples were shipped to the UK in ethanol. In the UK, DNA was extracted from each female and used as a template for PCR. Back up samples were also prepared by removing the abdomens, before gDNA extraction, which were used to confirm transgene positive samples. By analysing the PCR results, a genotype of homozygous, heterozygous or wild–type (WT) for the OX513A insertion was assigned to each individual. Real time PCR was also carried out on all of the heterozygous and homozygous individuals identified to confirm the copy number of the transgene. As expected, the majority of the females were wild-type, but a small number of homozygous individuals were detected in each test site.

11. Introduction

First developed in 2002 at Oxford University (Phuc *et al*., 2007), the *Aedes aegypti* OX513A strain was originally produced by transforming the Rockefeller strain using a *piggyBac*-based construct. This strategy allowed the integration of a repressible dominant lethal system in the *Ae. aegypti* genome whereby, in the absence of tetracycline, >95% of the heterozygous progeny of mating between OX513A males with wild type females (and of RIDL females with wild-type males) die before becoming functional adults due to the accumulation of high intracellular levels of tTAV protein produced by a positive feedback loop. However, if tetracycline is added to the larval rearing medium in sufficient quantities (e.g. to 30 µg/ml) tTAV expression is repressed, allowing for normal development to adulthood.

Recently, strain OX513A has been used in the first open releases of transgenic mosquitoes in the environment (Harris *et al*., 2011) and the first successful suppression of a field mosquito population by release of genetically sterile males (Harris *et al*., 2012). Due to the high penetrance of the lethal phenotype expressed by mosquitoes inheriting the OX513A construct, the overwhelming majority of the offspring of released transgenic males will die before reaching adulthood. However, under laboratory conditions a small percentage (≤5%) of these offspring survive to produce functional adults (Phuc *et al*., 2007), of which approximately half will be female. This survival rate may be even lower in heterozygous individuals produced in the field (due to the many environmental challenges that mosquitoes face in the wild relative to benign laboratory conditions).It is still possible that a few female individuals carrying the OX513A construct could survive to adulthood following a field release. Furthermore, although the sex-separation techniques used to eliminate females from the release cohorts are highly efficient

(well above 99% male, e.g. 0.066% female, Harris *et al*. 2012, 0.02% female, Carvhalo et al, 2013), they are not perfect, so there is potential for a small proportion of OX513A adult females to remain in the male populations released during a field trial.

To get a better idea of the actual numbers of surviving heterozygotes in a field situation, and the numbers of homozygous females that are actually released and survive, we genotyped samples of adults caught in traps coinciding with periods of OX513A release in field trials in Cayman and Brazil. During release periods, the vast majority of males are expected to be released OX513A males; analysis of these was not expected to be especially informative given the low proportion of the genotype of interest (heterozygous males); the same information can be obtained with much lower background by examining females. Males were therefore only genotyped from the post-release period in the Grand Cayman study while females were genotyped from all sites.

12. Methods

Field release

To date there have been three substantial evaluations of efficacy of sustained release of OX513A males for suppression of wild *Ae. aegypti* populations. The first was conducted in the Cayman Islands (Harris et al., 2012). Subsequently, two larger studies were conducted in and around the city of Juazeiro, Bahia, Brazil. The first of the Brazilian studies was in Itaberaba, a densely populated suburb of Juazeiro (Oxitec Report PH 2013 09), and the second in Mandacaru, a village of ~ 8k to the NE of Juazeiro (Oxitec Report PH 2013 11).

East End, Cayman Islands

A total of 16 BG Sentinel (adult traps) traps were installed in the treatment area for the duration of the trial, and trap catches were collected daily from 15/10/2010 when releases stopped. We chose the last period of the trial to conduct this analysis as this should be the period most sensitive to any lack of penetrance of the lethality trait as it is the period where the OX513A: wild male ratio is highest and hence the proportion of females with an OX513A father is also expected to be highest. Furthermore, were there any genetic variation in the wild population affecting penetrance of the lethal trait, this is the period where such hypothetical 'resistance' alleles would have been exposed to the longest period of selection. OX513A:wild male ratios in the field had been >18:1 for the preceding 7 weeks, therefore we expect most females to mate OX513A males (mean net mating competitiveness of released OX513A males measured in 2009 field experiment to be approximately 0.5 relative to wild type, so at 18:1 OX513A:wild male ratio we expect 90% of wild females to mate an OX513A male). We therefore expect approximately 90% of eggs laid to be heterozygous transgenic. Unfortunately this could not be directly assessed at this point in time as the strong suppression of the wild population by prior OX513A releases meant that very few eggs were collected, however earlier in the trial this proportion

had reached 70-90% with a lower OX513A:wild male ratio. Full details of trial are given in Harris et al 2012.

Itaberaba, Brazil

Releases in Itaberaba ran from early 2011 to the end of 2012, however adult traps were only installed permanently from May 2012 onwards with weekly collections. Prior to this point, traps were installed for discrete periods to coincide with cohorts of marked releases, and samples were collected daily. Females were analysed from 3 distinct periods (Table 2). Analysis of penetrance was carried out separately for each period as the underlying proportion of OX513A:Wild eggs differed. Periods 1 and 2 were conducted over a larger area (areas T1&T2 = 11 ha). In period 3, releases occurred only in area T2, therefore trap catch data was taken from this area only. Proportions of OX513A:wild eggs were estimated from analysis of ovitraps. Eggs recovered were hatched and larvae scored for the presence of the OX513A insert by the characteristic red fluorescence due to the expression of the DsRed2 marker (Lukyanov et al., 2000; Matz et al., 1999) (Clontech Laboratories Inc.) using a Leica MZ10 F epi-fluorescence microscope. Data for proportion OX513A eggs was taken from a period starting ~ 3-4 weeks preceding the adult collection to account for the inherent time delay between life stages.

Mandacaru, Brazil

For the purposes of the trial, Mandacaru village was divided into North East, North West and South areas. For the purpose of analysing trap samples the North East and North West areas were combined to form a North area. Releases began in the South on 19/3/2012, and in the North on 21/6/2012. Evaluation of penetrance was carried out over the initial period as later there were insufficient females and eggs recovered from ovitraps due to suppression of the wild population. BG traps were installed in the North and South at the same time as the start of releases in the South. Although there were no releases in the North before 21/6/2012 there was substantial movement of OX513A males into the North, from releases in the South, as indicated by the high proportion of OX513A eggs recovered. For completeness, females captured in traps in the North were also analysed. Data regarding the proportion of OX513A eggs was obtained from ovitraps placed between 3/4/2012 and 7/8/2012. Adult females samples were collected from BG traps recovered between 4/4/2012 to 19/8/2012.

Sample collection

Adult mosquitoes were collected from the traps and kept in separate bags for each trap catch. All mosquitoes were identified to species in the field laboratory, and *Aedes aegypti* further sorted by sex with the aid of a microscope, and stored in microfuge tubes containing 70% ethanol. Males and females, from each trap, were pooled in separate tubes. The microfuge tubes were stored at -20°C until they could be shipped to the UK.

Sample preparation in Oxitec, UK

The sex of each mosquito was confirmed by microscopic examination. The abdomen was removed and stored in a separate tube (B sample). The abdomens provided source material for additional second DNA extractions and PCR analysis, e.g. to confirm homozygous/heterozygous status.

Genomic DNA extraction

Genomic DNA (gDNA) was extracted from each female using the Invitrogen Purelink Genomic DNA purification kit (TD_SOP_00142) for the Brazil samples, or using the Fermentas Genejet genomic DNA extraction kit (TD_SOP_00094) for the Cayman samples, according to manufacturer's instructions and Oxitec standard operating procedures described in the associated SOPs.

PCR strategy and primers

The OX513A transgene is an insertion of the OX513 transposon into a region of the *Aedes aegypti* genome that shows a degree of polymorphism. This means that the corresponding region (without the transgene) on the wild-type chromosome may vary from one individual to another. In the Brazilian wild population we have detected 3 different sequences for the WT alleles that each required a different primer set to amplify. For the Cayman samples primer set 4 was not utilised as the corresponding WT allele was not detected.
Therefore, 2µl of each gDNA sample was used as a template for PCR, according to Oxitec standard procedure (TD_SOP_093v2) with the following primer sets:

1. 513: 1153)WT1 and 1155)IRVI amplify from the OX513A transgene into the flanking sequence, only when the OX513A transgene is present.
2. WT1: 1312)WT1c and 1154)WT2 amplify from WT DNA (WT allele 1).
3. WT2: 1177)513ins5R and 1215)CayaltF2 amplify from WT DNA (WT allele 2)
4. WT3: 1177)513ins5R and 1285)BrazilWT2 amplify from WT DNA (WT allele 3)

Primer details are given in Table 1:

Table 1: Details of primer sequences

Primer set number	Forward Primer name	Forward primer sequence	Reverse primer name	Reverse primer sequence	Amplicon length (bp)
1 (513)	1153)WT1	GAAATCCCCTAGTAAAATTCGCGGAGAAATTC	1155)IRV1	CGTCATTTTGACTCACGCGGTCGTTATAGTTC	400
2 (WT1)	1312)WT1c	GATGATCCTTGTTGAATTCTTGAGGTATCC	1154)WT2	CCAAGCGTTCTAACGATATTTTCAGCGTTC	354
3 (WT2)	1177)513ins5R	CTTAGACCGATAAAGAAGTGTAAATAGAGCATG	1215)CayaltF2	CTGCATTTGGTCCCTCGGTAGTG	715
4(WT3)	1177)513ins5R	CTTAGACCGATAAAGAAGTGTAAATAGAGCATG	1285)BrazilWT2	GTTTCCGGCATAATGTGGCTGAC	725

Gel electrophoresis of PCR products

3.5µl gel loading buffer (30% glycerol, 0.5% bromophenol blue) was added to each PCR product, and 9µl of this mixture was run on a 1% agarose TAE (Tris Acetate EDTA) gel, stained with Ethidium bromide, at 120V for 25 mins. The homozygous/ heterozygous/ wild-type state of each individual was determined, by the presence or absence of a gel band of the expected size with each primer set.

Real time PCR

Real time PCR can be used to determine relative copy numbers of a gene of interest (in this case OX513A) in a sample, by comparing the amount of OX513A PCR product, relative to the amount of PCR product from an endogenous single copy gene (in this case, Inhibitor of Apoptosis Protein 1, IAP1). Therefore, once normalised to IAP1, OX513A homozygous individuals will show twice as much OX513A PCR product as heterozygous individuals (i.e. a normalised Ct value ΔCt one lower, ΔΔCt=1, approximately). Real time PCR was carried out according to Oxitec standard procedures (TD/SOP/00125) on all samples positive for OX513A to confirm that the heterozygote/homozygote status inferred from the above endpoint PCR analysis described above.

Statistical analysis

In the laboratory we measure the penetrance of the lethal trait by assessing the proportion of eggs/larvae that successfully develop to form functional adults, relative to one or more comparator wild type control groups under the same conditions (Phuc et al 2007). This is not possible in the field where total eggs/larvae numbers and resulting numbers surviving to adult are not known. However we can infer the ratio of OX513A to wild eggs being laid in the environment, from the ratios observed in ovitrap collections. The ensuing adult population will contain surviving adults from these eggs, with any heterozygous OX513A resulting from survival due to incomplete penetrance[1]. If we had 100% penetrance and all OX513A failed to develop to adults, the relative number that would otherwise have been expected to survive can be derived from the number of wild adults collected and the fluorescence ratio of eggs laid in the appropriate prior period. We can therefore calculate the deviation from 100% penetrance in the

[1] Apparent incomplete penetrance in the field may be due to any or a combination of several factors including incomplete penetrance of the transgene as measured in the laboratory, presence of tetracycline in the environment, or the presence of genetic modifiers ('resistance alleles') in the wild population leading to disproportionate survival of OX513A/+ heterozygotes. Heterozygous females might also in principle be released from the rearing facility, if the mass-reared strain were not 100% homozygous, however the proportion of females present in the release population is very low; the number of heterozygous females trapped that originated from this source might be expected to be a small proportion (<1%) of the total number of homozygous females trapped. Since this was found to be a low number in this study, it is unlikely that the heterozygous females detected derived from this source.

field as the number of heterozygous adults captured divided by the expected number if there were no lethal trait. Hence, accounting for background mortality an adjusted proportion of incomplete penetrance of the lethal OX513A phenotype can be calculated, equation (1)

$$Penetrance = 1 - \left[\left(\frac{WT\ Eggs}{WT\ Adults}\right) \times \left(\frac{OX513A Heterozygote\ Adults}{OX513A\ Eggs}\right)\right] \quad 1.$$

Worked example:

- We observe a 1:1 WT:OX513A egg ratio in the field (eg 1000 WT eggs, 1000 OX513A eggs)
- 2-4 weeks later we catch 200 WT adults
- In the absence of any lethal effect we would expect to catch 200 OX513A heterozygote adults
- If, in fact we catch 1 OX513A heterozygote adult then the penetrance of lethality is 1-(1/200)=0.995.

Confidence intervals were calculated using likelihood ratio based methods. The maximum likelihood estimate (MLE) of penetrance was calculated. Likelihoods were then recalculated using estimates of penetrance increasing or decreasing in small increments away from the MLE. Penetrance values producing likelihood estimates such that $2(\text{maximum likelihood} - new\ likelihood) > \chi_1^2$ are considered outside of the 95% confidence interval.

13. Results

Example gels are shown in the figure below.

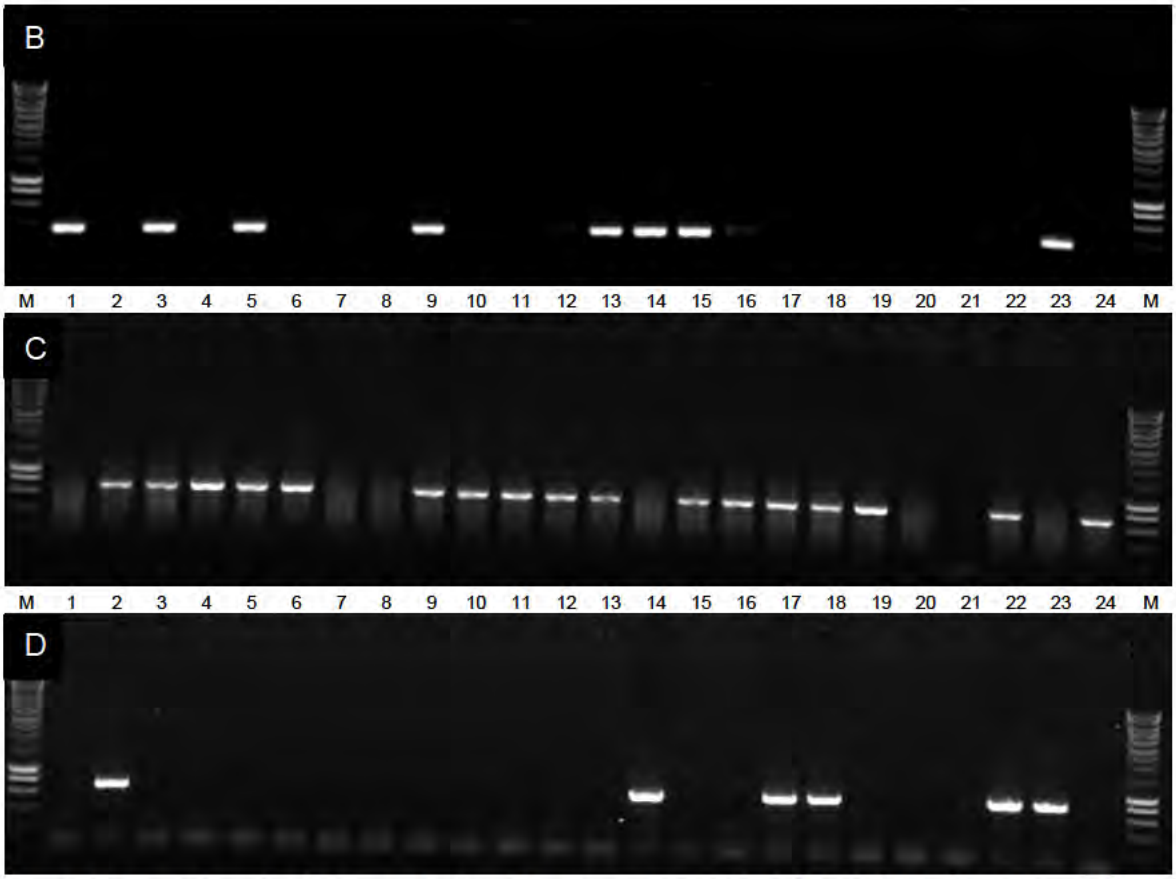
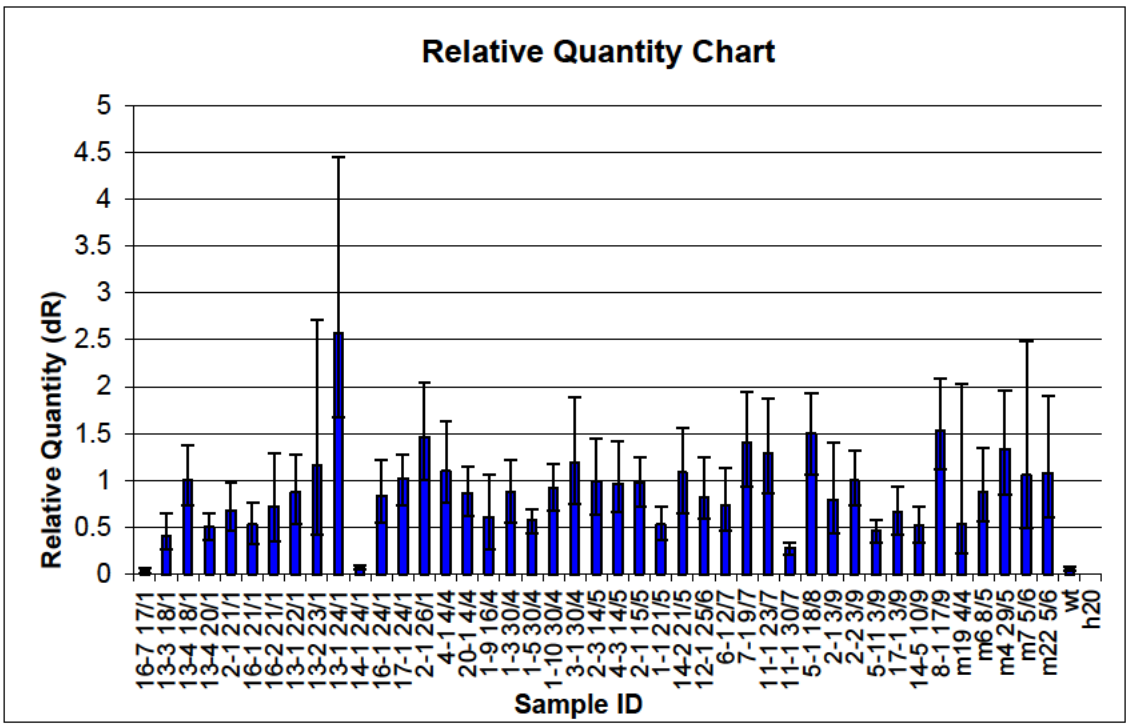


Figure 1: Example gels of PCR products with primers for OX513A (A), WT1(B), WT2 (C) and WT3 (D). In this example, samples 7, 8 and 20 are homozygous for OX513A (positive for this primer set only, gel A), and the rest are WT (positive for one or two of the WT primer sets, but not OX513A), except for sample 21 which is negative for all primer sets, probably due to in sufficient genomic DNA quality (subsequently confirmed by PCR using primers for endogenous Actin 4 sequence, data not shown).

For all of the samples which were negative in all PCR reactions (eg sample 21 above) and all of those positive for OX513A, PCRs were repeated. PCR was also carried out on the B samples for these individuals, using the same protocol.

For all of the samples confirmed positive for OX513A (homozygous or heterozygous), real time PCR was carried out. In theory, a heterozygote by PCR could be due to a WT individual, contaminated with a fragment of material, e.g. a leg, from a homozygous male, for instance. As explained in the methods section, use of real time PCR enables us to assess the relative copy number of the transgene, overcoming this kind of potential problem. Multiplex real time PCR was carried out on each sample, in duplicate, with primers and probe specific to the transgene, along with those specific to an endogenous autosomal gene (Inhibitor of Apoptosis Protein 1, IAP1), in the same well. Quantities (Ct value) of the transgene were normalised with respect to quantities of the endogenous gene, then compared to a homozygous individual. All of the

homozygotes have values of around 1, heterozygotes values around 0.5 and WT values of 0, as shown in the chart below.



The proportion of OX513A eggs and the genotype of corresponding adult population samples is summarised in Table 2 together with the estimated degree of incomplete penetrance (deviation from 100% lethality). It can be seen that there were no heterozygous adults from samples collected in Cayman and Mandacaru. In Itaberaba 12 heterozygous females were detected. Correspondingly this estimated incomplete penetrance as ranging between 0-4.28%. A small number of homozygous females were also detected.

Table 2: Incomplete penetrance estimates estimated from ratios of OX513A eggs and corresponding proportion of heterozygous adults genotyped.

Date	Location	Sex	Area(s)	Homozygous	Heterozygous	WT	Total eggs/larvae sampled	No RIDL +ve egg/larvae	% RIDL eggs/larvae	Incomplete penetrance (95% CIs)
Oct 2010	Cayman Island	♂	East End	145	0	41	100	90	90%	0 (0, 0.005)
Oct 2010	Cayman Island	♀	East End	1	0	92	100	90	90%	0 (0,2.0)
Nov 2011	Brazil - Itaberaba	♀	T1+T2	3	2	84	2869	1034	36%	4.3 (0.7,13.4)
Jan 2012	Brazil - Itaberaba	♀	T1+T2	9	3	149	3756	1813	48%	2.2 (0.6,5.6)
April-Sept 2012	Brazil - Itaberaba	♀	T2	14	7	140	3180	2174	68%	2.4 (1.0,4.5)
April-August 2012	Brazil - Mandaçaru	♀	North	0	0	15	276	625	44%	0 (0,<0.001)
April-August 2012	Brazil - Mandaçaru	♀	South	5	0	13	797	880	91%	0 (0,<0.001)

14. Discussion and Conclusions

We have successfully genotyped, by PCR, samples of adult mosquitoes caught in BG Sentinel adult traps from field sites where sustained release of OX513A was used to suppress wild populations of *Ae.aegypti.* OX513A homozygotes and heterozygotes identified by PCR were confirmed by real-time PCR for field collected samples.

Overall estimates of percentage incomplete penetrance ranged from 0-4.28%. It should be noted that sample size for some of the studies was low, but taking all studies together it is clear that the figure falls below the ~ 5% reported in laboratory studies. This is to be expected as

conditions in field are harsher that the benign conditions in which laboratory studies were conducted. These results support the hypothesis that the lethal phenotype is behaving as expected in the field and that incomplete penetrance is no higher than in the laboratory.

Furthermore, apparent incomplete penetrance in the field may be due to any or a combination of several factors including incomplete penetrance of the transgene as measured in the laboratory, presence of tetracycline in the environment, or the presence of genetic modifiers ('resistance alleles') in the wild population leading to disproportionate survival of OX513A/+ heterozygotes. That the presence of heterozygous females is no greater than expected from laboratory estimates of incomplete penetrance of the lethal phenotype of OX513A implies that other factors, e.g. hypothetical presence of tetracycline in larval habitats, or resistance alleles in the wild population, did not substantially affect the expression and effectiveness of the OX513A lethal phenotype in any of these trials sites.

15. Literature

Harris, A.F., McKemey, A.R., Nimmo, D., Curtis, Z., Black, I., Morgan, S.A., Oviedo, M.N., Lacroix, R., Naish, N., Morrison, N.I., *et al*. (2012). Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. Nature Biotechnology 30, 828-830.

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Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J., Pape, G., Fu, G., Condon, K.C., Scaife, S., Donnelly, C.A., *et al*. (2007). Late-acting dominant lethal genetic systems and mosquito control. BMC biology 5, 11.

OXITEC

INTERNAL RESEARCH REPORT

1. **Reference Number**: PH-2013-7-v1
2. **Issuing Date**: 5 February,2013
3. **Title**: Genetic stability of the transgenic elements in *Aedes aegypti* OX513A: Inheritance pattern
4. **Statement Of Data Confidentiality Claims:**

This document contains no confidential business information

5. Statement Concerning Good Laboratory Practices:

This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

6. Authors:

Study Coordinator (Signature):	Study Supervisor (Signature):
Study Coordinator (Name And Position):	**Study Supervisor (Name And Position):**
Senior Scientist	Chief Scientific Officer
Date Signed:	**Date Signed:** 08 April 2013

7. Associated Personnel:

Name	Tasks
	Study coordination, experimental design, data analysis, report writing
	Experimental design, report writing and approval
	Insectary work, data collection, report writing
	Molecular analysis
	Molecular analysis
	Insectary work, data collection
	Experimental design, study Sponsor

8. Test Facility:

This research was performed at Oxitec's research facility located at:
Buildings No. 46a and 71, Milton Park,
Abingdon, Oxfordshire,
OX14 4RX
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9. Objectives:

The objectives of this work are to test the following hypotheses:

a) The transgenic construct present in OX513A *Aedes aegypti* is inherited in a Mendelian fashion.

b) A similar pattern of inheritance is observed in two separate colonies (representing non-consecutive generational equivalents[1]) of *Aedes aegypti* OX513A.

10. Summary:

Oxitec's *Aedes aegypti* strain OX513A has recently been used in field trials that successfully suppressed field populations of *Ae. aegypti* by means of systematic releases of genetically sterile male mosquitoes (Harris *et al.*, 2012). Although a remarkable degree of stability has been previously reported for *piggyBac*-based constructs (such as the one present in strain OX513A) in *Ae. aegypti*, it is nonetheless conceivable, though highly unlikely, that genetic changes such as the loss or inactivation of a transgenic allele could have taken place during the time that strain OX513A has been maintained in the laboratory. In the current work, we test the hypothesis that the frequency with which the transgenic alleles found in homozygous OX513A individuals are inherited follows a Mendelian pattern, and that this pattern is similar in two separate colonies, representing non-consecutive generational equivalents of the strain. Our results indicate that the transgenic alleles present in strain OX513A remain stably integrated and functional until the present day.

11. Introduction:

First developed in 2002 at Oxford University, the *Aedes aegypti* RIDL strain OX513A was originally produced by transforming the Rockefeller strain using a *piggyBac*-based construct. This strategy allowed the integration of a repressible dominant lethal system in the *Ae. aegypti* genome whereby, in the absence of tetracycline >95% of the heterozygous progeny of mating between RIDL males with wild type females (and of RIDL females with wild-type males) die before becoming functional adults due to the accumulation of high intracellular levels of tTAV protein produced by a positive feedback loop. However, if tetracycline is added to the larval rearing medium in sufficient quantities (e.g. to 30 µg/ml), tTAV expression is repressed, allowing for normal development to adulthood (Phuc et al., 2007)).

Recently, strain OX513A has been used in the first open release of transgenic mosquitoes in the environment (Harris et al., 2011)) and the first successful suppression of field mosquito populations by the release of genetically sterile males (Harris *et al.*, 2012).

An understanding of the stability of the genetic elements used to create a transgenic strain assists in the evaluation of potential environmental risks associated with said strain. Although the re-mobilization of *piggyBac*-based constructs in transgenic insects of several species has been previously documented (Uchino *et al.*, 2008; Horn *et al.*, 2003; Lorenzen *et al.*, 2003), when artificially supplied with a source of *piggyBac* transposase, studies have suggested that in *Ae. aegypti* even under these circumstances the process of *piggyBac* remobilization is either completely inhibited or constitutes an exceedingly rare event (Sethuraman et al., 2007; O'Brochta et al., 2003).

Because strain OX513A was created several years ago and has been maintained and reared in the laboratory as a homozygous line ever since, it is theoretically possible, though highly

[1] A generational equivalent is defined as a time-based estimate of the rate of progress through generations dependant on insect lifecycle at a particular temperature as in large-scale rearing all life-cycle stages are present, and eggs collected at a particular point in time cannot be assigned to a particular generation by lineage or pedigree tracing.

unlikely, that genetic changes such as the loss or inactivation of a transgenic allele could have taken place during this time. If this were the case, nominally homozygous lines would contain alleles other than OX513A, i.e. mutant derivatives, at a frequency relating to the rate at which they appear (i.e. the degree of genetic instability), the number of generations for which the nominally homozygous lines had been held, and the fitness of the non-OX513A alleles relative to OX513A. Such alleles may be detected by out-crossing the nominally homozygous line to wild type – the expectation for a true homozygous line is that all progeny will inherit one copy of OX513A; non-OX513A alleles may be detected by failure to show dominant traits conferred by OX513A such as fluorescence, or the presence of specific DNA features detectable by PCR. . In the current work, we aim to establish (a) whether th e transgenic alleles present in homozygous OX513A *Aedes aegypti* are inherited following the pattern predicted by Mendelian genetic principles, and (b) whether the observed pattern of inheritance is maintained in two separate colonies (representing non-consecutive generations) of the transgenic strain.

12. **Methods:**

Strains

This study was performed using the following *Ae. aegypti* strains:

- **Latin wild-type:** This non-transgenic strain was originally collected in the region of Chiapas (Mexico) and was transferred to Oxitec from Mexico's Institute of Public Health in 2006 (D. Nimmo, personal communication). This strain will be henceforth referred to as WT.
- **OX513A (bi-sex lethal RIDL strain):** In the absence of tetracycline, this transgenic strain expresses high levels of the tTAV protein during immature stages through a positive feedback loop, resulting in high mortality. However, when reared in the presence of tetracycline, expression of tTAV in this strain is repressed, allowing for high survival levels (Phuc *et al.*, 2007). Individuals of this strain can be identified during immature stages by the expression of the fluorescent protein DsRed2 in their bodies in a characteristic punctate pattern driven by the Actin5C promoter (fig. 1). The particular strain used for this study was generated by introgressing the original OX513A strain into the aforementioned Latin WT genetic background.

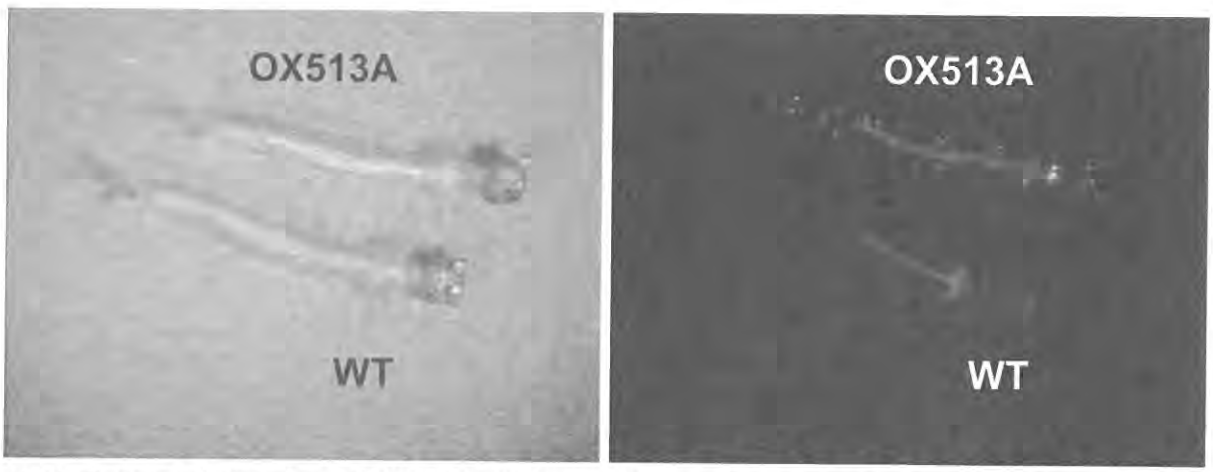

Fig. 1. **Fluorescent phenotype of OX513A individuals**. Left panel: WT and OX513A larvae viewed under white light. Right panel: Fluorescence micrograph showing the same larvae viewed using a filter set specific for the visualization of light emitted by the DsRed2 protein, which is seen as bright red spots spread throughout the body of the OX513A specimen. Auto-fluorescence (i.e. fluorescence not caused by DsRed2 expression) can be observed in the gut and head of both larvae.

Insect Rearing

All specimens were reared under standard insectary conditions: 27°C [+/- 1°C], 70% [+/- 10%] relative humidity, 12h: 12h light: darkness cycle. The offspring of crosses in which at least one parent carried the OX513A transgenic construct were reared with the addition of 30 µg/ml chlortetracycline hydrochloride (Sigma-Aldrich, USA) to the larval rearing water. Larvae were fed either finely ground Tetramin© fish flakes (Tetra GmbH, Germany) or Liquifry© liquid fish food (Interpet, England). Adults were provided with 10% sucrose solution (supplemented with 14 U/ml penicillin and 14 µg/ml streptomycin) *ad libitum*. To obtain eggs, mated females were provided with defibrinated horse blood (TCS Biosciences Ltd., UK) and given access to wet filter paper (Whatman, UK) as oviposition substrate.

Crosses

All crosses were performed by placing 100 WT female pupae and 50 OX513A male pupae in a cage. These were allowed to emerge as adults and mate, and were kept for one week prior to blood feeding.

Fluorescence screening

Larvae were visually screened using a Leica MZ95 dissecting scope equipped for fluorescence analysis with a M205FA/M165FC filtercube (excitation filter: ET546/10x, suppression filter: ET590 LP) (Leica Microsystems, Wetzlar, Germany). The expected phenotypes of WT and OX513A larvae are shown in fig. 1.

Experimental Design

The inheritance pattern of the OX513A construct was examined in two separate colonies (referred to as 'OX513A-A' and 'OX513A-B') which were derived from a common parental line in 2010 (approximately 2 years prior to their use in the present work) and kept as separate homozygous lines ever since, therefore representing non-consecutive generations of the transgenic strain[2].

For each one of these colonies, the process described below (and summarized in fig. 2) was followed in 4 separate replicates:

a) Homozygous OX513A males were crossed to WT females.
b) From the resulting offspring (hereafter referred to as G_1), 500 larvae were visually screened for the expression of the DsRed2 fluorescent protein. According to Mendelian laws of inheritance it is expected that all offspring of a homozygous parent will inherit one copy of the transgene. In addition, the fluorescence marker is considered to have high penetrance; it is therefore expected that all the screened larvae display the fluorescent phenotype indicating the presence of OX513A.
c) To validate the accuracy of the screening process, 10 larvae (selected at random from those visually screened) from each replicate were analysed by qPCR to confirm the

[2] Generation time for *Aedes aegypti* under these conditions is approximately 4 weeks. However, eggs may be stored for some months, so there can be fewer than 12 generations per year. Nonetheless, each strain undergoes no less than 2 generational equivalents per year.

presence of the OX513A construct. Details of the methodology used for qPCR are shown in annex 1.

d) G_1 males of each repeat were crossed to WT females.

e) From the resulting offspring (hereafter referred to as G_2), 500 larvae were visually screened for the expression of the DsRed2 fluorescent protein.

f) Using the chi-square test, the frequency of the fluorescent phenotype observed in the screened larvae was compared to the corresponding frequency expected for a genetic character inherited in a Mendelian fashion.

g) To validate the accuracy of the screening process, 10 randomly selected larvae of each observed phenotype from each replicate were screened by qPCR to confirm the presence/absence of the OX513A construct.

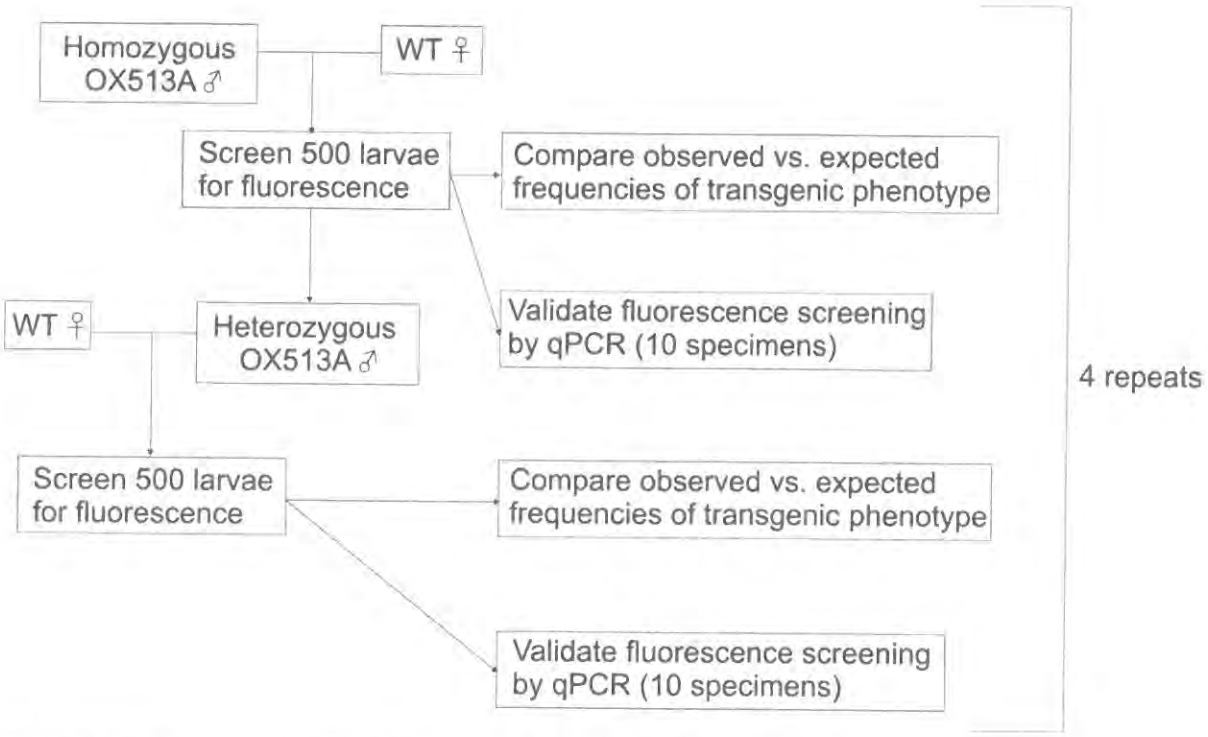

Fig. 2. **Schematic representation of the experimental design.** The process described in the diagram was applied to each of the two colonies tested. WT: wild-type.

Statistical analysis

Observed and expected frequencies of the transgenic phenotype were compared using Chi-square tests. Calculations were performed using Microsoft Excel version 2010 (Microsoft Corp, USA).

13. **Results:**

Fluorescence screening

The observed frequencies of transgenic and WT phenotypes were never significantly different from those expected for a genetic element inherited in a Mendelian fashion (table 1), suggesting that the transgenic alleles remain stably integrated and the fluorescent phenotype remains functional in both colonies.

qPCR validation

Screening by qPCR validated the results of screening by fluorescence in 100% of the G_1 larvae examined (10 samples/repeat x 4 repeats/colony x 2 colonies = 80 G_1 samples), showing that all larvae in this generation were heterozygous for the transgenic trait, as expected for the descendants of homozygous OX513A individuals crossed to WT mosquitoes. Among G_2 larvae, where a mixture of WT and OX513A genotypes are expected, the estimated efficiency for correctly identifying WT and OX513A specimens was 100% (78 out of 78) and 97.4% (75 out of 77), respectively (see Annex 1 for details).

Table 1. Summary of fluorescence screening results.

Colony	Generation	Repeat	No. Transgenic	No. WT	X^2	p-value
OX513A-A	G_1	1	500	0	*	*
		2	500	0	*	*
		3	500	0	*	*
		4	500	0	*	*
		Total	2000	0	*	*
	G_2	1	260	240	0.800	0.371
		2	254	246	0.128	0.721
		3	255	245	0.200	0.655
		4	259	241	0.648	0.421
		Total	1028	972	1.568	0.211
OX513A-B	G_1	1	500	0	*	*
		2	500	0	*	*
		3	500	0	*	*
		4	500	0	*	*
		Total	2000	0	*	*
	G_2	1	261	239	0.968	0.325
		2	251	249	0.008	0.929
		3	229	271	3.528	0.060
		4	254	246	0.128	0.721
		Total	995	1005	0.050	0.823

*Because only one phenotype is expected (and observed), it is not mathematically possible to perform the chi-square statistical test in these groups. However, the observed frequencies of the transgenic phenotype are exactly as expected for a genetic element inherited in a Mendelian fashion, with 100% of the screened larvae displaying the transgenic phenotype.

Of the G_1 putative heterozygotes, all (4000/4000) showed the expected fluorescent phenotype. From this we can derive confidence limit for the presence of non-fluorescent alleles at the OX513A locus. The upper bionomial 95% confidence limit is 0.0007-0.0009 (lower limit is obviously 0.0000) depending on the method used[3].

[3] JavaStat at http://statpages.org/confint.html using Clopper-Pearson and http://www.measuringusability.com/wald.htm using Adjusted Wald and other methods, both accessed 05 Feb 2013.

14. Discussion and Conclusions:

Our results support the hypothesis that the OX513A is inherited as expected from Mendelian genetic principles in both colonies tested. As these colonies were derived from the same ancestral line approximately 2 years ago (time equivalent to at least 4 generational equivalents), our results suggest that the genetic construct has remained stable and the elements controlling the fluorescent phenotype remain functional in these strains through several non-consecutive generational-equivalents. The absence of non-fluorescent alleles in the G_1 heterozygous progeny of OX513A putative homozygotes screened (0 from 4000 alleles 95% CI for non-canonical allele frequency 0.0000-0.0009) implies that the aggregate accumulation of such alleles has been less than 0.1% since the homozygous strain was constructed more than 51 generations earlier, so less than 0.002% per insect generation. Furthermore, this is likely an overestimate since loss-of-function mutations (or indeed wild type alleles entering the strain by contamination from another strain rather than internally generated through rather than by genetic instability) are likely to have higher fitness than the canonical OX513A allele (e.g. Bargielowski et al., 2011a; Bargielowski et al., 2012; Bargielowski et al., 2011b) and will therefore tend gradually to increase in frequency in the population if they should arise. Furthermore, the high degree of concordance between results obtained by qPCR and visual screening of the fluorescent phenotype gives us confidence in the use of this phenotype as a means of identifying the presence of the transgene.

15. Literature:

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Annex 1: Screening by qPCR:

a) Methodology

Genomic DNA extraction

Genomic DNA (gDNA) was extracted from each sample using the Quick non-enzymatic method, according to Oxitec Standard Operating Procedures (TD_SOP_00124)(Annex 2)

Real-Time PCR

1µl of each gDNA sample was used as a template for qPCR, according to Oxitec Standard Operating Procedures (TD_SOP_00125)(Annex 3) with the following primer sets:

- Calibrating probe AedesPro: AGACACCAGTCGGACTTGCAAAATCTG (5' labelled with HEX; 3' labelled with BHQ1)
- Insert probe K10Bea2: GACCACCGACGGCTCATTAGGGCTCGTGTGGTC (5' labelled with Fam; 3' labelled with BHQ1)
- Insert primer K10F155: CTCTGCTGACTTCAAAACGAGAAGAG
- Insert primer K10R266: ATTGGGTTTCACCGCGCTTAGTTACA
- Calibrating primer AedesF: CTGCAGTAGTGATGAAGATGAACCA
- Calibrating primer AedesR: GGGCGAAAATGCCGTATTGTACTCA

Specimens were considered as positive for the OX513A construct if the ΔCt value (Ct for internal comparison 'calibrating' primer set minus Ct of OX513A-specific primer set) was not more than 1.0 less than that of the OX513A heterozygous positive control, i.e. ΔΔCt >-1.0 [4]. The calibrator DNA used on all qPCR plates was extracted from sample 2-6T (G_2 generation). All samples were run as technical duplicates.

Actin 4 PCR

The samples where the scoring by qPCR differed from scoring by fluorescence (See Fig. A2), were screened for the Actin 4 gene by PCR.

Primer set:

- AeA4F2: CAATCGAAGCGAGGTATCCTCACCC
- AeA4R2: CTGGGTACATGGTGGTACCACCAGAC

Expected product size is 747bp

The reaction mix consisted of: 0.5µl AeA4F2 and AeA4R2 (10um), 4µl 5x PCRBIO Buffer, 0.5µl 10x BSA, 0.3µl PCRBIO Taq, 11.2µl Milli-Q water. Three microlitres of each gDNA sample were used as template in a 20ul reaction. There was also 1 x +ve control and 1 x –ve control. The thermal profile consisted of an initial denaturation step of 2 minutes at 95°C, followed by 2 cycles of the following steps: 7 seconds at 97°C, 40 seconds at 64°C, 90 seconds at 72°C,

[4] Due to the unavoidable variation in the amplification values observed among different individuals, the cut-off value of ΔΔCt >-1.0was empirically established based on preliminary experiments which showed that the relative quantities OX513A DNA in WT controls are normally well below this value. Presence of lower levels of OX513A in negative samples may be due to fragments of OX513A individuals, e.g. excreta or shed cuticle, contaminating wild type larvae and/or mis-priming and amplification of non-OX513A-derived amplicons.

followed by 35 cycles of the following steps: 7 seconds at 97°C, 40 seconds at 64°C, 15 seconds at 72°C. The final extension step was 1 minute at 72°C followed by a final holding temperature of 4°C.

To visualize PCR products, 4µl gel loading buffer (30% glycerol, 0.5% bromophenol blue) were added to each PCR product and 5µl of this mixture were run on a 0.8% agarose TAE (Tris Acetate EDTA) gel, stained with ethidium bromide, at 120V for 30 minutes. The presence/absence and strength of a gel band of the expected size will give an indication as to whether the gDNA is of adequate concentration to be used in subsequent downstream applications such as PCR or qPCR.

b) Results

qPCR results are presented in figures 3 and 4 below.

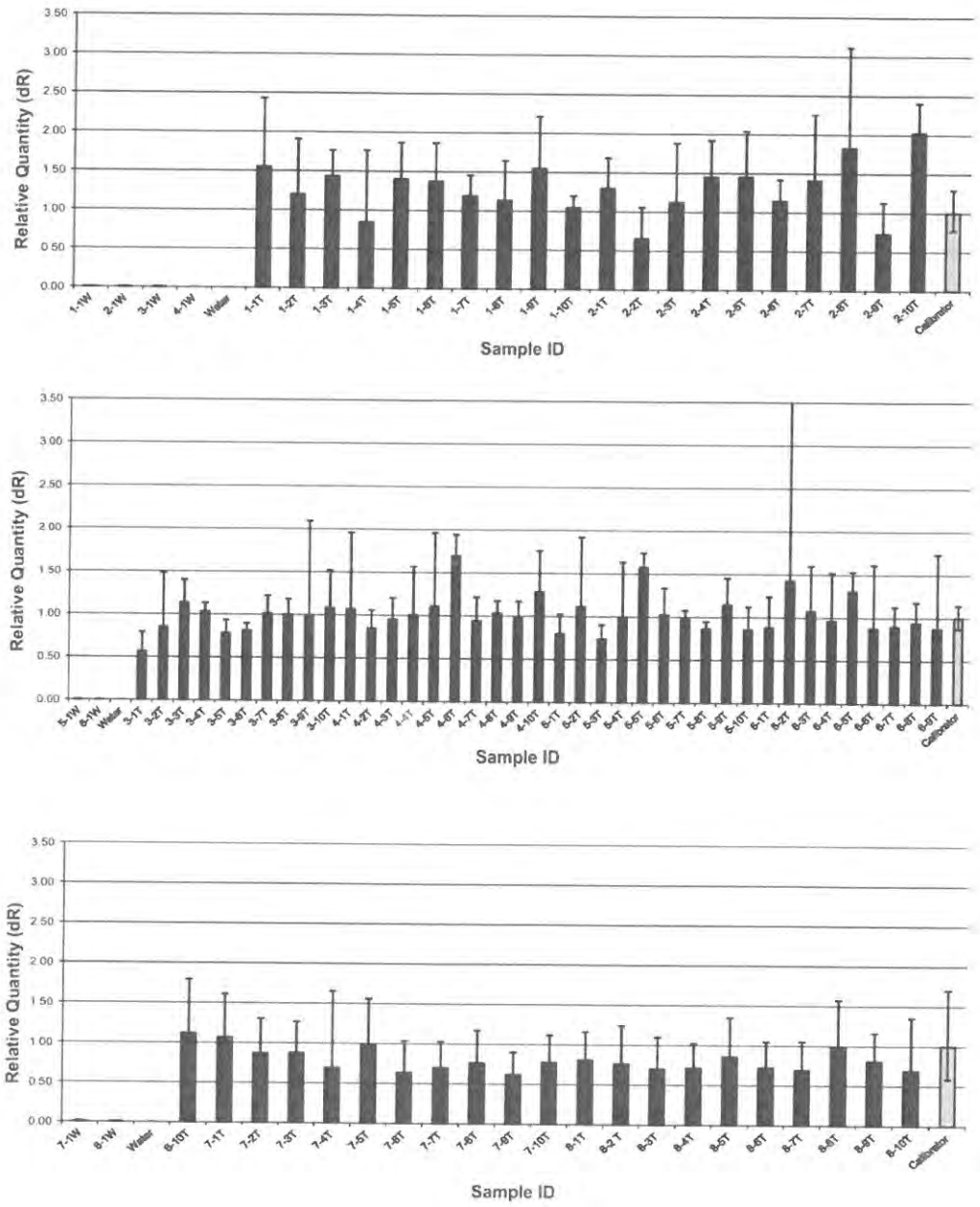


Fig. 3. **Results of qPCR of G_1 samples**. Bars represent relative quantities of OX513A DNA normalised against calibrator sample (heterozygous OX513A specimen; grey bar). Grouping of samples in the graphics reflects grouping of samples in qPCR plates. As expected, all G_1 samples produced estimated relative quantities of OX513A DNA which are ≥0.5 of the corresponding value in the calibrator sample, corresponding to ΔΔCt>-1.0. Sample ID code of all G_1 samples ends with a 'T'. Sample ID codes ending in 'W' indicate WT control samples (shown only for reference).

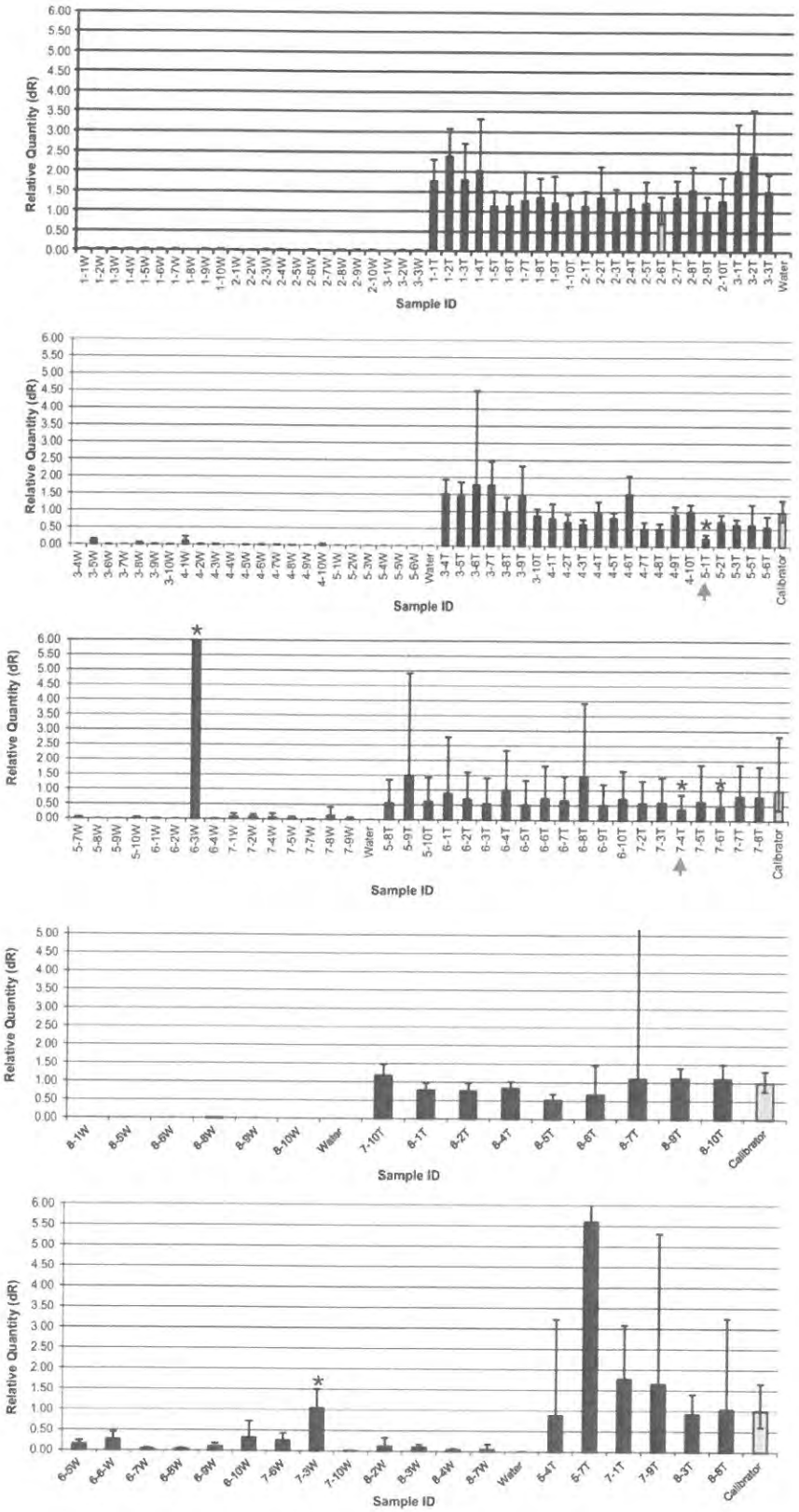
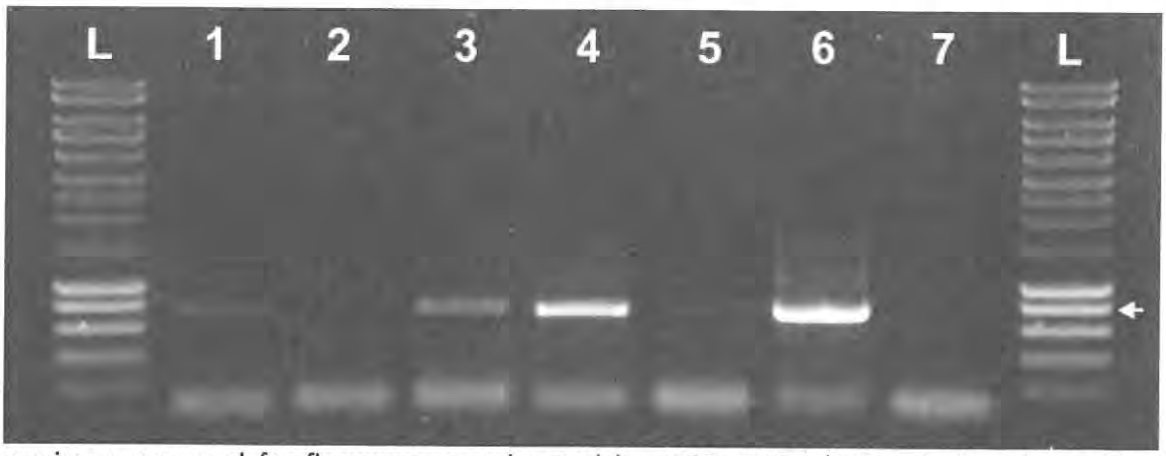


Fig. 4. **Results of qPCR of G_2 samples**. Bars represent relative quantities of OX513A DNA normalized against a calibrator sample (heterozygous OX513A specimen; grey bar). Grouping of samples in the graphics reflects grouping of samples in qPCR plates. Sample ID codes ending in 'W' indicate samples scored as WT during

fluorescence screening. Sample ID codes ending in 'T' indicate samples scored as OX513A during fluorescence screening. Bars marked with an asterisk represent samples where the scoring by qPCR differed from scoring by
Fig. 4 **(continued).** fluorescence. Samples marked with an arrow are those where the discrepancy between fluorescence and qPCR scoring is unlikely to be due simply to poor DNA quality, since samples produced a clear amplicon when screened for the Actin 4 gene by PCR (fig. 5). Therefore, samples marked with an arrow are likely to represent error in the fluorescence-scoring process. The low level of OX513A DNA detected in WT individuals (for example, samples 6-5W and 6-6W) can be explained by the fact that these DNA samples come from a generation expected to contain individuals of both genotypes. As a consequence, both WT and transgenic larvae must be reared together until the time when they are screened, which is likely to cause a certain level of contamination of WT larvae with biological material from OX513A individuals.

In total, screening by qPCR confirmed fluorescence scoring for 80 out of 80 (100%) of the G_1 larvae examined. Among G_2 larvae, two specimens scored as WT by fluorescence were scored as OX513A by qPCR (samples 6-3W and 7-3W)(fig. 4). One of these samples (sample 7-3W)(fig. 4) failed to produce a detectable band when scored by PCR for the Actin 4 gene (fig. 5), suggesting problems with its DNA quality. The other sample (sample 6-3W) produced an abnormally high relative value for OX513A DNA in the qPCR test (4743 9dR) fig. 4), suggesting technical issues (such as contamination with an auto-fluorescent substance) during the qPCR process. Similarly, three G_2 samples scored as OX513A by fluorescence screening failed to meet the criteria for being scored as OX513A by qPCR (samples 5-1T, 7-4T and 7-6T)(fig. 4). One of these (sample 7-6T) also failed to produce a detectable band when scored by PCR for the Actin 4 gene (fig. 5), suggesting problems with its DNA quality. Therefore, only two samples are likely to have been wrongly scored as transgenic during screening by fluorescence microscopy (samples 5-1T and 7-4T)(fig. 4). If we exclude the aforementioned samples which show evidence of either low DNA quality or technical issues with the qPCR process, the estimated efficiency for correctly scoring specimens from a mixed pool by visually screening the fluorescent phenotype is 100% (78 out of 78) for WT specimens, and 97.4% (75 out of 77) for OX513A specimens. Of total OX513A



specimens screened for fluorescence and tested by qPCR, 98.7% (155 out of 177) scored as fluorescent were confirmed as OX513A by qPCR.

Fig. 5. **Results of PCR reaction for the detection of Actin4 gene in G_2 samples showing discordance between results of screening by qPCR and fluorescence analysis.** L: Molecular size marker, 1: sample 6-3W, 2: sample 7-3W, 3: sample 5-1T, 4: sample 7-4T, 5: sample 7-6T, 6: positive control, 7: negative control. White arrow marks band in molecular size marker corresponding to 800bp.

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Annex 2: Quick non-enzymatic genomic DNA extraction

1.0 Purpose

To describe the process for quick non-enzymatic genomic DNA extraction.

2.0 Scope

This procedure applies to the preparation of genomic DNA, in particular from insect legs as part of the genotyping process

3.0 References / Associated Documents

-

4.0 Responsibilities

This procedure will be carried out by trained staff only

The owner of this document is responsible for maintaining and revising this document.

5.0 Equipment

PCR tubes / strips / 96 well plate (depending on the number of flies to be prepared), forceps, Bunsen burner, P200 or repetitive pipette, pipette tips, Solution A (100mM NaOH), Solution B (500mM HCl + 250mM Tris-HCl (pH 8.5) + 0.04% Phenol Red (0.02 g/50ml)), thermocycler, vortex, centrifuge, 1.5 ml Eppendorf tubes.

6.0 Precautions

Wear gloves, protection glasses when preparing solution A and protection glasses and mask when preparing solution B. Wear gloves when handling solutions A and B.

7.0 Instructions

7.1 Immobilise insects on ice or CO_2 plate.

7.2 Dispense 50 ?l solution A for whole insect or 25?l for leg extraction, in 0.2ml (PCR) tubes.

7.3 Place one pulled leg or whole insect per tube using forceps. If doing leg genotyping, store the adults matching each leg into adequately labelled 1.5 ml Eppendorf tubes. Burn forceps using Bunsen burner between each sample. Close tubes with lids.

7.4 Spin down briefly to get sure that the legs or whole insects are immersed inside solution A and then incubate in a thermocycler at 99°C for 30 minutes. Spin down briefly to clear lids and avoid contamination.

7.5 Add 10 ?l solution B for whole insect or 5?l for leg extraction. Vortex until the solution's colour is homogenous. Spin down briefly to clear lids and avoid contamination.

7.6 The DNA samples can be stored at -20°C until use.

7.7 Use 1 ?l for real-time PCR, 1 or 2 ?l for regular PCR.

8.0 Records / Attachments

-

9.0 Related Documents

- TD/SOP/ 00125: Genotyping *Ae. albopictus* using real-time PCR
- TD/SOP/00093 513 geonotyping PCR

10.0 Revision History

Page	Nature of Revision

Annex 3: Genotyping *Ae. albopictus* using real-time PCR

1.0 Purpose

To describe the process for genotyping insects using comparative real-time PCR.

2.0 Scope

This procedure applies to the genotyping of insects, in particular *Ae. albopictus*, to select individuals homozygous for a transgene including the K10 3'UTR, in the process of establishing a homozygous line. This protocol needs to be adjusted for other species by selecting an appropriate probe and primers for the calibrating reaction and possibly adjusting the volumes of primers and probes in the mastermix.

3.0 References / Associated Documents

- TD/SOP/000124: Quick non-enzymatic genomic DNA extraction

4.0 Responsibilities

This procedure will be carried out by trained staff only.

The owner of this document is responsible for maintaining and revising this document.

5.0 Equipment

PCR tubes / strips / 96 well plate (depending on the number of flies to be prepared), pipettes, filtered pipette tips, real-time PCR machine, plus following reagents:

- TaqMan® Gene Expression Master Mix (Applied Biosystems, Warrington, UK)
- Insert probe K10Bea2: GACCACCGACGGCTCATTAGGGCTCGTGTGGTC (5' labeled with Fam; 3' labelled with BHQ1)
- Calibrating probe AedesPro: AGACACCAGTCGGACTTGCAAAATCTG (5' labeled with HEX; 3' labelled with BHQ1)
- Insert primer K10F155: CTCTGCTGACTTCAAAACGAGAAGAG
- Insert primer K10R266 primer: ATTGGGTTTCACCGCGCTTAGTTACA
- Calibrating primer AedesF: CTGCAGTAGTGATGAAGATGAACCA
- Calibrating primer AedesR: GGGCGAAAATGCCGTATTGTACTCA
- MilliQ (ultra-pure) water

6.0 Precautions

Keep probes in the dark as they are light-sensitive.

Handle the real-time machine gently as this is a very expensive and sensitive piece of equipment.

Wear gloves, use filtered tips and take care avoiding contamination.

Use optically-clear lids on the PCR tubes and do not write on lids as the machine reads fluorescence in the tubes through the lids.

7.0 Instructions

Preliminary extract gDNA from legs following TD/SOP/000124: Quick non-enzymatic genomic DNA extraction.

Switch on real-time PCR machine and Mx-Pro software, select "comparative quantitation" and pre-warm the lamp by clicking on the lamp button in the toolbar.

7.1 Always do the following controls:

No DNA (replace DNA with MilliQ water)

Confirmed homozygous or heterozygous DNA (if possible)

WT DNA

7.2 Prepare the following mix (volumes for a full 96-well plate):
76.8 µl K10F155 primer (10µM)
76.8 µl K10R266 primers (10µM)
57.6 µl AedesF primer (10µM)
57.6 µl AedesR primer (10µM)
57.6 µl K10Bea2 probe (10µM)
57.6 µl AedesPro probe (10µM)
576 µl MiliQ water
Total = 960 µl.
7.3 Add 960 µl TaqMan® Gene Expression Master Mix.
Dispense 19 µl in each tube.
Dispense 1 µl leg gDNA in each tube (or MilliQ water for the No DNA control).
Place lids on tubes, ensuring they remain optically clear.
7.4 (optional) centrifuge briefly to collect all liquid to the bottom.
7.5 Place the plate in the real-time machine.
In the software Plate Setup screen, select all the wells and set well-type to "unknown". Tick the box next to Hex and Fam.
In the Thermal Profile Setup screen, right-click and select "import". Select the reaction dated "7.09.2011" in the folder Storage\genevieve. This should load a thermal profile with an initial denaturation step of 10 minutes at 95°C followed by 43 cycles of the following steps: 11 seconds at 94°C, 15 seconds at 60°C, 30 seconds at 54°C, 30 seconds at 60°C.
Click "RUN" on the top right corner and save the reaction in the appropriate folder. Tick "turn off lamp at the end of run".
7.5 When the reaction is finished, look at the amplification curves in the "run" screen. Ignore data from wells with flat curves.
In the Plate Setup screen, highlight all the wells and normalize on Hex. Also select the well with your known homozygote or heterozygote control and set this well as calibrator in the well-type box.
In the Analysis screen, highlight all the wells except the ones with flat curves. Go to results and select "Relative quantity chart". The calibrator well should have a value of 1. If it is a heterozygote, homozygous individuals should have a value around 2 and heterozygous around 1. If the calibrator is a homozygote, homozygous individuals should have a value around 1 and heterozygous around 0.5. WT control should have a value around 0.
Write down the wells holding homozygous legs and start your homozygous strain with the corresponding adults.

8.0 Records / Attachments

-

9.0 Related Documents

- TD/SOP/ 000125: Quick non-enzymatic genomic DNA extraction

10.0 Revision History

Page	Nature of Revision

OXITEC

INTERNAL RESEARCH REPORT

1. **Reference Number**: PH-2013-11-V1

2. **Issuing Date: 31 August 2016**

3. **Title:**
Suppression of field populations of *Aedes aegypti* with sustained release of OX513A males in Mandacaru, Brazil

4. **Statement Of Data Confidentiality Claims:**

This document contains no confidential business information

5. Statement Concerning Good Laboratory Practices:

This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

6. Authors:

Study Coordinator (Signature):	**Study Supervisor (Signature):**
Study Coordinator (Name And Position): Head of Field Studies	**Study Supervisor (Name And Position):** Chief Scientific Officer
Date Signed:	**Date Signed:**

7. Associated Personnel:

Name	**Tasks**
	Study coordination, experimental design, data collection and analysis, report writing
	Experimental design, report editing, study supervisor
	Data analysis, report writing
	Data collection and analysis
	Study Sponsor

8. Test Facility:
This research was conducted as part of the evaluation of the technology in Juazeiro, Bahia Brazil, in collaboration with local partners Moscamed:
Biofábrica Moscamed Brasil
Av. C1, 992 - Quadra D 13, Lote 15
Distrito Industrial do São Francisco
Juazeiro - BA - Brasil - CEP 48.908-000

9. Objectives:
The objective of this study was to assess the effectiveness of periodic release of OX513A males as a method for suppressing a wild population of *Aedes aegypti*. This study was conducted between 2012 and 2013 in Mandacaru, Bahia, Brazil.

10. Summary:

OX513A was found to be a potent suppressor of wild *Aedes aegypti* populations in Mandacaru, an isolated village near the city of Juazeiro, Brazil. The wild populations, measured using ovitraps, in treated areas were reduced by 91 and 95% over a 6 month period relative to similar untreated control areas. The adult population, estimated using mark release recapture, showed a reduction of 96% in the treated area following sustained release of OX513A males. The study area comprised 735 houses, equating to a human population of 2,793. An overall mating competitiveness value for the strain in the field, which incorporates the rearing, release, mate-searching, and breeding processes was found to be 0.012. That the observed suppression of the *Ae. aegypti* population was even greater in this study relative to previous studies (cf 80-85% reduction in ovitrap index in Grand Cayman 2010 and Itaberaba 2011-2012) is likely due to the isolation of the study area and the presumed absence of immigration of *Ae. aegypti* from neighbouring untreated areas.

11. Introduction:

This study represents a field evaluation of male-only releases of a strain (OX513A) of *Aedes aegypti* (Linnaeus) carrying a lethal (also known as autocidal) transgene, as a tool for suppressing wild pest populations of *Ae. aegypti*. Released males are homozygous for a dominantly inherited transgene that when transmitted to an embryo via the sperm, leads to death of the zygote during either the late larval or pupal stages of development (Alphey et al., 2010; Catteruccia et al., 2009). Simulation modelling has suggested that this method would be effective and economical against *Ae. aegypti* (Alphey et al., 2011; Atkinson et al., 2007), and field testing in the Cayman Islands demonstrated successful competition of OX513A for wild mates and that repeated male releases suppressed a wild population of *Ae. aegypti* (Harris et al., 2012; Harris et al., 2011) by over 80%. Approximately 80% suppression of wild population has also been demonstrated in Itaberaba, Juazeiro Bahia (Oxitec Report PH-2013 09). This was a densely inhabited suburb with high *Ae aegypti* infestation. In both these previous demonstrations, the area treated with sustained release of OX513A males was immediately adjacent to untreated area from which immigration of wild *Ae aegypti* provided constant source of infestation. This study sought to select a field site which was geographically isolated with regard to *Ae aegypti* dispersal, thereby removing interference from immigration, and opening possibility of achieving localised elimination. In addition, it provides another set of environmental parameter in which to evaluate sterile OX513A release for population control.

12. Methods:

Study area

The study was conducted in the village of Mandacaru, 8 km North-East from the city of Juazeiro, Bahia in the semi-arid North East of Brazil (latitude – 9.389, longitude - 40.430). The site consisted predominately of residential housing of low average to average economic status and was identified by local public health officials as having *Ae. aegypti* with incidence of dengue. In general housings were of a higher standard compared to Itaberaba (Oxitec Report PH2013 09) and had reliable piped water supply, reducing reliance on stored water. These factors are consistent with comparatively lower *Ae. aegypti* infestation than Itaberaba. Ovitrap results prior to suppression revealed that the *Ae. aegypti* population was in the Medium/low range (~10% ovitrap index). *Ae. aegypti* population is detectable year round with higher number occurring in the warmer summer months of November to April. The village is surrounded by mixture of farm land and dry uninhabited areas that are not hospitable to *Ae. aegypti* and thus constituted a likely geographical barrier both for immigration and emigration. This provided an ideal scenario to evaluate impact of sustained release of OX513A without the potential interference of immigration of wild *Ae. aegypti* from adjacent areas, as was the case in previous field evaluations (PH 2013 09, Harris et al., 2012).

Baseline monitoring was initiated in April 2011 using ovitraps. All eggs from initial 4 collections were reared to adult for species identification to assess the presence/absence of *Aedes albopictus* (Skuse) and *Ae. aegypti*. No *Ae. albopictus* were detected, and subsequent identification of adult trap catches from Mandacaru were also negative for *Ae. albopictus.* A detailed census of Mandacaru revealed an average of 3.8 residents/house and a total 735 houses, equating to an estimated human population of 2,793. For planning and experimental purposes, the trial site was subdivided into three areas: South, North-East and North-West (Figure 1) which are described in Table 1. Unlike the Itaberaba study which had a uniform population density, these areas had substantially different human population density ranging from 32 to 118 inhabitant/Ha. Juazeiro and its surroundings have a semi-arid climate with average annual precipitation of 536mm falling mostly in warmer summer

months (November-April). Average min/max daily temperatures range from 18-25°C and 22-28°C for winter and summer respectively[1].

Two villages similar to Mandacaru were selected as untreated comparator control sites. Care was taken to match sites in terms of habitat and other environmental factors such as housing type. As with Mandacaru, sites were selected in consultation with local vector control department and were known to have *Ae. aegypti* and reported incidence of dengue. Carnaiba is located 23 km South of Mandacaru (latitude - 9.590, longitude - 40.424) and has an estimated population of 2,861. Maniçoba village is 15 Km North East of Mandacaru (latitude - 9.314, longitude - 40.307) with an estimated 3,861 inhabitants. Each had similar *Ae. aegypti* population levels as Mandacaru during our baseline studies, thus making them ideal comparator control sites.

All villages, including Mandacaru, fell within the Juazeiro vector control department jurisdiction receiving the same conventional mosquito control programs. Release of OX513A and monitoring was conducted independently, and did not change or interfere with the vector control authority's standard practice. This typically consisted of teams of public health agents visiting homes between 4 and 6 times per year, where they physically destroyed some breeding sites and treated others with the organophosphate larvicide, temephos.

Community Engagement/regulatory

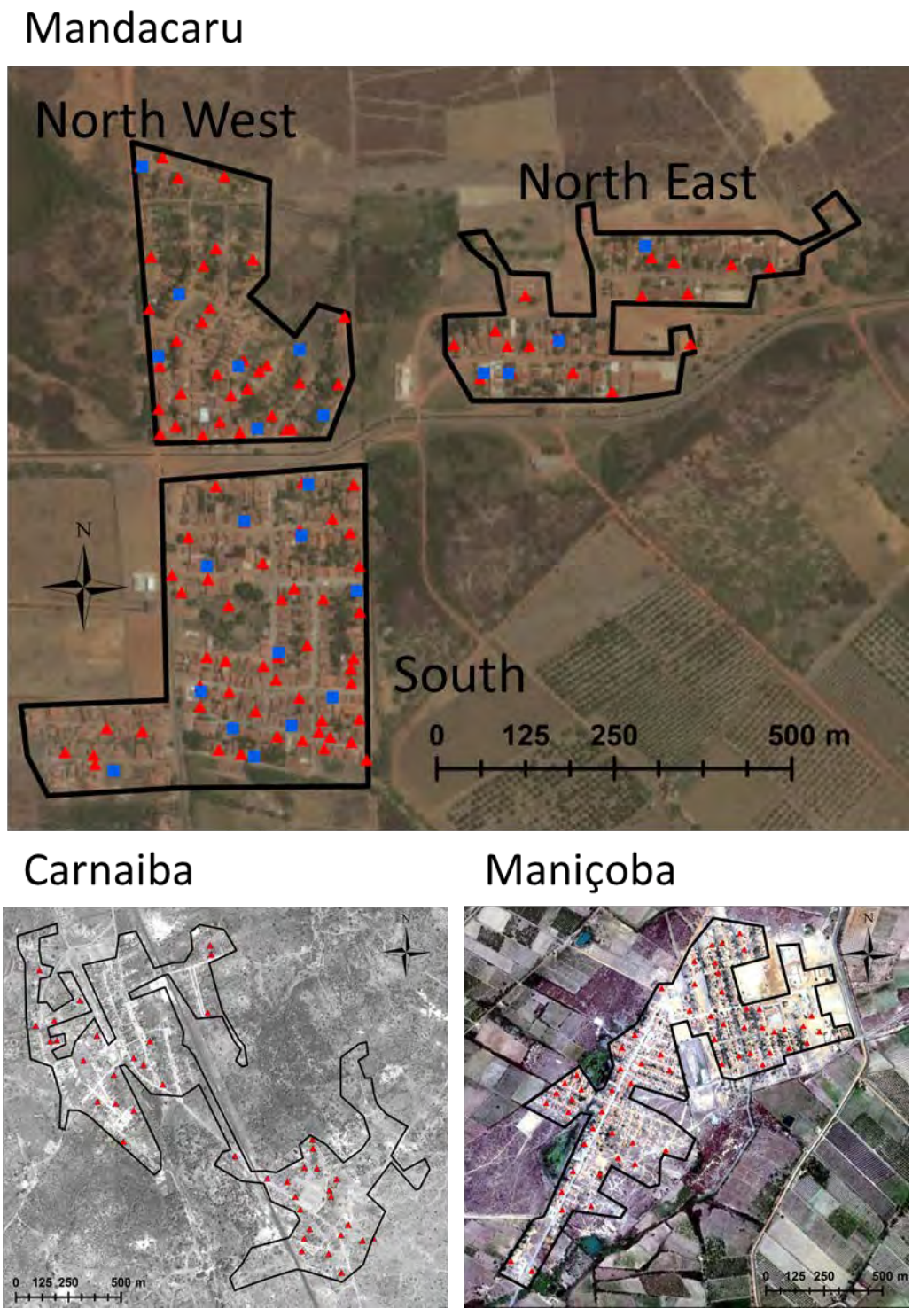
From its inception the project sought to adopt full transparency with a vigorous and proactive community engagement (CE) campaign. A local social scientist was engaged to provide expert advice on best communication strategy specifically tailored to local community. In addition a full time journalist was hired to facilitate the CE program. Implementation included communication via local media (radio, TV and press), community meetings, printed information (posters and leaflets), school presentations, carnival parades, use of small vans with loudspeakers and social media (websites and blogs). Dedicated door to door campaigns and ongoing contact with field technicians working in the community provided face-to-face interaction on an individual basis, allowing specific questions to be addressed and for direct feedback and concerns to be aired. Consent and support came from national and regional administration and local community leaders. Prior to establishment of the transgenic OX513A strain in the mass rearing facility and subsequent open releases, regulatory approvals were obtained from the appropriate Brazilian national regulatory body (CTNBio, Processo nº: 01200.002127/2010-50; Processo nº: 01200.002644/2010-29).

OX513 A: transgenic *Ae. aegypti* strain with the OX513A insertion. This strain was formed through introgression of LA513A[2] (Phuc et al., 2007) into a Mexican-derived genetic background through five generations of backcrossing, prior to a selection process for homozygous individuals only. This was the same strain previously used for field evaluation in the Cayman Islands (Harris et al., 2012; Harris et al., 2011). The breeding line was originally imported from Oxitec Ltd. by the University of São Paulo (Import Permit number: Processo nº: 01200.000785/1997-79) where it underwent laboratory evaluations against Brazilian *Ae. aegypti* lines before being transferred to Moscamed (Moscamed.com), Juazeiro City.

[1] http://www.worldweatheronline.com/Juazeiro-weather-averages/Bahia/BR.aspx

[2] The strain referred to as LA513A in Phuc et al. (2007) was subsequently renamed OX513A; LA513A and OX513A refer to the same transgene insertion; the different designations reflect a name change only.

Figure 1 – Field sites. Black line denotes boundary of inhabited area. Red triangle = Ovitrap location, Blue square = BG traps location.



Mass rearing of OX513A

Subsequent to obtaining regulatory approval, male mosquitoes were produced at the Moscamed facility in an 84m^2 laboratory adapted specifically for the purpose. Mass rearing insectaries were maintained at 27°C (+/- 2) with a RH 70-90% and a 12 hrs day/night cycle. A filter colony of homozygous OX513A was established producing sufficient eggs to supply male mosquito production for release. Mosquitoes destined for release were reared to pupae where they were mechanically sorted to remove females (Ansari et al., 1977; Focks, 1980). For quality control a minimum of 1500 male pupae from every release batch were individually checked using a microscope to ensure < 1% female contamination. In practice, sorting efficiency averaged 0.02% female contamination (SEM 0.003%), equivalent to 1 female for every 4,300 males. A separate laboratory was used to maintain locally derived wild type colonies and process field traps, thereby reducing the potential for contamination of the homozygous OX513A breeding line. Weekly quality control checks were made of the transgenic phenotype i.e. expression of the fluorescent marker and lethality in the absence of tetracycline. Detailed methods for production of male pupae followed procedures that are detailed in Carvalho et al., 2013 (Carvalho et al., in preparation).

Eclosion and Release

Male pupae were aliquoted into combined pupal eclosion/adult release devices (RD). Each RD consisted of a 1.8 litre (14cm high x 13 cm diameter) clear plastic cylindrical container (Produtos Prafesta®, Brazil) with a large hole in the lid that was covered with fine mesh to permit air exchange. Sugar feeding was provided *via* cotton wool soaked in 10% sucrose solution placed on top of the mesh. 1,000 pupae were added to each RD. Once adults had eclosed, any remaining water was drained from pots through a slit in the side of the container before release, which took place approximately 48-72 hrs after pupation. Releases started on 19/3/2012 with 3 releases a week, reduced to 2 releases after 19/11/2012 once suppression of wild population had been achieved. RD's were transported by truck in large cool boxes to the release site and opened according to a scheduled release plan. Total numbers of adults released were estimated by subtracting the numbers of dead adults and pupae remaining in RD's after release from the initial numbers of pupae added.

Suppression Phase

For planning treatment regimen and release rates, Mandacaru was sub-divided into 3 areas: South, North East and North West (Table 1). Human population density varied substantially between these areas from 32 people/ha in North East to 118 people/ha in the South. As *Ae. aegypti* populations are closely associated with human populations, target release rates were normalised in terms of numbers released/person/week, rather than numbers released/ha/week. Initial release rates in Mandacaru were based on experience from an earlier suppression trial in Itaberaba where a release rate threshold of approximately 30,000/ha/week (= 182/person/week) was needed before suppression of wild population was detected (Oxitec Report PH-2013 09). However, ovitrap index in Mandacaru was substantially lower at ~10% compared with ~40% in Itaberaba. Taking this into account we conservatively selected an initial OX513A release rate/person of approximately 100 OX513A/person/week.

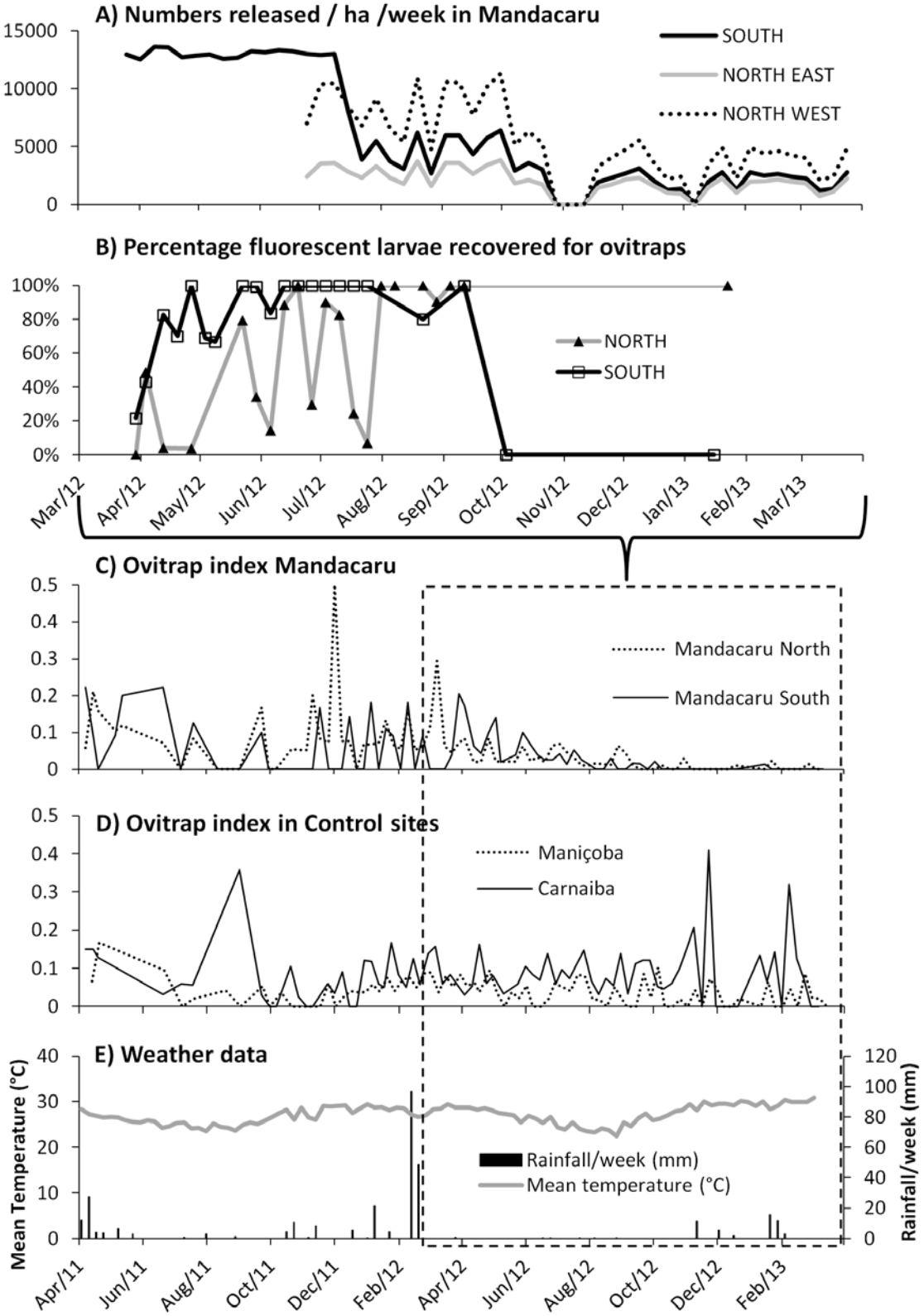
Initiation of sustained releases of OX513A was staggered, beginning in the South on 19/3/2012 followed by the North 3 months later on the 21/6/2012 (Table 1, Figure 2 A). Release rates were reduced twice in the South and once in the North in line with reduction in wild population, approximately halving each time (Table 1).

Table 1: Size and population density in different areas of Mandacaru, and mean OX513A male release rates over Periods A-C.

Site characteristics				Mean OX513A release rates per week					
				Period A		Period B		Period C	
Area	Size (ha)	Human Population	Population density/ha	Per ha	Per Person	Per ha	Per Person	Per ha	Per Person
South	15.0	1,763	118	13,007	111	4,737	40	2,040	17
NE	7.0	224	32	-	-	2,787	87	1,590	50
NW	8.5	806	95	-	-	8,164	86	3,846	41

Period A (South 19/03/2012-08/07/12), Period B (South 09/07/12-20/10/12; North 21/06/12-20/10/12), Period C (15/11/12-24/03/13)

Figure 2: (A) Weekly numbers of adult males released per person in different areas of Mandacaru (B) Percentage of larvae recovered from ovitraps in North and South areas with the OX513A transgene as detected by fluorescence. After the initial release period the mosquito population was strongly suppressed, such that most weekly egg collections produced no larvae (in some cases eggs were observed which failed to hatch, hence the larval data are a subset of the ovitrap data, which is based on egg numbers). Points on the graph represent those weeks when at least one larva was recovered. (C) Ovitrap Index in North and South areas (D) Ovitrap index in Control sites. (E) Weather Data. Note that the time periods are different for panels A and B relative to C-E, as indicated.


A) Numbers released / ha /week in Mandacaru
15000
10000
5000
0
SOUTH
NORTH EAST
NORTH WEST
B) Percentage fluorescent larvae recovered for ovitraps
100%
80%
60%
40%
20%
0%
NORTH
SOUTH
Mar/12
Apr/12
May/12
Jun/12
Jul/12
Aug/12
Sep/12
Oct/12
Nov/12
Dec/12
Jan/13
Feb/13
Mar/13
C) Ovitrap index Mandacaru
0.5
0.4
0.3
0.2
0.1
0
Mandacaru North
Mandacaru South
D) Ovitrap index in Control sites
Maniçoba
Carnaiba
E) Weather data
Mean Temperature (°C)
40
30
20
10
0
120
100
80
60
40
20
0
Rainfall/week (mm)
Rainfall/week (mm)
Mean temperature (°C)
Apr/11
Jun/11
Aug/11
Oct/11
Dec/11
Feb/12
Apr/12
Jun/12
Aug/12
Oct/12
Dec/12
Feb/13

Monitoring

Ovitrapping was used as the main measure of presence and abundance of wild *Ae. aegypti* population. Ovitraps were deployed in Mandacaru and control sites. Traps were initially deployed between end of January 2011 to beginning of April 2012. Numbers of traps increased in Mandacaru with start of release from 30 to 120, and a further 80 traps were added two months later (15/5/2012, Table 2). Trap number deployed increased with addition of new locations/houses, and by placing two traps per house. Where a house had two traps, one was located inside and the other in the peridomestic area outside. In Maniçoba, 60 traps were deployed throughout, and in Carnaiba the initial 40 traps were doubled to 80 traps for the period 15/5 – 16/11/2012 by increasing from one to two traps per house. Following initiation of releases, all eggs recovered from Mandacaru were hatched and larvae scored for the presence of the OX513A insert by the characteristic red fluorescence due to the expression of the DsRed2 marker (Lukyanov et al., 2000; Matz et al., 1999) (Clontech Laboratories Inc.) using a Leica MZ10 F epi-fluorescence microscope. Throughout the release period, non-fluorescent larvae were reared to adults as an additional check for the presence of *Ae. albopictus*; none were detected. Ovitrap index (number of positive traps/total number of traps recovered) based on the number of egg positive traps, rather than larvae identified as *Ae. aegypti*, was used as a representative statistic. This avoids the possibility of inconsistencies in calculated ovitrap index due to variation in egg hatch. Ovitraps are designed to mimic natural oviposition sites (Silver, 2008) and consisted of black plastic pots (11.5 x 11 cm) three-quarters filled with clean water with a fibre board paddle (12 x 3cm) protruding above the water line to provide an oviposition substrate. Traps were checked and replaced weekly.

BG-Sentinel adult traps (Biogents, Regensburg, Germany) were installed to coincide with start of releases in Mandacaru providing continuous adult monitoring (Table 3). Traps were checked weekly, although sometimes access to properties prevented this for a small proportion of traps. As trap catches were cumulative over time, the mean catch per day was used for comparative purposes where different service intervals were used. Data from BG-Sentinel traps that had not been serviced for more than 2 weeks were disregarded.

Table 2: Deployment of ovitraps in treated and control sites.

Period		Mandacaru				Control area	
		NE	NW	S	Total	Carnaiba	Maniçoba
27/01/2011* to 15/03/2012	Houses	9	10	11	30	40	60
	Traps	9	10	11	30	40	60
15/03/2012 to 15/05/2012	Houses	13	17	30	60	40	60
	Traps	26	34	60	120	40	60
15/05/2012 to 16/11/2012	Houses	18	32	50	100	40	60
	Traps	36	64	100	200	80	60
16/11/2012 to 12/03/2013	Houses	18	32	50	100	40	60
	Traps	36	64	100	200	40	60

**Traps set up; Carnaiba = 27/1/2011, Mandacaru = 8/2/2011, Maniçoba = 6/4/2011*

Table 3: Deployment of BG-Sentinel adult traps in Mandacaru.

	Mandacaru			
Period	NE	NW	S	Total
16/03/2012 - 23/03/2012	3	5	8	16
23/03/2012 - 30/03/2012	3	7	9	19
30/03/2012 - 09/04/2012	3	7	10	20
09/04/2012 - 11/08/2012	4	7	11	22
11/08/2012 - 19/03/2013	4	7	12	23

Statistical analysis:-

Statistical analyses were performed using R freeware (R Core Team, Vienna, Austria), and Excel (Microsoft, Redmond, Washington, USA).

Mating Competitiveness estimates

Mating competitiveness was derived from the ratio of OX513A to wild males achieved in field and the corresponding proportion of fluorescent larvae hatched from eggs collected in ovitraps. Methods used are described in more detail in Oxitec Report PH 2013 09. A 10,000 permutation bootstrap was conducted on the trapping data to estimate the 95% confidence interval for the mating competitiveness estimates.

Equation 1: Mating competitiveness *(C)*

$$C = \frac{PM_W}{(1-P)M_R}$$

Where:

P is the proportion of fluorescent larvae $P = \frac{L_R}{L_R+L_W}$

$\boldsymbol{M_w}$ is the density of wild type mosquitoes

$\boldsymbol{M_R}$ is the density of OX513A mosquitoes

$\boldsymbol{L_W}$ is the number of wild larvae screened

$\boldsymbol{L_R}$ is the number of OX513A larvae (fluorescent) screened

Population Estimates

We used mark-release-recapture (MRR) statistics (Lincoln, 1930; Petersen, 1896) in order to calculate adult wild populations, based on estimated standing crop of OX513A, and the corresponding ratio of released OX513A males to wild males recovered in BG Sentinel traps. The standing crop of OX513A was estimated from known release numbers and estimated survival in environment. Longevity values (Daily survival probability = 0.49) used were based on the mean of multiple MRR studies conducted in Itaberaba (Oxitec Report PH-2013 09) using OX513A males from the same rearing facility. Ratio of released OX513A to wild males was derived from change in sex ratio (male:female). We used an underlying sex ratio value of 0.69 males/females obtained in Itaberaba from BG traps catches in areas where there were no releases; this is a composite of the true adult population sex ratio and the relative attractiveness of the BG traps to each sex. Full details of calculation and formulae are given in Oxitec Report PH 2013 09. A 10,000 permutation bootstrap was conducted on the trapping data to estimate the 95% confidence interval for the population estimates.

13. Results and discussion

Ovitrap monitoring was initiated one year before releases began, providing baseline data regarding *Ae. aegypti* population dynamics in Mandacaru and control sites over a full seasonal cycle (Table 2, Figure 2 C and D). *Ae. aegypti* population was detectable year round with ovitrap indices broadly in the range of 5-20% which is indicative of a moderate infestation level, corroborating the historical and anecdotal experience of the Juazeiro vector control agents. There was some evidence of typical seasonality for the region with higher populations corresponding with warmer and wetter summer period on November to April (Figure 2), although this was not very pronounced. This may be due to the nature of ovitrap surveys which will tend to under-represent (dampen) some types of seasonal fluctuation. Here, in the wetter summer season when *Ae. aegypti* population is typically higher, there will tend to be a greater abundance of alternative breeding sites reducing probability of a female *Ae. aegypti* selecting an ovitrap to lay eggs. The converse is true in the drier winter season where a lower number of alternative breeding sites will likely result in a greater probability of any given female laying eggs in one or more ovitraps.

Care was taken in selection of control sites to be as closely matched to Mandacaru as possible. Baseline ovitrap results confirm *Ae. aegypti* population dynamics were comparable within Mandacaru (North and South) and between Mandacaru and comparator control sites (Maniçoba and Carnaiba). This validates the choice of comparator control sites to provide comparable populations dynamics, against which any changes resulting from OX513A in Mandacaru can be assessed. It is clear from overview of ovitrap indices that the sustained release of OX513A resulted in a collapse of the *Ae. aegypti* wild population over 6 months to the point where it was undetectable for much of the summer (Nov 2012-May2013), when typically one would expect higher populations to be detected. There was no evidence of a similar collapse of populations in control sites so the observed decline of the wild *Ae. aegypti* population in Mandacaru can be attributed to the sustained release of OX513A (Figure 2).

Indeed, the relative ovitrap index of Mandacaru to the control sites, i.e. Mandacaru ovitrap index divided by control site ovitrap index, decreased sharply after the second half of 2012 until the beginning of 2013 (Figure 3). When the average relative ovitrap index is calculated before suppression (April 2011-March 2012) and after suppression (September 2012-March 2013) a 91% and 95% significant decrease was observed for Mandacaru/Maniçoba (Welch t-test: $t = 4.1594$, $df = 10.418$, $p = 0.0018$) and Mandacaru/Carnaiba (Wilcoxon rank sum test: $W = 0$, $p < 0.001$) respectively (Figure 4).

Mating competitiveness is a key measure of efficacy for released males. We estimated net field mating competitiveness of the released males in the South of the site as 0.012 (95% CI 0.0052-0.021, Table 5). Mating competitiveness was not evaluated for the North as it was not possible to decipher the proportion of % fluorescence derived from releases in North and from immigration of releases in South. The value obtained from the South part of the site was in the same range (0.017-0.074) observed in the Itaberaba study before suppression was achieved Oxitec Report (PH 2013 09). Mating competitiveness as measured by this approach includes any effect of the transgene on the released males, the effect of artificial rearing, handling and distribution, and the effect of migration both of pre-mated females into the area and of released males and mated females out of the area. It may be that at relatively low wild population densities as significant proportion of the released males are released in areas that have few or no females; this may further depress the apparent mating competitiveness of the released males relative to wild males, which are likely to have a similar initial distribution to wild females. Relatively few estimates of mating competitiveness under open-field conditions have been published, despite the long history of sterile-male methods. In large-scale, successful Sterile Insect Technique(SIT) programmes, field

competitiveness of sterile males was estimated at 0.1 for New World screwworm (*Cochliomyia hominivorax*) (Mayer et al., 1998; Vreysen, 2005) and <0.01 for Mediterranean fruit fly (*Ceratitis capitata*) (Rendon et al., 2004; Shelly et al., 2007).

Table 5. Mating Competitiveness calculation for the South area of Mandacaru. The field data were collected between the 09/04 2012 to 22/05/2012 at the beginning of the study.

	Formula	Mandacaru South area (09/04 2012 to 22/05/2012)
Total male	MT	3,955
Total Female	FT	16
Sex Ratio	ST = MT/FT	247
Sex Ratio (M/F) in untreated area	SC	0.69
Estimated # wild male	MW = FT*SC	11.03
Estimated # OX513A	MR = MT-MW	3,944
Overflooding ratio	O = MR/MW	357
OX513A larvae	LR	309
Wild larvae	LW	71
Proportion OX513A larvae	P = LR/(LR+LW)	0.813
Mating competitiveness	**C = (P*MW)/((1-P)MR)**	**0.012**
Confidence interval 95%		[0.0052; 0.0210]

Wild population estimates in terms of adult standing crop were calculated using MRR statistics (Table 6). Results show a 96% reduction from estimated *Ae. aegypti* per ha before and after treatment in the South.

Table 6. Population estimates before and after suppression for the South area of Mandacaru. The calculations follow the Petersen-Lincoln method (Lincoln, 1930; Petersen, 1896). The field data used for the calculations were collected before suppression (09/04 2012 to 22/05/2012) and after suppression (05/11/2012 to 31/03/2013).

Date	Formula	Before Suppression South (09/04 2012 to 22/05/2012)	After suppression South (05/11/2012 to 31/03/2013)
Mean RIDL male Standing Crop/Ha	RS	1810	258
Estimated WT male standing Crop/ha	WM = RS/O	5.0652	0.2005
Male adult Ae aegypti/ha -95%CI		2.2911	0.0000
Male adult Ae aegypti/ha + 95% CI		8.2753	0.8746
Estimated WT female standing Crop/ha	WF = WM/SC	7.3	0.29
Female adult Ae aegypti/ha -95%CI		3.3205	0.0000

Female adult Ae aegypti/ha + 95% CI		11.9932	1.2675
Wild adult Ae aegypti /ha	**WT**	**12.4**	**0.5**
Confidence interval 95%		[5.61; 20.27]	[0.00; 2.14]
Population reduction			**-96.04%**

Figure 3: Monthly relative ovitrap Index (Mandacaru/control sites) over time

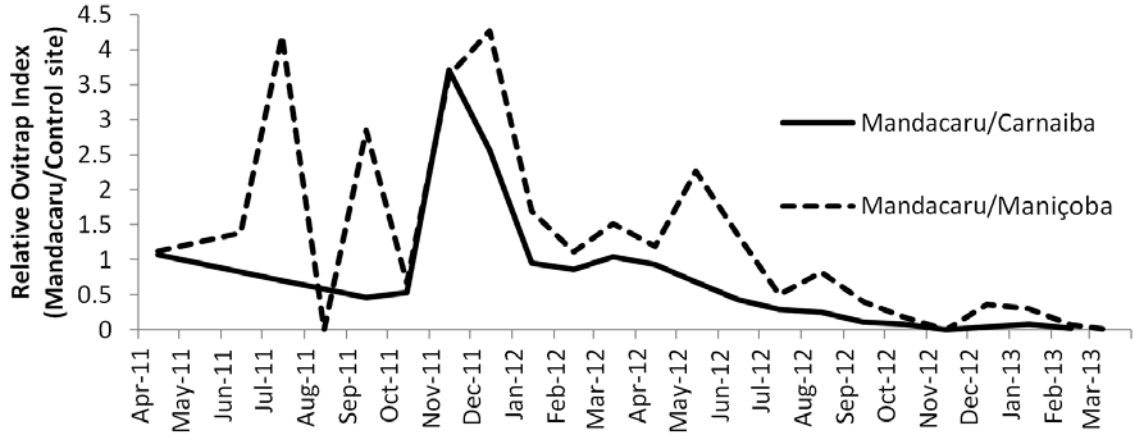
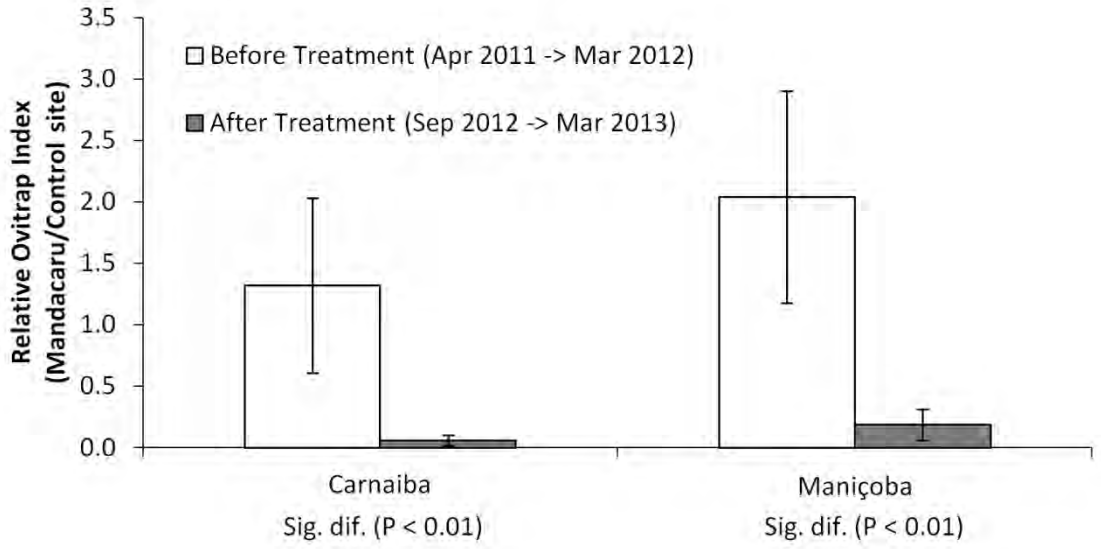


Figure 4: Mean relative ovitrap index for Mandacaru compared with Carnaiba and Maniçoba from before and after suppression of wild Ae. aegypti population in Mandacaru



14.Conclusion

Sustained release of OX513A was shown to be even more effective for suppressing the local population of *Ae. aegypti* in the isolated area of Mandacaru than in the previous non-isolated release areas (East End, Grand Cayman, and Itaberaba, Brazil). Indeed, in Mandacaru, an *Ae. aegypti* population reduction of 90 to 95% was achieved over a 6 month period and successfully maintained for a further 7 months, using reduced release rates. In principle, much lower release rates should be adequate to prevent re-infestation once the target population is suppressed or eliminated than are required to suppress the relatively high-density pre-treatment population. 'Re-infestation' in this context may relate to the immigration of wild type mosquitoes into the programme area, perhaps as eggs or adult inadvertently moved by humans. It may also relate to hatching of eggs laid at an earlier period, though the viability of such eggs is expected to decline over time such that this will be a source of reinfestation for a limited period only. This two-phase high-low release rate strategy was used successfully in this programme, indicating that it is feasible. However, we did not attempt to demonstrate that the lower release rate found to be adequate for maintaining the target population after suppression would have been inadequate for initial suppression. This study highlights that choosing a site that has limited immigration of females from non-treated areas facilitates an enhanced efficacy of OX513A at controlling *Ae. aegypti* populations; consequently large scale programmes which cover large urban areas are more likely to cause a greater population reduction. However, irrespective of potential immigration from untreated areas the decreases in the wild population of *Ae. aegypti* afforded by the use of OX513A are substantial for vector control operations. Estimates from Dana Focks and others of the degree of suppression required to reduce *Ae. aegypti* populations indicate that the degree of suppression observed in the present study would be sufficient to prevent epidemic dengue in almost all settings studied (Focks and Alexander, 2006; Focks et al., 2000). Field-based mating competitiveness values were above those estimated for successful conventional SIT programmes for other pest species, reinforcing the potential of OX513A as an effective and economical solution.

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OXITEC

Study Report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins

[Confidential business information (CBI) deleted]

SR-00004 Edition 2.b

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

Table of Contents

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

ACKNOWLEDGEMENTS

Oxitec would like to extend their gratitude to Dr Eric Calvo of the National Institutes of Health (NIH), Bethesda, USA for training Oxitec staff in mosquito saliva collection techniques, providing purified recombinant Aegyptin and providing rabbit anti-sera against recombinant Aegyptin.

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

1 STATEMENT OF DATA CONFIDENTIALITY

Confidential business information (CBI) has been deleted from this report.

2 STATEMENT CONCERNING GOOD LABORATORY PRACTICES

This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

3 ASSOCIATED PERSONNEL

Name	Affiliation	Role/Tasks
	Oxitec Ltd, UK	Study Sponsor, report editing and approval
	Oxitec Ltd, UK	Responsible for insect rearing and saliva extraction
	Oxitec Ltd, UK	Insect rearing and saliva extraction
	Oxitec Ltd, UK	Insect rearing and saliva extraction
	Oxitec Ltd, UK	Insect rearing and saliva extraction
	Oxitec Ltd, UK	Study co-ordination, experimental design, data collection
	Oxitec Ltd, UK	Data collection, report editing
	Oxitec Ltd, UK	QMS support, report writing
	Oxitec Ltd, UK	Report editing and approval

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

4 TEST FACILITY

This research was performed at the laboratory of Oxitec Limited located at:

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5 OBJECTIVES

The objective of this study was to investigate whether there is a detectable presence of the tetracycline-controlled transactivator protein (tTAV) or the marker protein DsRed2 in the saliva of adult female *Aedes aegypti* homozygous for the OX513 rDNA construct, known as OX513A.

6 SUMMARY

To test whether tTAV and/or DsRed2 protein is present in the saliva, which is a secretion of the salivary glands, of homozygous adult female *Aedes aegypti* expressing the OX513 construct, OX513A *Aedes aegypti* were reared in the presence of doxycycline hyclate to adulthood. Saliva was collected from bloodfed adult females between 10 and 15 days post-eclosion. Saliva was collected from these insects as well as from wild type *Aedes aegypti* females and two pools (OX513A and WT) created that were used for the entire study. Western blot analysis using a polyclonal tTAV antibody (anti VP16 tag antibody) and a polyclonal DsRed2 antibody was carried out, using an Enhanced Chemiluminescence (ECL) based detection method. Sample integrity was confirmed using an antibody detecting a secreted salivary protein in mosquitoes, Aegyptin. Aegyptin detection was also used as a basis to determine that equivalent amounts of saliva were loaded in control and sample lanes between the test saliva samples of OX513A and the WT control saliva samples.

The Limit of Detection (LOD) for tTAV and DsRed2 on the western blots was determined using recombinant tTAV and recombinant DsRed2. Purified tTAV and DsRed2 proteins from OX513A could not be used as sufficient quantity cannot be extracted from the insects for this study.

Results from western blot analyses were captured using the ChemiDoc-IT 500 Imaging System (UVP), and signals were quantified by relative densitometry, using the VisionWorks LS Acquisition and Analysis Software (UVP).

The Limit of Detection (LOD) for recombinant tTAV was determined to be 0.8 ng and the LOD for recombinant DsRed2 was determined to be between 5.0 and 2.5 ng.

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

The introduced proteins, tTAV and DsRed2 were not detected in OX513A *Aedes aegypti* saliva at and above these LODs in the 5 µl of saliva analysed. 5 µl of OX513A saliva equates to the quantity of saliva collected from approximately 5.5 female adult mosquitoes based on this study (270 µl of pooled saliva collected from approximately 300 *Aedes aegypti* adult females homozygous for OX513A rDNA construct).

7 INTRODUCTION

Genetically engineered *Aedes aegypti* strain OX513A carrying the rDNA construct OX513 was developed by Oxitec Ltd in 2002. This strain carries a genome integrated tetracycline transcriptional activator gene (tTAV), whereby the tTAV is produced in the absence of tetracycline or analogues and accumulated to levels lethal to those mosquitoes[1]. The OX513 rDNA construct also carries a gene coding for the DsRed2 protein as a marker.

In this study, pooled saliva samples collected from homozygous OX513A adult females (OX513A saliva) were investigated for the presence of tTAV and DsRed2 proteins. Pooled saliva samples from non-genetically engineered wild type *Ae.aegypti* adult females (WT saliva) were used as negative controls.

Western blotting detection[2] was carried out to determine the absence or presence of these proteins in OX513A saliva samples using an Enhanced Chemiluminescence (ECL) approach. Purified tTAV and DsRed2 proteins from OX513A are unavailable as sufficient quantity cannot be extracted from the insects to conduct the study. Therefore recombinant tTAV (rtTAV) and DsRed2 (rDsRed2) proteins were used to determine the limit of detection (LOD) of the western blot analysis in detecting these proteins in OX513A saliva.

For the purpose of this study, the LOD was defined as "the lowest quantity of a protein that an operator can visibly discern on a developed western blot image and create a user defined-region (UDR) and meets the acceptance criteria such that D_{SLC} is less than 10% of D_S" (Figure1). To be valid, all pixels on the complete western blot image must be less than a pixel intensity of 65,536 for a 16-bit grey scale image (as measured by the VisionWorks LS Acquisition and Analysis Software (UVP) used). 65,536 is the maximum pixel intensity of a 16-bit grey-scale image.

An endogenous 30 kDa saliva protein (Aegyptin)[3] was used as positive control in both OX513A and WT saliva to ensure their integrity and the equivalence of the saliva samples used throughout this study. Aegyptin protein is found in the saliva of females of a number of mosquito species including Aedes, Anopheline and Culicine species[4]. The detection signal obtained from the specific binding of the anti-Aegyptin antibody to the Aegyptin protein in the saliva was used to ensure that comparable amounts of total protein were loaded between the OX513A and WT saliva samples. A recombinant version of Aegyptin (rAegyptin[3]) was used as a positive control for the anti-Aegyptin antibody.

The LOD for tTAV and DsRed2 was determined on replicate blots using quantified amounts of recombinant proteins (rtTAV and rDsRed2). To ensure that the LODs obtained using the recombinant proteins were applicable for the detection of tTAV and DsRed2 in OX513A saliva, LODs were determined in the presence of WT saliva.

8 MATERIALS AND EQUIPMENT

8.1 Saliva

8.1.1 Saliva from approximately 300 *Ae. aegypti* adult females homozygous for OX513A rDNA construct (OX513A saliva) pooled in PBS to give a total volume of 270 µl saliva [therefore 5 µl OX513A saliva equals approximately 5.5 adult mosquitoes].

8.1.2 Saliva from approximately 1000 wild type *Ae. aegypti* (Latin) adult females (WT saliva) pooled in PBS to give a total volume of 890 µl [therefore 5 µl WT saliva equals approximately 5.5 adult mosquitoes]

8.2 Equipment

8.2.1 Microcapillary tube (Sigma-Aldrich, Cat # P1299)

8.2.2 Trans-Blot® Turbo™ Transfer System (BioRad Cat# 170-4155)

8.2.3 ChemiDoc-IT 500 Imaging System and VisionWorks LS Acquisition and Analysis Software (UVP)[5].

8.3 Recombinant proteins

8.3.1 Recombinant Aegyptin (rAegyptin) obtained from Eric Calvo National Institutes of Health (NIH), Bethesda, USA [expressed and purified in the Laboratory of Malaria and Vector Research (NIAID/DIR) by affinity size exclusion chromatography column as described by Calvo[3]]

8.3.2 Recombinant DsRed2 (rDsRed2) protein (Clontech, Cat # 632436)

8.3.3 Recombinant tTAV (rtTAV) protein (produced in *Escherichia coli* by Oxitec, according to Study Report SR-00003)[6]

8.4 Reagents and materials

8.4.1 4x Laemmli Sample Buffer (BioRad, Cat #161-0747)

8.4.2 Defibrinated horse blood (TCS Biosciences, UK, Cat #HB035)

8.4.3 Precision Plus Protein™ WesternC™ Pack (Mwt Marker, BioRad, Cat #161-0385)

8.4.4 Mini-PROTEAN® TGX gel (4-15%, BioRad, Cat #456-1086)

8.4.5 Nitrocellulose membrane (BioRad, Cat #170-4270)

8.4.6 Clarity™ Western ECL Substrate (BioRad, Cat #170-5060)

8.4.7 Tris/Glycine/SDS buffer (BioRad, Cat #161-0732)

8.4.8 Pierce™ Clear Milk Blocking Buffer (Life Technologies, Cat #37587)

8.4.9 Restore™ Western Blot Stripping Buffer (Life Technologies Cat #21059)

8.4.10 Tween 20 (Pierce, Cat # 28320)

8.4.11 PBS (Phosphate buffered saline, 0.01 M phosphate, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4) tablets (SIGMA, Cat # P4417)

8.4.12 TBS-T (Tris-buffered Saline Tween 20, 20 mM Tris, 150 mM NaCl, pH 7.5, 0.1% Tween 20)

8.5 Antibodies

8.5.1 Rabbit Anti-Aegyptin Antibody provided by Eric Calvo - Laboratory of Malaria and Vector Research (NIAID/DIR) (Chagas *et al*[7])

8.5.2 Rabbit Anti-VP16 tag polyclonal antibody (Abcam, Cat #ab4808)

8.5.3 Goat Anti-Rabbit IgG (whole molecule)-Horse Radish Peroxidase Polyclonal Antibody (Abcam, Cat # ab97051)

8.5.4 Living Colors®DsRed Polyclonal Antibody (Clontech, Cat # 632496)

9 METHODS

9.1 Strains

The following *Aedes aegypti* strains were used for this experiment:

OX513A: In the absence of doxycycline hyclate, this strain expresses the tTAV protein during immature stages through a positive feedback loop, resulting in high mortality (>95%). However, when reared in the presence of doxycycline hyclate, expression of the tTAV in this strain is repressed, allowing the OX513A mosquitos to

complete all parts of their lifecycle. Individuals of this strain can be identified during immature stages by the expression of the fluorescent protein DsRed2 in their bodies in a characteristic punctate pattern driven by the Actin5C promoter[1, 8].

Wild Type (WT): The non-genetically engineered background strain of OX513A, originating from Chiapas, Mexico in 2007.

9.2 Insect Rearing

All strains were reared under standard insectary conditions: 26°C [± 2°C], 75% [± 15%] relative humidity, 12h: 12h light: dark cycle. Adults were provided with 10% sucrose solution *ad libitum*. Larvae were reared with the addition of doxycycline hyclate to the water at a final concentration of 1 µg/ml, and fed finely ground Tetramin© fish flakes (Tetra, GmbH, Germany).

9.3 Saliva Collection

The WT and OX513A strains are homozygous for their respective genotypes and so female pupae were collected and directly placed into cages. One week post eclosion, females were offered a blood meal of warmed, defibrinated horse blood (TCS Biosciences, UK). Saliva samples were collected between 10 and 15 days post-eclosion as described below.

Females were cold-anesthetised in the freezer at ≤ -15°C for 15-20 seconds. Legs were removed before mounting them on a microscope slide using double sided tape. Each female's proboscis was inserted into a microcapillary (0.5 µl) containing mineral oil. A 15 minute interval was allowed for salivation at which point females were discarded and pools of 10 samples were collected into 1.5 mL microcentrifuge tubes containing 10 µL of phosphate buffered saline (PBS) pH 7.5 and frozen at ≤ -15°C. Samples were defrosted at room temperature and pooled before being used in this study. [DEV02: Saliva samples were frozen at ≤ -15°C prior to pooling. Saliva samples were thawed at room temperature and pooled. Protocol SP00002 stated that the saliva samples were to be pooled prior to freezing. This does not affect the analysis or results and so no further action was required].

9.4 Production of Recombinant tTAV (rtTAV)

Recombinant tTAV was produced by Oxitec using pET Express and Purify Kit – HisTALON, according to Study Protocol SP_00001[9] and Study Report SR-00003[6].

9.5 Densitometry Methods

Images of the western blot membrane were captured using the ChemiDoc-IT 500 Imaging System (UVP), and signals were quantified by relative densitometry, using the VisionWorks LS Acquisition and Analysis Software (UVP)[5]. To achieve this, the operator defined a rectangular box around a 'Band' of the expected size for the protein being analysed. This selection is referred to as the user defined region ('UDR', Figure 1a). The pixel density within the 'UDR' is referred to as 'Total Density', and is automatically normalised for background by the software, which subtracts the sum of the pixel intensity values of all pixels in an area within three pixels outside of the 'UDR' ('Total Background'), from the sum of the pixel intensity values for all pixels within the 'UDR' ('Total Raw Density').

The 'Total Density' of the 'UDR' containing a 'Band' representing specific binding of the antibody to the target protein is referred to as the 'Specific Density (D_S)'. The 'Total Density' of a sample lane control (D_{SLC}) was measured similarly by defining a rectangular box of identical dimensions to the 'UDR' within the same lane, in an area without specific antibody binding, either above or below the specific signal. 'Total Density' for a 'Blank Lane

Control (D_{BLC})' was defined by creating rectangular box of the same dimensions as the 'UDR' in a nearby blank lane on the same gel (i.e. no protein sample, Figure 1b). 'D_{BLC}' was expected to be low compared to 'D_S' and served as a control for the level of non-specific binding of antibodies to the blocked membrane.

The 'predictable range of the detection method' was accommodated to ensure that the signals from the recombinant proteins and saliva proteins were falling within the range of signal intensity that changed in a predictable way with concentration.

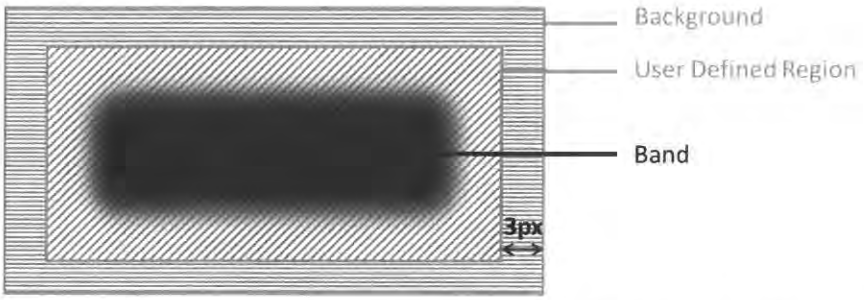

Figure 1a: Schematic representation of a 'UDR' (diagonal stripes), the 'Background' which is a perimeter 3 pixels wide surrounding the 'UDR' (horizontal stripes) and a 'Band' within the 'UDR'. 3px abbreviation denotes the three pixel perimeter.

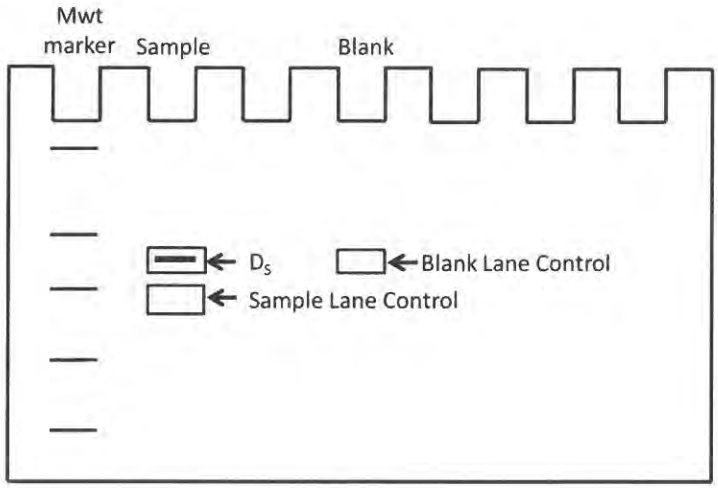

Figure 1b: Diagram of a gel showing how a user defined the UDR's which will give the Total Densities for the Band (D_S), a Sample Lane Control (D_{SLC}) and the Blank Lane Control (D_{BLC}).

9.6 Determination of Endogenous Aegyptin Signals in OX513A and WT Saliva

Two replicate gels (4-15% Mini-PROTEAN® TGX) were run each containing 2-fold serial volumes of both OX513A saliva and WT saliva (8.0, 4.0, 2.0 and 1.0 µl) and a positive control of rAegyptin (2.5 ng), each in 1x Laemmli buffer (diluted in PBS from 4X Laemmli buffer) with 5.5 % β-mercaptoethanol and made up to 12 µl in PBS. All gels were separated at 200V in 1X Tris/Glycine/SDS buffer for approximately 30 minutes. On each gel Molecular weight Marker (Mwt Marker) was loaded onto the outside wells and blank lanes were loaded with PBS in 1X Laemmli Sample Buffer (Table 1). The gel loading plan differed from that detailed in SP_00002[10], see section 12 for deviation (DEV01) details.

Gels were blotted onto nitrocellulose membrane. All gels were transferred using the TransBlot Turbo transfer system using the '1 MiniTGX mixed MW' setting. Blots were probed using polyclonal Rabbit Anti-Aegyptin as the

primary antibody at a 1/2,500 dilution in 10ml TBS-T. All antibody stains were performed at room temperature, shaking, for 1 hour. Polyclonal Goat Anti-Rabbit IgG (whole molecule)-Horse Radish Peroxidase Antibody (HRP) and StrepTactin-HRP (a component of the Precision Plus Protein™ WesternC™ Pack) were used as a secondary detection markers at a 1/30,000 and 1/10,000 dilution in 15 ml TBS-T respectively. HRP-conjugated secondary detection markers were detected using Clarity™ Western ECL Substrate according to the manufacturer's instructions, using 2 ml of substrate per membrane. Following incubation with the primary antibody, all blots were washed 4 times, for 5 minutes at room temperature, in TBS-T. Following incubation with the secondary markers, all blots were washed 3 times, for 5 minutes at room temperature, in TBS-T followed by a 10 minute wash under the same conditions Blots were digitally captured as a 16-bit greyscale image using ChemiDoc-IT 500 Imaging System (UVP) to assess the intensity of signals in the different lanes using VisionWorks LS Analysis Software (UVP). All blots were imaged in increasing exposures, starting at 10 seconds and doubling with each capture, until the image was over exposed (i.e. contained pixels with an intensity of 65,536 - see LOD definition in section 13). The longest exposure, which was not overexposed, was then used for analysis.

A standard curve was plotted for the D_S values of the endogenous Aegyptin signal in both OX513A and WT saliva for each blot to determine equivalence of the total protein levels of the two saliva samples.

Table 1: Gel loading plan for determination of endogenous Aegyptin in OX513A and WT saliva

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Mwt Marker/µl	5.0														5.0
rAegyptin/ng								2.5							
WT Saliva/µl			1.0	2.0	4.0	8.0									
OX513A Saliva/µl										1.0	2.0	4.0	8.0		

Lanes 2, 7, 9 and 14 are blank lanes and loaded with PBS in 1X Laemmli Sample Buffer

9.7 Determination of Limit of Detection (LOD) for rtTAV and Detection of tTAV in OX513A Saliva

Two replicate gels were run containing 5 µl of OX513A saliva and 5 µl of WT saliva and the following amounts of rtTAV; 12.5, 6.3, 3.1, 1.6, 0.8 and 0.4 ng (this range was determined empirically in range-finding studies prior to this study. rtTAV was quantitated using the Bicinchoninic acid assay (BCA) assay in SR_00001[6]) mixed with 5 µl of WT saliva with 1x Laemmli buffer with 5.5 % β-mercaptoethanol and made up to 12 µl in PBS. On each gel Mwt. Marker was loaded in the outside wells and blank lanes were loaded with PBS in 1X Laemmli Sample Buffer (Table 2). Gels were blotted onto nitrocellulose membrane. Blots were probed using polyclonal anti-VP16 tag antibody as the primary antibody at a dilution of 1/500 in 10 ml TBS-T, and the same secondary markers and substrate described in section 9.6.

To ensure equivalence of the saliva samples loaded, the membrane was then stripped of all bound antibodies using Restore™ Western Blot Stripping Buffer. Membranes were incubated with 20 ml stripping buffer at room temperature, shaking, for 15 minutes. Membranes were then re-probed using the anti-Aegyptin antibody as described in section 9.6.

Table 2: Gel loading plan for determination of LOD of rtTAV and detection of tTAV in OX513A saliva

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Mwt Marker/µl	5.0														5.0
rtTAV/ng			12.5		6.3	3.1	1.6	0.8	0.4						
WT Saliva/µl			5.0		5.0	5.0	5.0	5.0	5.0		5.0				
OX513A Saliva/µl													5.0		

Lanes 2, 4, 10, 12 and 14 are Blank Lanes and are loaded with PBS in 1X Laemmli Sample Buffer

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

9.8 Determination of Limit of Detection (LOD) for rDsRed2 and Detection of DsRed2 in OX513A Saliva

Two replicate gels were run containing 5 µl of OX513A saliva and 5 µl of WT saliva and the following amounts of rDsRed2; 40.0, 20.0, 10.0, 5.0, 2.5 and 1.3 ng (this range determined empirically in range-finding studies prior to this study) mixed with 5µl of WT saliva with 1x Laemmli buffer with 5.5 % β-mercaptoethanol and made up to 12 µl in PBS. On each gel Mwt. Marker was loaded in the outside wells and blank lanes were loaded with PBS in 1X Laemmli Sample Buffer (Table 3). Immunoblotting was carried out using Living Colors® DsRed Polyclonal Antibody as a primary antibody at a 1/1,000 dilution in 10 ml TBS-T, and the same secondary markers and substrate described in section 9.6.

To ensure equivalence of the saliva samples loaded, the membrane was then stripped of all bound antibodies using Restore™ Western Blot Stripping Buffer (as described in section 9.7) and re-probed using the anti-Aegyptin antibody as described in section 9.6.

Table 3: Gel loading plan for determination of LOD of rDsRed2 and detection of DsRed2 in OX513A saliva

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Mwt Marker/µl	5.0														5.0
rDsRed2/ng			40.0		20.0	10.0	5.0	2.5	1.3						
WT Saliva/µl			5.0		5.0	5.0	5.0	5.0	5.0		5.0				
OX513A Saliva/µl													5.0		

Lanes 2, 4, 10, 12 and 14 are Blank Lanes and are loaded with PBS in 1X Laemmli Sample Buffer

10 RESULTS

10.1 Determination of Endogenous Aegyptin Signals in OX513A and WT Saliva

OX513A and WT saliva samples were analysed by western blot with an anti-Aegyptin antibody to assess the comparative levels of endogenous Aegyptin in the saliva pools. The outermost lanes in these figures appear bowed, as the lower part of the TGX gel expands prior to the transfer, therefore the lane numberings are aligned to the lane boundaries close to the signals of interest on each blot.

Visually, the signals increased in intensity in a linear fashion with increasing saliva volume for both saliva types, and the strengths of the endogenous Aegyptin signals are equivalent (Figures 2A and 3A). The lower-molecular weight (MW) band of the endogenous Aegyptin signal (the dominant band) was further analysed by densitometry, and the D_S values plotted against saliva volume (Figures 2B and 3B). This analysis also revealed a linear relationship between D_S and saliva volume for both OX513A and WT saliva, both best fit lines are co-linear, with R^2 values of between 0.96 and 0.99, suggesting that the signals from the endogenous Aegyptin from these blots fall within the range of signal intensity that change in a predictable way with amount of protein loaded. Although not identical, the curves were considered similar by the operator, considering the typical intra-blot variations observed with western blots of this nature in range finding studies.

The rAegyptin protein displayed a distinct migration pattern compared to secreted endogenous Aegyptin found in the saliva. rAegyptin migrated slower than the secreted endogenous Aegyptin protein, and produced a single band at approximately 37 kDa, whereas two bands of similar MW (approximately 27 and 30 kDa) were detected by the anti-Aegyptin antibody in the saliva samples. One of the two bands may be a result of cross reactivity of the anti-Aegyptin antibody with another saliva protein, or more likely, differentially processed forms of Aegyptin. The observation that the rAegyptin construct migrates slower than the endogenous Aegyptin is likely due to differences in either protein folding or post-translational modifications, as a result of protein processing pathways in the mammalian 293-F cell expression system in which the rAegyptin construct was expressed[3], compared to the secretory system of expression in the saliva glands of *Ae. aegypti*. Specifically, the difference in

molecular weight between the recombinant Aegyptin and endogenous Aegyptin, is most likely explained as differences in the secretion pathway for Aegyptin, as in a previous study, intracellular (non-secreted) Aegyptin from the excised salivary glands of *Ae. aegypti* appears as a single band on western blot, and migrates at approximately 37kDa, similar to the recombinant construct[3].

Collectively, these data suggest that the endogenous Aegyptin levels (and therefore total protein levels) are equivalent in both OX513A and WT saliva, and therefore equal volumes (5 µl) of OX513A and WT saliva were loaded on the gels for the detection of tTAV and DsRed2 in OX513A saliva in sections 9.7 and 9.8.

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

Figure 2

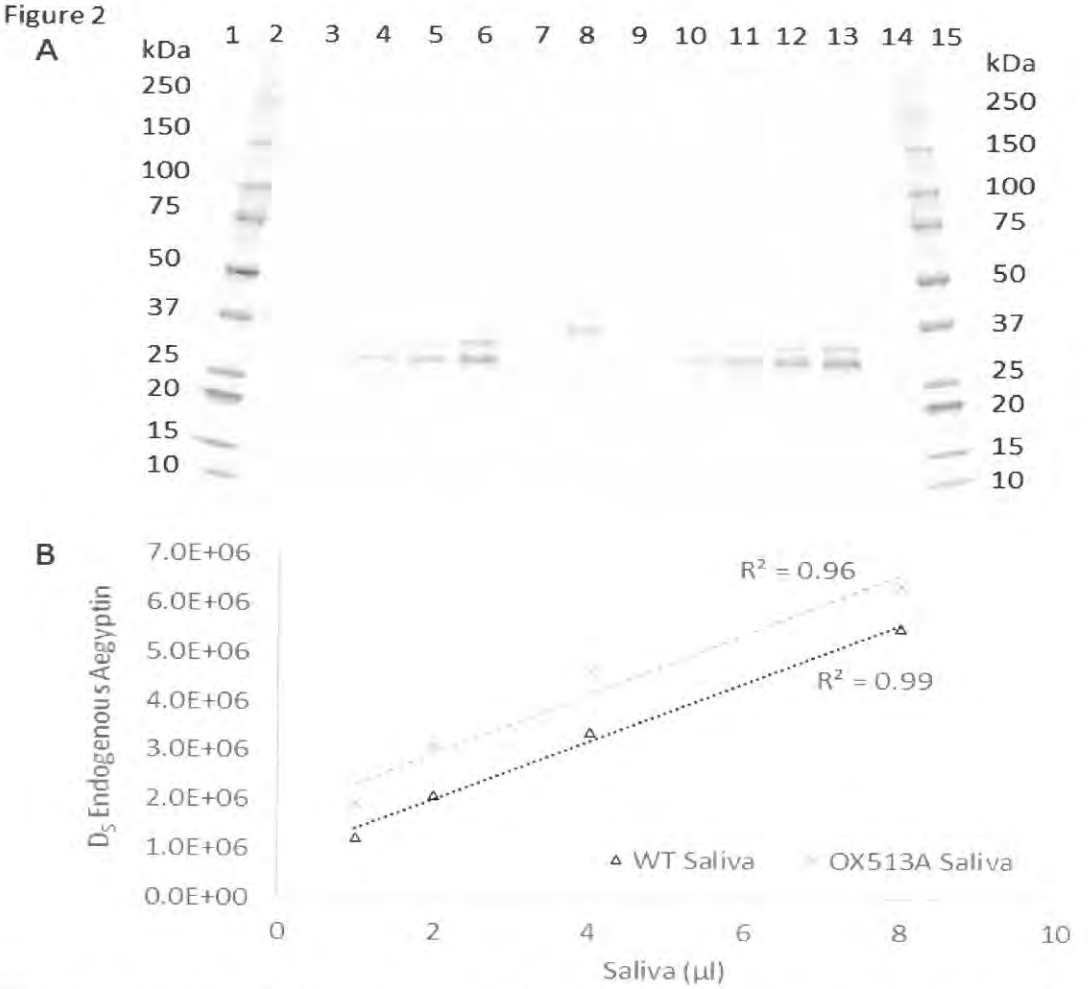

Figure 2: Western blot analysis demonstrating equivalence of endogenous Aegyptin signals in OX513A and WT Saliva

The endogenous Aegyptin in OX513A and WT saliva samples was analysed by western blot. (A) 2-fold serial dilutions of OX513A and WT saliva and 2.5ng of rAegyptin were separated on replicate gels by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with a rabbit anti-Aegyptin antibody to detect the presence of Aegyptin protein. (B) D_s values for the lower-MW band were calculated using VisionWorks LS Acquisition and Analysis Software (UVP). A linear trend line was plotted, and R^2 values calculated, using Microsoft Excel. Δ represents WT Saliva, X represents OX513A saliva. Lane designations are as follows (Blank lanes contain PBS in 1X Laemmli buffer):

1: 5µl Mwt marker
2: Blank lane
3: 1µl WT Saliva
4: 2µl WT Saliva
5: 4µl WT Saliva
6: 8µl WT Saliva
7: Blank lane
8: 2.5ng rAegyptin
9: Blank lane
10: 1µl OX513A Saliva
11: 2µl OX513A Saliva
12: 4µl OX513A Saliva
13: 8µl OX513A Saliva
14: Blank lane
15: 5µl Mwt Marker

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

Figure 3

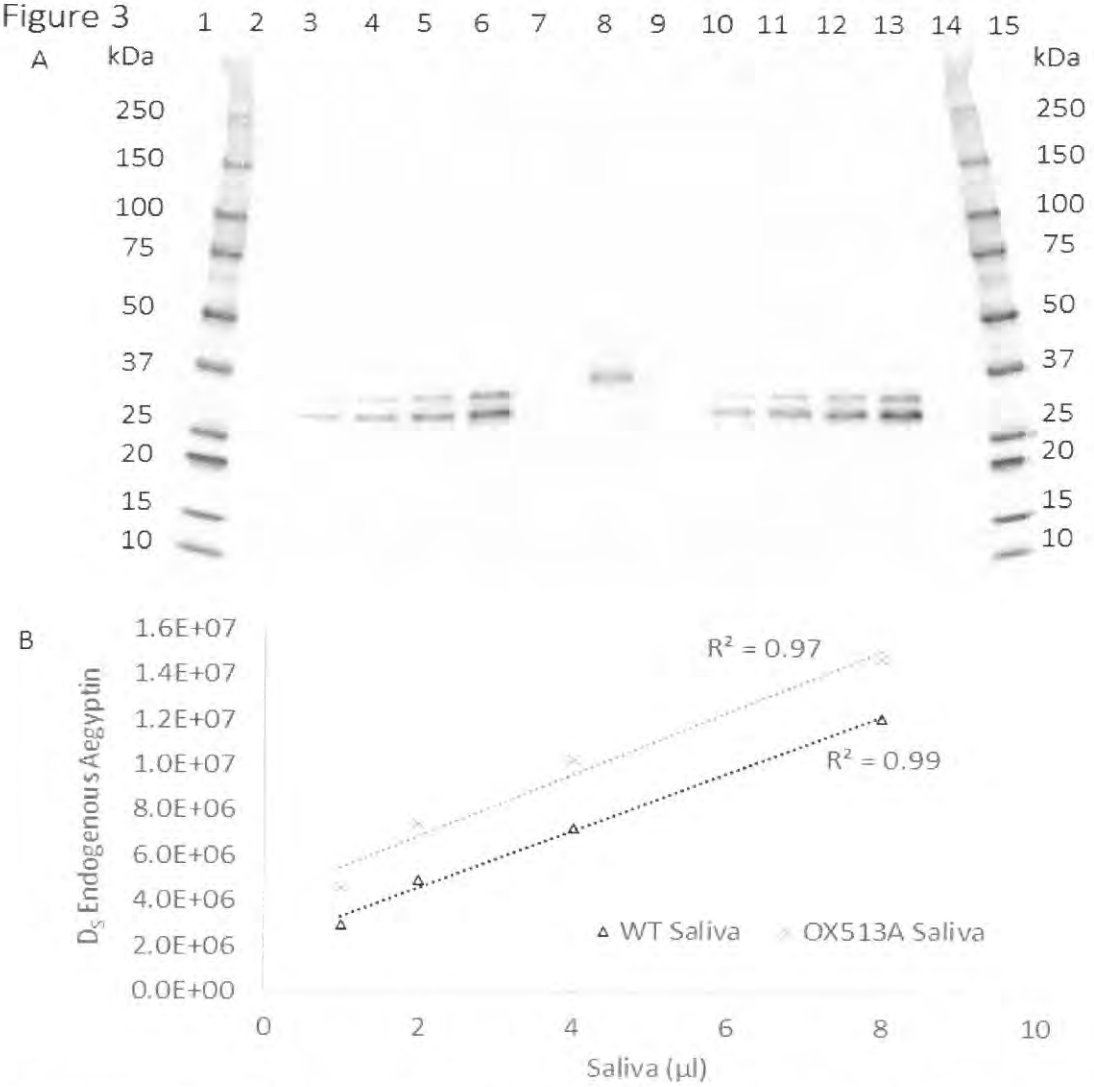

Figure 3: Western blot analysis demonstrating equivalence of endogenous Aegyptin signals in OX513A and WT Saliva (Replicate)

The endogenous Aegyptin in OX513A and WT saliva samples was analysed by western blot. (A) 2-fold serial dilutions of OX513A and WT saliva and 2.5ng of rAegyptin were separated on replicate gels by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with a rabbit anti-Aegyptin antibody to detect the presence of Aegyptin protein. (B) D_s values for the lower-MW band were calculated using VisionWorks LS Acquisition and Analysis Software (UVP). A linear trend line was plotted, and R^2 values calculated, using Microsoft Excel. Δ represents WT Saliva, X represents OX513A saliva. Lane designations are as follows (Blank lanes contain PBS in 1X Laemmli buffer):

1: 5µl Mwt marker
2: Blank lane
3: 1µl WT Saliva
4: 2µl WT Saliva
5: 4µl WT Saliva
6: 8µl WT Saliva
7: Blank lane
8: 2.5ng rAegyptin
9: Blank lane
10: 1µl OX513A Saliva
11: 2µl OX513A Saliva
12: 4µl OX513A Saliva
13: 8µl OX513A Saliva
14: Blank lane
15: 5µl Mwt Marker

10.2 LOD for Recombinant tTAV and Recombinant DsRed2 and Detection of tTAV and DsRed2 in OX513A Saliva

LODs for rtTAV and rDsRed2 were determined by western blot analysis where recombinant proteins were loaded with 5µl of WT saliva as the background matrix to be consistent with the test material (OX513A saliva) and control material (WT Saliva). rtTAV has a predicted MW of 39.9kDa and migrates at approximately 46-50kDa when separated by SDS-PAGE, likely due to the helical nature of this protein. This is discussed in more detail in SR_00003[6].

The western blots shown in figure 6A and 7A show rDsRed2 supplied by Clontech gives two bands when analysed by western blot (Figures 6A and 7A). The dominant band, representing monomeric DsRed2, migrates at approximately 30kDa, slightly slower than it's predicted MW (25.7kDa) would suggest. This migration pattern is also documented in the manufacturer's Certificate of Analysis for this protein [ref: http://www.clontech.com/xxclt_ibcGetAttachment.jsp?cItemId=27589]. A higher MW band is also apparent, migrating at approximately 60kDa, approximately twice that of the monomer. DsRed2 natively forms homodimeric and homotetrameric complexes[11], and so this band likely represents dimeric DsRed2. Although samples are denatured prior to SDS-PAGE analysis, complete disruption of all subunit interactions of multimeric complexes is not always achieved. For this study the dominant monomeric band was used for all subsequent analysis.

According to the acceptance criteria detailed in section 7, both replicate blots had the same LOD for rtTAV, 0.8 ng (Figures 4A and 5A). The replicate blots for rDsRed2 had different LODs this protein, 5.0 ng and 2.5 ng for each blot (Figures 6A and 7A). The endogenous Aegyptin signal visually appears to slightly increase in strength from left to right across the blot. This pattern is not apparent in any of the other blots, and is likely due to typical intra-blot variations observed with western blots of this nature, as seen in range finding studies.

D_S values were determined for the signals from the recombinant proteins, and when the D_S values for 'visible' bands are plotted against protein amount loaded reveal a linear relationship, with R^2 values between of 0.96 and 1.00 (Figure 8), suggesting that the signals from the recombinant proteins from these blots fall within the range of signal intensity that change in a predictable way with amount of protein loaded.

Specific bands representing antibody binding to DsRed2 and tTAV proteins, equivalent in size to their recombinant counterparts or otherwise, were not detected in OX513A saliva in either of the replicate blots. The rabbit anti-VP16 tag polyclonal antibody cross-reacts with a 30 kDa protein in WT and OX513A saliva, producing a very slight, but consistent, signal which is difficult to visualise on the western blot images (Fig 4A and Fig 5A). As this protein is also present at a consistent level in WT saliva, it does not represent anti-VP16 antibody binding to tTAV. This cross-reactivity is likely caused by shared epitope between VP16 and an endogenous saliva protein which is being recognised by the polyclonal anti-VP16 antibody. Polyclonal antibodies are by definition a mixture of more than one antibody molecule so multiple epitopes will be recognised by the anti-VP16 polyclonal antibody. The Living Colors®DsRed rabbit polyclonal antibody does not appear to cross react with any saliva proteins.

To ensure equivalence of saliva samples loaded, membranes were stripped of all antibodies using stripping buffer, and re-probed with rabbit anti-Aegyptin polyclonal antiserum to detect endogenous Aegyptin protein in the saliva samples (Figures 4B, 5B, 6B and 7B). Although stripping of the anti-VP16 and anti-DsRed2 antibodies was not complete and faint bands remained, the stripping was sufficient so that these remaining signals did not interfere with the endogenous Aegyptin signal. This analysis revealed that the endogenous Aegyptin signals were visually equivalent across each blot, and therefore demonstrates that equivalent volumes of saliva were loaded in each sample lane.

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

Figure 4

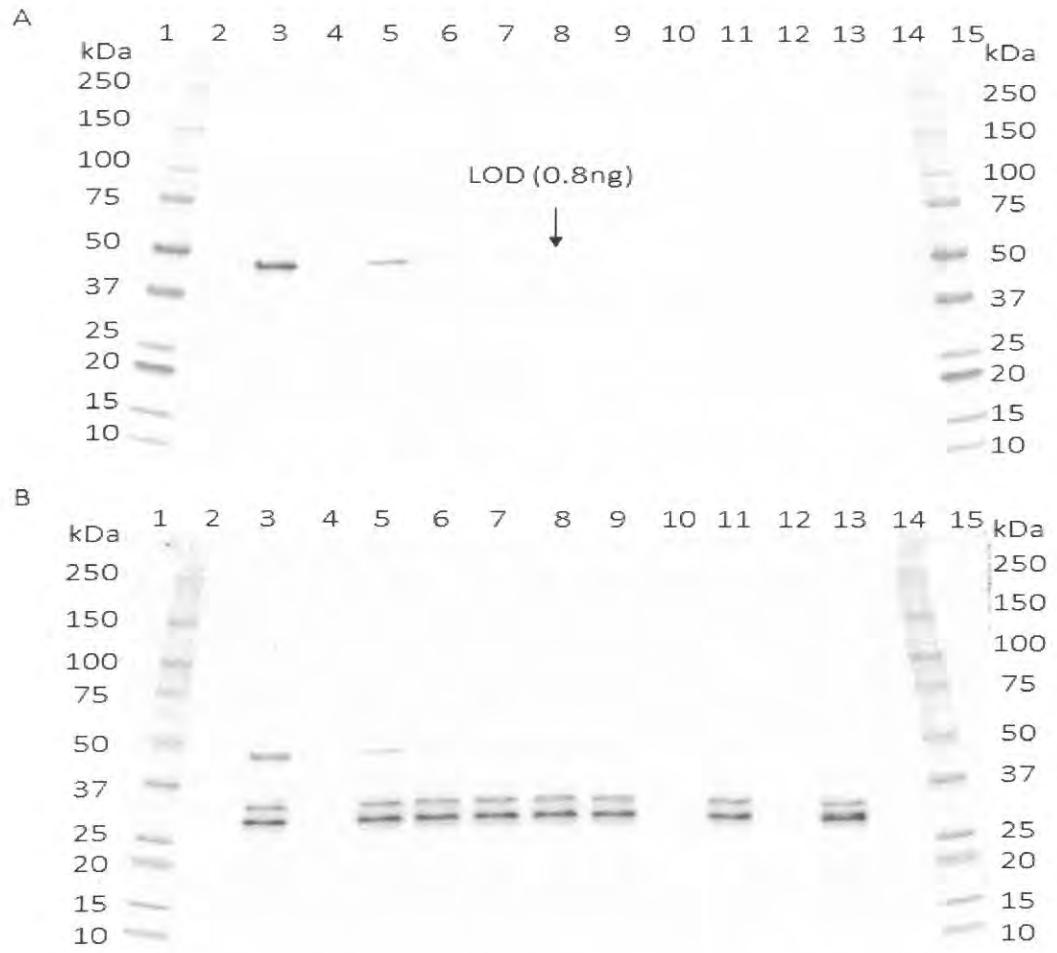

Figure 4: tTAV protein cannot be detected in OX513A saliva using western blot analysis

A 2-fold serial dilution series of rtTAV (mixed with 5µl WT saliva), 5µl of WT saliva and 5µl of OX513A saliva was separated by SDS-PAGE and transferred to a nitrocellulose membrane. (A) The membrane was initially probed with the rabbit anti-VP16 tag polyclonal antibody to detect tTAV protein. (B) The membrane was then stripped of all antibodies and re-probed with rabbit anti-Aegyptin polyclonal antiserum to detect the presence of endogenous Aegyptin protein in the saliva. Lane designations are as follows (Blank lanes contain PBS in 1X Laemmli buffer):

1:	5 µl Mwt marker
2:	Blank lane
3:	12.5ng rtTAV + 5µl WT Saliva
4:	Blank lane
5:	6.2ng rtTAV + 5µl WT Saliva
6:	3.1ng rtTAV + 5µl WT Saliva
7:	1.6ng rtTAV + 5µl WT Saliva
8:	0.8ng rtTAV + 5µl WT Saliva
9:	0.4ng rtTAV + 5µl WT Saliva
10:	Blank lane
11:	5µl WT Saliva
12:	Blank lane
13:	5µl OX513A Saliva
14:	Blank lane
15:	5 µl Mwt Marker

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

Figure 5

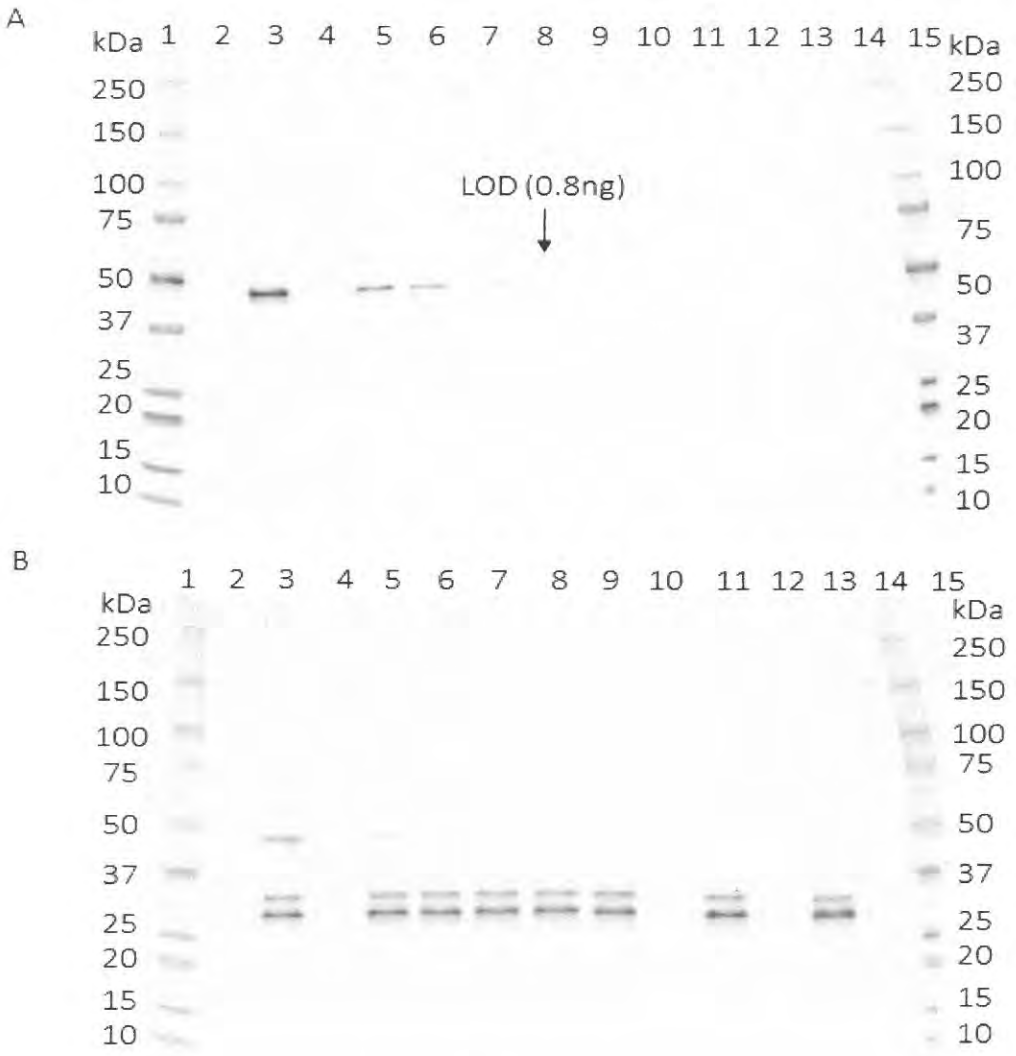

Figure 5: tTAV protein cannot be detected in OX513A saliva using western blot analysis (Replicate)

A 2-fold serial dilution series of rtTAV (mixed with 5µl WT saliva), 5µl of WT saliva and 5µl of OX513A saliva was separated by SDS-PAGE and transferred to a nitrocellulose membrane. (A) The membrane was initially probed with the rabbit anti-VP16 tag polyclonal antibody to detect tTAV protein. (B) The membrane was then stripped of all antibodies and re-probed with rabbit anti-Aegyptin polyclonal antiserum to detect the presence of endogenous Aegyptin protein in the saliva.

1: 5 µl Mwt marker
2: Blank lane
3: 12.5ng rtTAV + 5µl WT Saliva
4: Blank lane
5: 6.2ng rtTAV + 5µl WT Saliva
6: 3.1ng rtTAV + 5µl WT Saliva
7: 1.6ng rtTAV + 5µl WT Saliva
8: 0.8ng rtTAV + 5µl WT Saliva
9: 0.4ng rtTAV + 5µl WT Saliva
10: Blank lane
11: 5µl WT Saliva
12: Blank lane
13: 5µl OX513A Saliva
14: Blank lane
15: 5 µl Mwt Marker

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

Figure 6

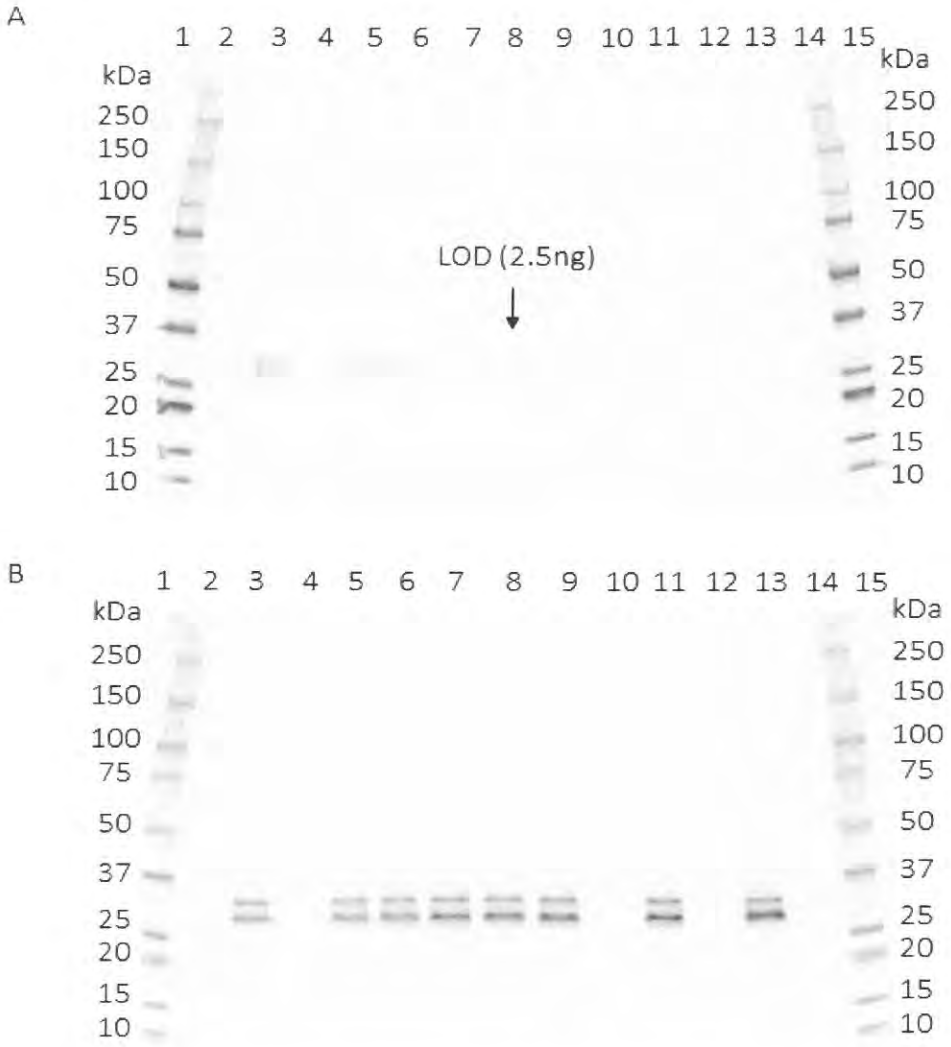

Figure 6: DsRed2 protein cannot be detected in OX513A saliva using western blot analysis

A 2-fold serial dilution series of rDsRed2 (mixed with 5µl WT saliva), 5µl of WT saliva and 5µl of OX513A saliva was separated by SDS-PAGE and transferred to a nitrocellulose membrane. (A) The membrane was initially probed with the Living Colors® DsRed rabbit polyclonal antibody to detect DsRed2 protein. (B) The membrane was then stripped of all antibodies and re-probed with rabbit anti-Aegyptin polyclonal antiserum to detect the presence of endogenous Aegyptin protein in the saliva. Lane designations are as follows (Blank lanes contain PBS in 1X Laemmli buffer):

1: 5 µl Mwt marker
2: Blank lane
3: 12.5ng rtTAV + 5µl WT Saliva
4: Blank lane
5: 6.2ng rtTAV + 5µl WT Saliva
6: 3.1ng rtTAV + 5µl WT Saliva
7: 1.6ng rtTAV + 5µl WT Saliva
8: 0.8ng rtTAV + 5µl WT Saliva
9: 0.4ng rtTAV + 5µl WT Saliva
10: Blank lane
11: 5µl WT Saliva
12: Blank lane
13: 5µl OX513A Saliva
14: Blank lane
15: 5 µl Mwt Marker

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

Figure 7

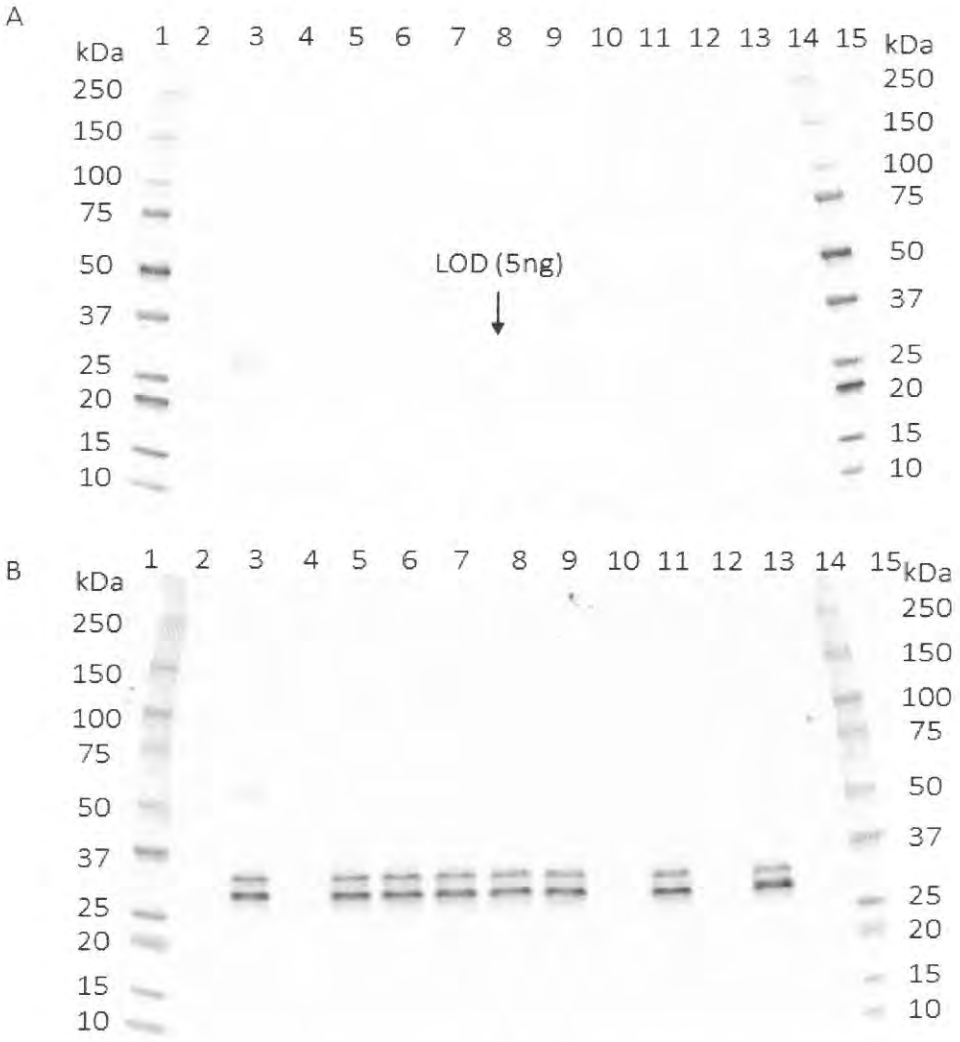

Figure 7: DsRed2 protein cannot be detected in OX513A saliva using western blot analysis (Replicate)

A 2-fold serial dilution series of rDsRed2 (mixed with 5µl WT saliva), 5µl of WT saliva and 5µl of OX513A saliva was separated by SDS-PAGE and transferred to a nitrocellulose membrane. (A) The membrane was initially probed with the Living Colors® DsRed rabbit polyclonal antibody to detect DsRed2 protein. (B) The membrane was then stripped of all antibodies and re-probed with rabbit anti-Aegyptin polyclonal antiserum to detect the presence of endogenous Aegyptin protein in the saliva. Lane designations are as follows (Blank lanes contain PBS in 1X Laemmli buffer):

1:	5 µl Mwt marker
2:	Blank lane
3:	12.5ng rtTAV + 5µl WT Saliva
4:	Blank lane
5:	6.2ng rtTAV + 5µl WT Saliva
6:	3.1ng rtTAV + 5µl WT Saliva
7:	1.6ng rtTAV + 5µl WT Saliva
8:	0.8ng rtTAV + 5µl WT Saliva
9:	0.4ng rtTAV + 5µl WT Saliva
10:	Blank lane
11:	5µl WT Saliva
12:	Blank lane
13:	5µl OX513A Saliva
14:	Blank lane
15:	5 µl Mwt Marker

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

Figure 8

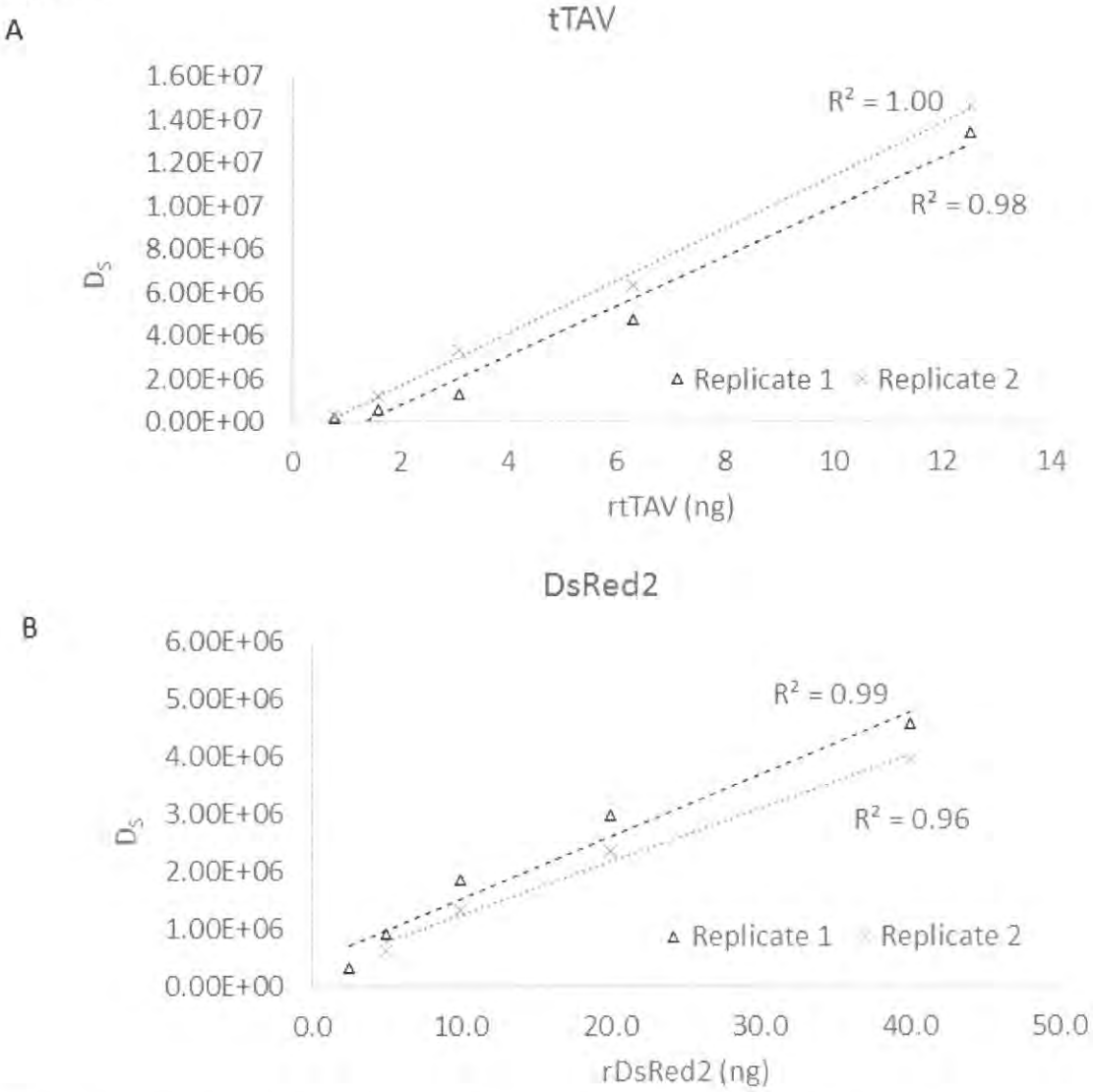

Figure 8: rtTAV and rDsRed2 signals fall within the predictable, linear range for the detection method
D_S values for rtTAV and rDsRed signals from replicate blots for the detection of (A) tTAV and (B) DsRed2 proteins in OX513A saliva were calculated using VisionWorks LS Acquisition and Analysis Software (UVP) and plotted against amount of protein loaded (ng). A linear trend line was plotted, and R^2 values calculated, using Microsoft Excel. Δ represents WT Saliva, X represents OX513A saliva.

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

11 REFERENCE TO LOCATION OF RAW DATA

Raw data for this study will be stored for 5 years and can be found in the following locations;

- Experimental write-up, deviation details and print outs; Laboratory Notebook OX230 pages 30-41
- Raw 16-bit grey-scale images (TIFF files); Oxitec server location S:\G. Regulatory\1.0 USA\FDA\saliva study 2015\Raw data

12 DEVIATIONS

DEV01	Saliva volumes in section 9.6 were incorrectly loaded in ascending order (left to right) instead of descending order as detailed in SP_00002[10]. No action required as this deviation has no impact on the results or analysis.
DEV02	Saliva samples were frozen at ≤ -15°C prior to pooling. Saliva samples were thawed at room temperature and pooled. Protocol SP00002 stated that the saliva samples were to be pooled prior to freezing. This does not affect the analysis or results and so no further action was required.

13 DISCUSSION AND CONCLUSIONS

The data presented in this report shows that tTAV and DsRed2 proteins are not detectable in the saliva of OX513A females by western blot analysis where the limits of detection were determined as 0.8ng for rtTAV and 5.0 - 2.5ng for rDsRed2 in WT saliva. 5 µl of saliva was analysed which equates to the quantity of saliva collected from approximately 5.5 female adult mosquitoes based on this study (270 µl of pooled saliva collected from approximately 300 *Aedes aegypti* adult females homozygous for OX513A rDNA construct).

The equivalence of Aegyptin signal between OX513A and WT saliva was confirmed both visually and by densitometry. Given the subjective nature of the analysis performed, it could be argued that the endogenous Aegyptin signal is marginally stronger in the OX513A saliva compared to WT. If this was the case, OX513A saliva was the test material, and so if there were higher levels of total protein in these samples, it would make it more likely that tTAV and DsRed2 could be detected relative to the WT negative control, and therefore would be unlikely to impact the analysis performed, or the conclusions, drawn from this study.

14 LIST OF ACRONYMS, ABBREVIATIONS AND TECHNICAL TERMS (ALPHABETICAL)

Area	The total number of pixels within the user-defined region 'UDR'.
Background	The area surrounding the perimeter of the user defined-region 'UDR', three pixels wide.
Band	A region of antibody staining clearly visible and discernible by the human eye.
BCA	Bicinchoninic acid.
Blank lane	A lane loaded with PBS in 1X Laemmli loading buffer + 5.5% β-Mercaptoethanol and no protein sample.
D_{BLC}	Density (Blank Lane Control) defined as 'Total Density' for an area of equal size to the 'UDR', in a similar position, in a nearby blank lane representing non-specific binding of antibody to an area where no protein is present.
D_S	Density (Specific) defined as 'Total Density' for the 'UDR' containing a 'band' representing specific antibody binding to the protein of interest
D_{SLC}	Density (Sample Lane Control) defined as 'Total Density' of a region, for an area of equal size to the 'UDR' and situated immediately above or below a 'band' representing antibody

	binding to an area where proteins are likely to be present other than the specific protein of interest.
DsRed2	Fluorescent marker gene from *Discosoma* species.
ECL	Enhanced Chemiluminescense.
kDa	KiloDalton.
LOD	Limit of detection: the lowest quantity of a protein that an operator can visibly discern on a developed western blot image and create a 'UDR' that meets the acceptance criteria such that D_{SLC} is less than 10% of D_S. For the image analysis to be valid, all pixels on the complete western blot image must fall within the dynamic range of the ChemiDoc-IT 500 Imaging System (UVP). This is a pixel intensity within 0 – 65,536 for a 16-bit grey scale image. If this is not the case this image is rejected and an image of a lower exposure time is selected.
MW	Molecular Weight
Mwt Marker	Molecular weight marker.
OX513A saliva	Pooled saliva from homozygous OX513A females.
PBS	Phosphate-buffered saline.
Pixel Intensity	An integer between 0 and 65,536 (for a 16bit grey-scale image) representing the intensity for each pixel
Predictable range	The predictable range of the detection method was be determined by using a linear best fit equation in Microsoft Excel program.
rDsRed2	Recombinant DsRed2.
rtTAV	Recombinant tTAV.
TBS-T	Tris-buffered Saline Tween 20
Total Background	Sum of the pixel intensity values for all pixels within the 'Background'.
Total Density	'Total Raw Density' minus the 'Total Background'.
Total Raw Density	Sum of the pixel intensity values for all pixels within the 'UDR'.
tTAV	Tetracycline-controlled transactivator.
UDR	User defined-region: A rectangle drawn around an antibody specific 'band' or equivalent area by the user, large enough to enclose the entire 'band' or equivalent area.
VP16	*Herpes simplex* Protein 16.
WT saliva	Pooled saliva from wild-type *Aedes aegypti* females.

15 REFERENCES

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16 APPROVALS

Approval below indicates agreement with information presented in this study report. Raw data has been checked against summary information presented within this report.

Name	Position	Signature	Date Signed
	Senior Scientist, Study Co-ordinator		5 Aug 2015
	Senior Quality Systems Manager, Report Author		05 AUG 2015
	Head of Regulatory Affairs, Study Sponsor		5 Aug 2015
	Chief Scientific Officer		5 Aug 2015

OX513A *Aedes aegypti*: Suppression Project
Pedra Branca neighbourhood, Jacobina (Bahia), Brazil

Introduction

OX513A *Aedes aegypti* is a revolutionary and environmentally friendly new tool that brings an unprecedented ability to suppress infestations of the wild *Aedes aegypti* mosquito, the main vector of dengue and chikungunya. In four separate trials, regular releases of OX513A *Aedes aegypti* have resulted in a substantial and sustained reduction in the wild *Aedes aegypti* population, of over 90% as measured by the number of mosquito eggs recovered from ovitraps. Moreover theoretical models indicate that the control achieved is sufficient to prevent epidemics.

Since June 2013 Moscamed has been conducting a project using the strain OX513A in Jacobina, Bahia. The project started in the neighbourhood of Pedra Branca in the northwest of the city (Figure 1).

Pedra Branca, Brazil	
Start date	July 2013
Residents	1,144
Area	23 Ha
Suppression	92%

Location and project

Pedra Branca is a densely populated urban neighbourhood with a semi-arid climate and limited seasonality of *Aedes aegypti*. Populations of the mosquito are present all year round although lower during the dry season (May to October; 23°C) and higher during the rainy season (November to April; 26°C). The site was identified by local vector control agency as a problem hotspot with consistently high vector population and dengue incidence. The rest of Jacobina served as a control area, except the neighbourhood closest to Pedra Branca (Figure 1)..

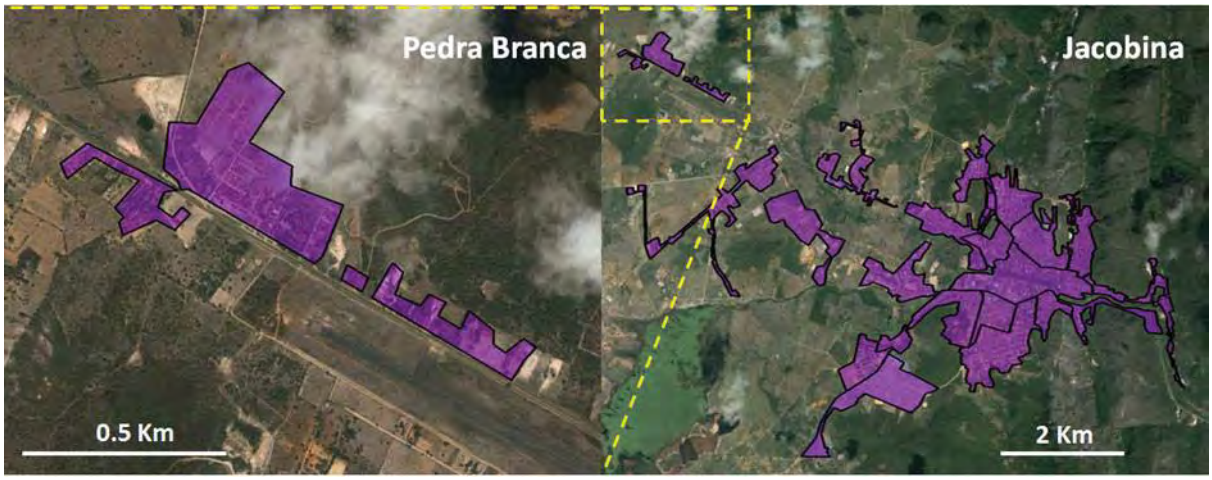

Figure 1. *Aerial photograph of Jacobina with an enlargement of the OX513A treated area, Pedra Branca. Populated areas are shaded.*

The objective of the project was to achieve substantial suppression of the local *Aedes aegypti* population and subsequently maintain that control during the expansion of the project to the other neighbourhoods of the city.

Results achieved

Throughout the study changes in release rate preceded corresponding changes in the percentage of progeny obtained from ovitraps that carried the fluorescent marker (Figure 2A). This value is indicative of the proportion of local females mating OX513A males (mating fraction), a factor that ultimately guides the rate of population suppression.

The release of sufficient numbers of OX513A to target suppression of the local *Aedes aegypti* population began on 14th July 2013. A substantial reduction of this local population occured in the following six months (Figure 2). The local population was then maintained at this low level despite the fact that the plot was relatively small, and susceptible to immigration from adjacent untreated areas where *Aedes aegypti* populations remained high.

A number of different metrics were used to assess local *Aedes aegypti* populations, before and after suppression, allowing the percentage suppression to be ascertained. Ovitrap surveys were the principal monitoring tool deployed in both treated and untreated sites. Any change in the local population of the OX513A-treated area was quantified relative to the untreated area. This was done by dividing the values for the treated area by those of the untreated area to give relative mean number of eggs caught per trap and relative ovitrap index (Figure 2B).

Adult population sampling in areas receiving treatment provided a ratio of recaptured OX513A to local *Aedes aegypti* males, allowing the population density of adult local *Aedes aegypti* to be estimated. Pupae per person were calculated according to the method described in Focks *et al.*[1] with site-specific parameters for the temperature and human population density in the treated area. For all metrics a substantial suppression of local *Aedes aegypti* population was observed following treatment with OX513A males, with percentage reduction estimate ranging from 77%-95%.

Results						
Metric	Eggs/trap (treated area)	Relative eggs/trap (treated/control)	Ovitrap index (treated area)	Relative ovitrap index (treated/control)	Local *Aedes aegypti* (adults/ ha)	Local *Aedes aegypti* (pupae/person)
Before supressão	35.0	2.8	44%	1.50	N/A	N/A
After supressão	1.9	0.22	10%	0.31	23	0.05
Reduction	**95%**	**92%**	**77%**	**79%**	**N/A**	**N/A**

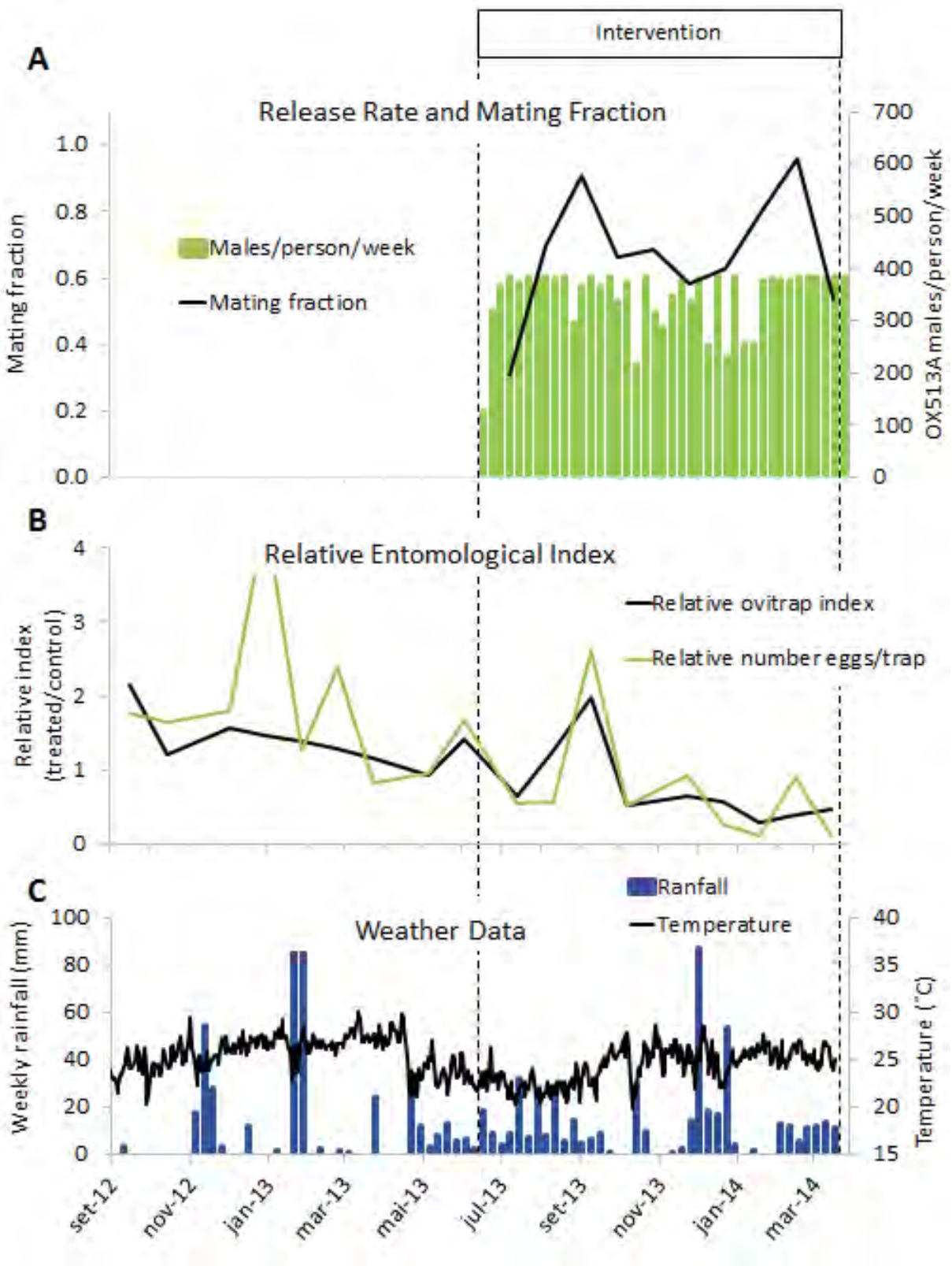

Figura 2. *(A) Number of OX513A males released per person per week and estimated mating fraction during the study. (B) Relative number of eggs per trap and ovitrap index. (C) Average daily temperature and weekly rainfall during the study.*

Commentary

A primary consideration for public health agencies is maintaining the vector population below the level required for sustained disease transmission. Disease transmission thresholds are dynamic and dependent upon multiple factors. These factors vary spatially and temporally making predictions for specific localities difficult. However, using the temperature dependent model proposed by *Focks et al*, a generic prediction for dengue transmission thresholds that are related to initial serotype prevalence (also termed herd immunity) is possible.

Despite the potential negative effects imposed by migration (as outlined below), during this demonstration study the local *Aedes aegypti* population was reduced to below the transmission threshold predicted for when there is no previous immunity in the population (seroprevalence rate of 0%). This demonstrates the strong potential for this technology to reduce dengue transmission rates, even in areas where the human population is totally susceptible to the disease.

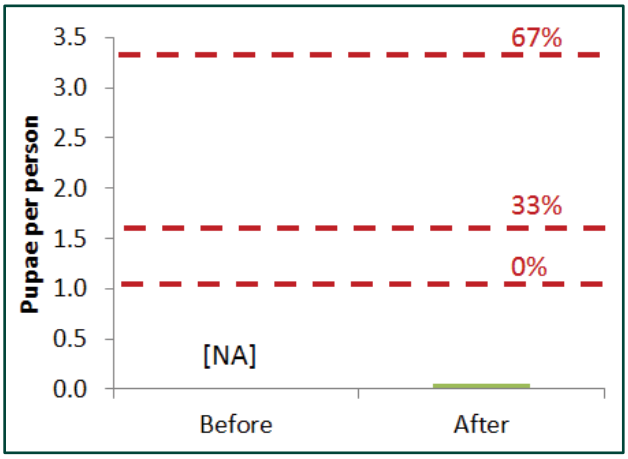

Figura 3. *Suppression of* Aedes aegypti *populaition in pupae per person relative to predicted dengue transmission thresholds at three initial seroprevalence rates of 0 (1.05 pupae per person), 33 (1.55), and 67% (3.41).*

The adult mosquito numbers before suppression were not recorded, due to an absence of adult traps. Nevertheless, ovitrap data indicates that local *Aedes aegypti* levels were 13 times higher before suppression by OX513A (based on relative numbers of eggs per trap).

After suppression, the estimated number of pupae per person was 0.05, which is well below the predicted transmission threshold for this area, regardless of the initial level.

The exceptional control of *Aedes aegypti* during this study show that, even in small areas with high migration, sustained releases of OX513A are highly effective.

Version.1.0, September 2014.

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List of Standard Operating Procedures (SOP)- OX513A

00031_01 OX513A Fluorescence Screening of Larvae

QD-SOP-00011 OX513A Quality Control Protocol for Assessment of Penetrance and Doxycycline Sensitivity

QD-SOP-00012 OX513A Quality Control Protocol for Assessment of Mating Competitiveness

QD-SOP-00013 OX513A Quality Control Protocol for Colony Genotyping

R_SOP_00001 Protocol for the detection of the OX513A transgene by polymerase chain reaction

00058_02 OX513A Identification of BG-Sentinel catches

HS-P-00057 Rearing Unit Emergency Procedures Policy

HS-SOP-00053 Cleaning and Waste Procedures

HS-SOP-00054 Room or Area Decontamination

HS-SOP-00059 Rearing Unit Entry and Exit

HS-SOP-00061 Rearing Unit Biosafety Procedures

R-SOP-00002 Package and Transport of OX513A Eggs

TD-SOP-00194 OX513A Egg Hatching - For Release

TD-SOP-00195 OX513A Calculation of Aliquot - For Release

TD-SOP-00284 OX513A Pre and Post Shipment Checks Procedure

TD-SOP-00291 OX513A Larval Rearing (Tray System)

TD-SOP-00292 OX513A Larvae Pupae Sorting - For Release

TD-SOP-00293 OX513A Sex Sorting of Pupae for Release

TD-SOP-00294 Male Eclosion in Release Device

TD-SOP-00295 OX513A Releases

TD-SOP-00299 OX513A Pupae Larvae Chemical Sorting

00013_02 OX513A Egg Receipt and Storage

00027_01 OX513A Identification of Trap Sites

00028_01 OX513A Placing and Collection of Ovitraps

00030_01 OX513A Hatching Larvae from Ovitrapped Eggs

00031_01 OX513A Fluorescence Screening of Larvae

00032_01 OX513A Species screening of ovitrapped larvae

00039_02 Chlortetracycline HCl Working Solution Preparation

00054_01 OX513A Hurricane Preparedness Policy

00057_01 OX513A Placing and Collection of BG-Sentinel traps

00031_01 OX513A Fluorescence Screening of Larvae

PURPOSE

To ensure that larvae hatched from field-trapped eggs are consistently and accurately sorted into OX513A larvae and Wild Type (WT) larvae.

SCOPE

Monitoring field-traps collected following release of *Aedes aegypti* OX513A.

QD-SOP-00011 OX513A Quality Control Protocol for Assessment of Penetrance and Doxycycline Sensitivity

PURPOSE

This procedure will serve as the standard method for assessing the mating competitiveness of the *Aedes aegypti* OX513A strain as a part of routine quality control testing.

It will be used to analyse the ability of OX513A homozygous males to compete for mates with a WT strains reared under the same conditions and of similar size.

The purpose of this procedure is, by comparing data from this assay to a baseline characterisation, to assess any changes in mating competitiveness of the OX513A strain that would warrant further investigation.

SCOPE

Quality control of *Aedes aegypti* OX513A strain throughout Oxitec's operations worldwide.

QD-SOP-00012 OX513A Quality Control Protocol for Assessment of Mating Competitiveness

PURPOSE

This procedure will serve as the standard method for assessing the mating competitiveness of the *Aedes aegypti* OX513A strain as a part of routine quality control testing.

It will be used to analyse the ability of OX513A homozygous males to compete for mates with a WT strains reared under the same conditions and of similar size.

The purpose of this procedure is, by comparing data from this assay to a baseline characterisation, to assess any changes in mating competitiveness of the OX513A strain that would warrant further investigation.

SCOPE

Quality control of *Aedes aegypti* OX513A strain throughout Oxitec's operations worldwide.

QD-SOP-00013 OX513A Quality Control Protocol for Colony Genotyping

PURPOSE

This procedure serves as the standard method for routine genotyping of Aedes aegypti OX513A strain to confirm the strain from mass rearing facility is homozygous for the OX513A allele and without wild-type alleles. If wild-type alleles are detected at the OX513A locus, this is a reportable event.

This procedure also serves as the standard method for genotyping of samples collected from traps in *Aedes aegypti* OX513A strain field release sites.

SCOPE

Quality control of *Aedes aegypti* OX513A strain throughout Oxitec's operations worldwide. Monitoring of OX513A control programmes.

R_SOP_00001 Protocol for the detection of the OX513A transgene by polymerase chain reaction

Purpose and scope:

To provide a protocol to detect the presence or absence of the OX513A transgene in a variety of OX513A insect samples (field, mass-rearing and laboratory). The same protocol can also be used to provide evidence of stability of the OX513A transgene over time. Successful amplification of the OX513A transgene over time provides evidence of its stability, as one primer anneals to the transgene, the other to the flanking genomic sequence, so mobilisation of the transgene results in a negative PCR.

00058_02 OX513A Identification of BG-Sentinel catches

PURPOSE

To ensure that BG-Sentinel catches species and sex are properly identified and counted.

SCOPE

Monitoring field-traps collected following release of *Aedes aegypti* OX513A.

HS-P-00057 Rearing Unit Emergency Procedures Policy

PURPOSE

To ensure that responses required during emergency situations are documented and followed.

SCOPE

Operations involved with *Aedes aegypti* OX513A strain in cabin/ rearing unit facilities worldwide.

HS-SOP-00053 Cleaning and Waste Procedures

PURPOSE

To ensure that cleaning is planned, recorded and effective. To ensure that waste is handled and treated correctly.

SCOPE

Operations at Oxitec Limited in the UK facility and Oxitec rearing facilities worldwide.

HS-SOP-00054 Room or Area Decontamination

PURPOSE

To ensure that rooms or areas in which live transgenic insects have been present are effectively decontaminated so as to present very low risk of environmental contamination.

SCOPE

Facilities used to rear Oxitec transgenic insects throughout Oxitec's operations worldwide.

HS-SOP-00059 Rearing Unit Entry and Exit

PURPOSE

To ensure that entry and exit into and from the mobile rearing unit is consistently performed to a set standard.

SCOPE

Rearing of Oxitec transgenic insects within mobile rearing units throughout Oxitec's operations worldwide.

HS-SOP-00061 Rearing Unit Biosafety Procedures

PURPOSE

To ensure that biosafety requirements are documented and maintained.

SCOPE

This procedure covers biosafety requirements to be established for operations involved with *Aedes aegypti* OX513A strain in cabin facilities worldwide. Any specific country requirements will be clarified within this procedure or as an appendix to this procedure.

R-SOP-00002 Package and Transport of OX513A Eggs

PURPOSE

To ensure that Aedes aegypti OX513A strain eggs produced in the UK Mass Rearing Unit are consistently packed, labelled and despatched in such a way as to ensure that:

- The environment is protected from accidental release.
- Local importation laws and restrictions are complied with.
- Viable eggs are delivered to the recipient.

SCOPE

Packaging and despatch of OX513A eggs from the UK Mass Rearing Unit to the Cayman facility.

TD-SOP-00194 OX513A Egg Hatching - For Release

PURPOSE

To ensure that Enumeration and Aliquoting of Larvae is consistently performed to a set standard.

SCOPE

Rearing of *Aedes aegypti* OX513A strain throughout Oxitec's operations worldwide.

TD-SOP-00195 OX513A Calculation of Aliquot - For Release

PURPOSE

To ensure that Enumeration and Aliquoting of Larvae is consistently performed to a set standard.

SCOPE

Rearing of *Aedes aegypti* OX513A strain throughout Oxitec's operations worldwide.

TD-SOP-00284 OX513A Pre and Post Shipment Checks Procedure

PURPOSE

To ensure that OX513A Larvae are subject to consistent conditions when reared.

SCOPE

Rearing of *Aedes aegypti* OX513A strain in the rearing unit

TD-SOP-00291 OX513A Larval Rearing (Tray System)

PURPOSE

To ensure that OX513A Larvae are subject to consistent conditions when reared.

SCOPE

Rearing of *Aedes aegypti* OX513A strain in the rearing unit

TD-SOP-00292 OX513A Larvae Pupae Sorting - For Release

PURPOSE

To ensure that separation of larvae from pupae is consistently performed to a set standard.

SCOPE

Rearing of *Aedes aegypti* OX513A strain for release.

TD-SOP-00293 OX513A Sex Sorting of Pupae for Release

PURPOSE

To ensure that sex sorting of pupae is consistently performed to a set standard.

SCOPE

Rearing of *Aedes aegypti* OX513A strain for release.

TD-SOP-00294 Male Eclosion in Release Device

PURPOSE

To ensure that sex-sorted pupae are eclosed and matured in Release Devices in such a way that they are suitable for release.

SCOPE

Rearing of *Aedes aegypti* OX513A strain throughout Oxitec's operations worldwide.

TD-SOP-00295 OX513A Releases

PURPOSE

To ensure that *Aedes aegypti* OX513A adult males are consistently transported to the release site in such a way as to ensure that the environment is protected from accidental release.

To ensure that the OX513A releases are performed to a set standard.

SCOPE

Transportation and release of *Aedes aegypti* OX513A strain adult males reared in Oxitec's operations.

TD-SOP-00299 OX513A Pupae Larvae Chemical Sorting

PURPOSE

To ensure that pupae are consistently separated from larvae to a set standard.

SCOPE

Rearing of *Aedes aegypti* OX513A strain throughout Oxitec's operations worldwide

00013_02 OX513A Egg Receipt and Storage

PURPOSE

To ensure that *Aedes aegypti* OX513A Egg deliveries are received, recorded and processed so as to ensure traceability and stored under the correct conditions so as to ensure consistent supply of high viability.

SCOPE

Rearing of *Aedes aegypti* OX513A strain for Release.

00027_01 OX513A Identification of Trap Sites

PURPOSE

To ensure that are placed in suitable sites in the field.

SCOPE

Monitoring wild population of *Aedes aegypti* following release of *Aedes aegypti* OX513A.

00028_01 OX513A Placing and Collection of Ovitraps

PURPOSE

To ensure that ovitraps are collected and replaced consistently.

SCOPE

Monitoring wild population of *Aedes aegypti* following release of *Aedes aegypti* OX513A.

00030_01 OX513A Hatching Larvae from Ovitrapped Eggs

PURPOSE

To ensure that eggs from ovitraps are consistently hatched to yield larvae suitable for screening.

SCOPE

Monitoring wild population of *Aedes aegypti* following release of *Aedes aegypti* OX513A.

00031_01 OX513A Fluorescence Screening of Larvae

PURPOSE

To ensure that larvae hatched from field-trapped eggs are consistently and accurately sorted into OX513A larvae and Wild Type (WT) larvae.

SCOPE

Monitoring field-traps collected following release of *Aedes aegypti* OX513A.

00032_01 OX513A Species screening of ovitrapped larvae

PURPOSE

To ensure that larvae hatched from field-trapped eggs are consistently and accurately sorted into OX513A larvae and Wild Type (WT) larvae.

SCOPE

Monitoring field-traps collected following release of *Aedes aegypti* OX513A.

00039_02 Chlortetracycline HCl Working Solution Preparation

PURPOSE

To ensure that the chlortetracycline stock solution is prepared accurately.

SCOPE

Rearing of *Aedes aegypti* OX513A strain throughout Oxitec's operations worldwide.

00054_01 OX513A Hurricane Preparedness Policy

PURPOSE

To ensure that a policy is established and maintained for biosafety in the event of a hurricane or tropical storm.

SCOPE

As an example policy for the type of policy which would be developed if a programme is undertaken in Saba, this document describes the safe handling practices and procedures for the use of OX513A *Aedes aegypti* in the laboratories at the FKMCD building in Marathon, FL for the duration of the investigational new animal drug test (INAD 012-109) under the FFDCA in the event of a hurricane or tropical storm. The HRU and ML are located within a Hurricane Category 4 rated building.

00057_01 OX513A Placing and Collection of BG-Sentinel traps

PURPOSE

To ensure that BG-Sentinels are collected and replaced consistently.

SCOPE

Monitoring wild population of *Aedes aegypti* following release of *Aedes aegypti* OX513A.

Appendices

Submission to the GMO Office of the National Institute of Public Health and the Environment of the Netherlands (RIVM) for the technical evaluation of the release of *Aedes aegypti* OX513A in Saba

Molecular characterisation and lineage of Aedes aegypti OX513A

Information submitted to The National Institute for Public Health and the Environment (RIVM) of the Netherlands on the Molecular Characterization of the rDNA construct and the lineage for a specific homozygous diploid line (OX513A) of *Aedes aegypti*, containing a single integrated copy of the OX513 rDNA construct, located at the OX513A site, directing the expression of an insect-optimised tetracycline-repressible transactivator protein (tTAV), and a red fluorescent protein (DsRed2) to aid in field monitoring. Appendix 1 contains specific details on the construction of the insert, molecular characterisation, table of genetic elements and phenotype of the strain.

Appendix 2

OX513A Part A - Bioinformatics tTAV-DsRed2-Sept 2013

Study Title

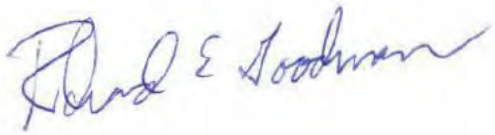
Bioinformatics analysis for risks of allergenicity and toxicity of proteins encoded by the two genes introduced into genetically engineered mosquitos (*Aedes aegypti*), strain OX513A for production of sterile males to reduce vector transmission of important human diseases

Authors

Richard E. Goodman

Study Completed On

5 September, 2013



Performing Laboratory

Food Allergy Research and Resource Program
Food Science and Technology
University of Nebraska
143 Food Science & Technology
Lincoln, NE 68583-0955

Laboratory Project ID

Study Number: REG Oxitec OX513A

Summary:
Genetically modified (GM) *Aedes aegypti* mosquitoes were developed by Oxitec Limited by the insertion of a single contiguous DNA segment comprising two genes to produce male mosquitoes that carry a lethal dominant trait under control of a promoter that allows successful reproduction only in cultured conditions in the presence of tetracycline. One gene encodes the fluorescent red protein (DsRed2) from an Anthozoan species (corals and sea anemones) that has been used as a visible selection marker in a number of plant transformation events (Jach et al., 2001; Dietrich and Maiss, 2002; Wenck et al., 2003; Mirabella et al., 2004; Stuitje et al., 2003). The second gene regulates the reproductive development of the mosquitoes by production of a protein with a tetracycline-repressible transcriptional activator fused to and controlling a segment of a *Herpes simplex* virus VP16 protein. The second gene is a dominant lethal trait as the large majority of males and females carrying this trait die before functional adulthood. The engineered male mosquitoes are released into the environment to breed with normal females, but production of the next generation of progeny fails to develop to adulthood (Gossen and Bujard, 1992; Gong et al., 2005; Phuc et al, 2007; Kongmee et al., 2010, Fu et al., 2010).

Regulatory agencies in countries where the genetically modified mosquitoes might be released will have to evaluate potential human safety issues that might be presented by the GM mosquitoes. Although the safety assessment process for genetically modified organisms (GMOs) is normally applied in consideration of the safety of the organism for food use, as that is the majority of GMO's currently seen by regulators, in this case regulators may consider risks to humans that may be exposed to mosquito proteins through bites by female GM mosquitoes (males do not bite). Potential exposure routes include: worker exposure to female mosquitoes during mosquito rearing; incomplete sex separation leading to release of some homozygous female OX513A mosquitoes together with the males; incomplete penetrance of the lethal trait leading to production of some functional heterozygous female adult OX513A mosquitoes among the offspring of the released homozygous OX513A males and wild females. The exposure would be expected to occur through bites and saliva, not through dietary exposure (in humans). The primary risk of severe reactions would be assumed to be from the transfer of a protein that causes systemic allergic reactions in allergic individuals rather than sensitization de novo. Thus a bioinformatics evaluation to ensure the newly expressed proteins are not allergens and are not highly identical to allergens as described here, was used as an important evaluation step to minimize potential risks for humans. Finally, an evaluation was performed to consider if the protein has any properties that would be considered toxic in the context of human exposure to a mosquito bite. The bioinformatics searches performed and reported here did not uncover any concerns of potential allergenicity, allergenic cross-reactivity or potential toxicity that would demonstrate a need for further testing regarding safety. The conclusion of the bioinformatics evaluation and the evidence of expression patterns demonstrated that the DsRed2 and tTAV proteins do not present a risk of allergy or toxicity to humans.

1.0 Introduction

Oxitec Limited, UK has developed genetically modified *Aedes aegypti* mosquitoes by inserting DNA encoding two proteins in transgenic strain OX513A, using the LA513 transposon - also known as OX513 (Phuc et al., 2007). One protein DsRed2 (Matz et al., 1999; Jach et al., 2001; Dietrich and Maiss, 2002; Wenck et al., 2003; Stuitje et al., 2003; Mirabella et al., 2004; Phuc et al., 2007) was used as a marker protein for efficient selection of recombinant mosquitoes. The second protein is the recombinant tetracycline repressible transcriptional activator protein (tTAV) described by Phuc et al. (2007). The purpose is to produce homozygous male mosquitoes carrying a dominant lethal gene that prevents the successful development of progeny from wild-type females mating with the GM males into adults Phuc et al , 2007).. Since this species is a vector for Dengue Fever and Yellow Fever as well as other arboviral diseases, there is an urgent need to reduce the reproductive success of this mosquito as one mechanism to reduce human disease. Laboratory as well as field trials are currently in progress to evaluate the effectiveness of this system (Bargielowski et al., 2011; Lacroix et al., 2012, Harris et al, 2011, Harris et al, 2012).

The human safety component of GM organisms (GMOs) normally focuses on the safety of food produced from the GMO (Codex, 2003) and mosquitoes are not consumed by humans. However, the female mosquitoes must feed on the blood of mammal hosts (usually humans in the case of *Aedes aegypti*) in order to provide nutritional requirements for egg production. Proteins in the saliva of mosquitoes are known to cause allergic reactions in humans. In addition, there is a potential for a toxic protein to be introduced through a mosquito bite.

While it would be important to consider whether one or both of the newly introduced proteins (DsRed2 or tTAV) in this GM mosquito might be present in saliva of GM mosquitoes, the primary evaluation should be whether the proteins are known to be allergens or toxins, or whether they are nearly identical to any known allergen or toxic protein. That is why Oxitec Limited requested a bioinformatics analysis of the two expressed proteins. In 2011 a bioinformatics study was performed and reported on tTAV regarding potential similarities to allergens and toxins (Goodman, 2011). The current report provides results from a new updated search that includes the DsRed2 marker protein and tTAV compared to all compiled allergens or toxins in public databases. This report describes the sequences, the datasets, the methods and the results of the bioinformatics evaluation of the DsRed2 and tTAV proteins, using the amino acid sequence information provided by Oxitec as the query sequences.

2.0 Purpose

The purpose of this study is to perform an evaluation of the potential allergenicity and toxicity of the DsRed2 and tTAV proteins that are encoded by the genes introduced in OX513A mosquitoes (*Aedes aegypti*) based on published literature about the source of the genes and bioinformatics (sequence comparisons) of proteins with known allergens and toxins. The intent is to guide decisions regarding whether additional safety tests would be needed for evaluating these proteins as potential sources of allergy or toxicity if there is any human exposure through the bite of the female mosquitoes.

3.0 Methods

3.1 Scientific literature search strategies. The PubMed database (http://www.ncbi.nlm.nih.gov/pubmed) maintained by the U.S. National Library of Medicine was used as the primary data source for scientific literature on allergy and toxicity. The primary question is whether the source of the gene is a common cause of allergy or toxicity. The data (authors, publication, date and abstracts) from searches were saved to files for review. All publication abstracts were manually reviewed and any likely relevant publications suggesting adverse health risks were investigated further by reading the journal articles.

3.1.1 Search for allergenicity. Search terms "gene source" AND "allergen" as well as "gene source" AND "allergy" were used on 10 August, 2013.

3.1.2 Search for toxicity. Search terms "gene source" AND "toxin" as well as "gene source" AND "toxicity" were used on 10 August, 2013.

3.2 Amino acid sequences of query proteins. The mosquito transfection clone construct used to develop OX513A was described in Phuc et al., 2007.

3.2.1 DsRed2. The DsRed2 amino acid sequence from OX513A was supplied by Oxitec Limited (Table 1) and is identical to the protein expressed by the transient expression vector pX-DR, GI:237652127 (Chen et al., 2009) except for an additional three amino acids at the N-terminus that was added in constructing the mosquito insertion transposon (personal communication, C. Beech, 17 May, 2013).

3.2.2 tTAV. The tTAV amino acid sequence from OX513A was supplied from Oxitec Limited (Table 1) and is 100% identical to GI: 60542785, Accession AJ865387, from Gong et al., 2005 and Phuc et al., 2007.

Table 1 Amino acid sequences of the novel OX513A transgenic event mosquito proteins.

Protein (in OX513A) Common name of nearest source organism *Latin name*	**Protein name** Nearest published sequence GI: Protein length (aa) Percent ID to Oxitec [native publication]	**Protein sequence for OX513A proteins (supplied by Oxitec Ltd.)**
DsRed2 Coral *Discosoma sp.*	**DsRed** GI:55976617 225 amino acids 97% (219/225 aa) [Matz et al., 1999]	1 MARMASSENV ITEFMRFKVR MEGTVNGHEF EIEGEGEGRP YEGHNTVKLK 51 VTKGGPLPFA WDILSPQFQY GSKVYVKHPA DIPDYKKLSF PEGFKWERVM 101 NFEDGGVATV TQDSSLQDGC FIYKVKFIGV NFPSDGPVMQ KKTMGWEAST 151 ERLYPRDGVL KGETHKALKL KDGGHYLVEF KSIYMAKKPV QLPGYYYVDA 201 KLDITSHNED YTIVEQYERT EGRHHLFL
tTAV GI: 60542785 Synthetic construct from two proteins Bacterial tetracycline repressor *Escherichia coli* *Herpes simplex* virus 1 (human)	**Tetracycline repressor protein** (3-208 aa) GI:486188873 **Transactivating tegument protein** (211-338 aa)	1 MGSRLDKSKV INSALELLNE VGIEGLTTRK LAQKLGVEQP TLYWHVKNKR 51 ALLDALAIEM LDRHHTHFCP LEGESWQDFL RNNAKSFRCA LLSHRDGAKV 101 HLGTRPTEKQ YETLENQLAF LCQQGFSLEN ALYALSAVGH FTLGCVLEDQ 151 EHQVAKEERE TPTTDSMPPL LRQAIELFDH QGAEPAFLFG LELIICGLEK 201 QLKCESGSGP AYSRARTKNN YGSTIEGLLD LPDDDAPEEA GLAAPRLSFL 251 PAGHTRRLST APPTDVSLGD ELHLDGEDVA MAHADALDDF DLDMLGDGDS 301 PGPGFTPHDS APYGALDMAD FEFEQMFTDA LGIDEYGG

3.3 Sequence database search strategies.

The AllergenOnline version 13 (http://www.allergenonline.org/) and the NCBI Entrez Protein (http://www.ncbi.nlm.nih.gov/BLAST/) databases were used as the protein amino acid data sources for the sequence comparisons for allergens and toxins (31,601,460 sequences on 14 August, 2013). The AllergenOnline database was updated in 12 February 2013 and is maintained by the Food Allergy Research and Resource Program of the University of Nebraska. Protein entries in the Entrez search and retrieval system is compiled and maintained by the NCBI of the

National Institutes of Health (U.S.A.). The database is potentially updated or modified daily, and therefore the date of sequence searches by BLASTP is relevant to the dataset used in the BLASTP searches. BLASTP and FASTA3 are unique computer algorithms that provide similar local alignments and results if the appropriate scoring matrices and criteria are used.

3.3.1 FASTA3 overall search of AllergenOnline. The potential sequential and inferred structural similarities of the DsRed2 and tTAV proteins were evaluated using version 13 of AllergenOnline.org.

3.3.2 FASTA3 of AllergenOnline by 80 aa segments. This short segment search is based on the recommendation of Codex (2003). The rationale is that this might help in identifying structural motifs, much shorter than the intact protein, which might contain a conformational IgE binding epitope. It should also help to identify potentially cross-reactive proteins that are not true homologues of an allergen that have significant local identities that might provide an immunological target for IgE antibodies in those with allergies to the matched allergen. A match of >35% with a known allergen will suggest further testing for possible cross-reactivity.

3.3.3 BLASTP of NCBI Entrez with "allergen" as keyword limit. The BLASTP is available on the NCBI Entrez website (http://www.ncbi.nlm.nih.gov/BLAST/). The current version is BLASTP 2.2.28+ (28 July, 2013). A BLASTP search was used comparing the DsRed2 and tTAV query sequences against the entire Entrez Protein database, with a limit option selected to query entries for "allergen", to align only with proteins identified as allergens. The purpose of this BLASTP search is to ensure that a significant match with a newly discovered allergenic sequence that has not yet been entered into AllergenOnline is not overlooked. Evaluation of the *E* value, the length of the alignment and the percent identity of any identified match is necessary to judge the significance of any alignment using BLASTP.

3.3.4 BLASTP of NCBI Entrez without keyword limit. The purpose of this BLASTP search is to compare the DsRed2 and tTAV proteins to all known protein sequences to evaluate whether there are other similar proteins from other organisms that might provide information of safe exposure to homologues of this protein.

3.3.5 BLASTP of NCBI Entrez with "toxin" as keyword limit. The purpose of this BLASTP search is to identify matches to known toxic proteins (toxins) and if alignments share significant identities, to determine potential risks that would require further testing. The sequences of the DsRed2 and tTAV proteins were compared to the NCBI Entrez database using "toxin" as a keyword search limit.

4.0 Results and Discussion. The summary results for the PubMed search using the various protein sources and search terms, and the amino acid sequences of the DsRed2 and tTAV proteins, are presented here.

4.1 PubMed Searches. The PubMed scientific literature database was searched for evidence that the DsRed2 and tTAV proteins are linked to allergy or toxicity. The search demonstrated that there is no published evidence that the two proteins are allergens or toxins for mammals and no evidence that implies there might be an association with allergenicity or toxicity. An important consideration of the safety assessment related to potential toxicity, is an understanding of the mechanism of action of the newly expressed protein. If the protein is an enzyme, potential biological impacts of any new metabolites should be considered. If the inserted DNA or new protein is a transcription or translational regulator, potential targets or measured effects in source organisms or other host organisms should be considered. Identical or nearly identical genes introduced in OX513A mosquitoes have been have been inserted in transgenic animals, plants or arthropods by many scientists and the proteins have been expressed within cells of various tissues of the hosts, without adverse impacts (e.g., for DsRed2 see Ryu et al., 2013; for tetracycline repressible transactivator protein see Gong et al., 2005). Since these proteins have been produced directly within the cells of diverse eukaryotic species without obvious toxic effects, it is highly unlikely that incidental exogenous exposure by any route (ingestion, inhalation or injection) would have adverse biological impacts on human or mammalian health.

4.1.1 Allergenicity.

The terms "*Discosoma*" AND "allergen" as well as "*Discosoma*" AND "allergy" were used to search PubMed for evidence of allergy from the source organism of the DsRed2 protein, *Discosoma sp.* No references were listed when "allergen" was used. Two references were listed when "allergy" was used (Teterina et al., 2010; Tawfik et al., 2008), but in both cases the studies used the DsRed protein as a fluorescent label to study disease processes and there was no causal relationship with allergy.

Literature searches to evaluate the potential allergenicity (and toxicity) of the source for the TAV protein are somewhat complex. The source of the gene/protein for the tetracycline repressor protein (amino acids 3-208 of TAV) part of the protein is the Tn10 plasmid in *E. coli* (Gossen and Bujard, 1992; Altschmeid et al., 1988), which is produced as a fusion protein with the *Herpes simplex* virus protein 16 C by the design of the gene construct (Gong et al., 2005, Phuc et al., 2007).

The *E. coli* bacterium has used as a cloning and expression host for many allergens and toxins. In addition, some strains of *E. coli* are known to produce toxins. Therefore it was expected that simple searches of "*E. coli*"

AND "allergen" or "toxin" would find many irrelevant publications. A search of the terms "*Escherichia coli*" AND "allergen" in PubMed returned 665 publication references. Since it is not possible to efficiently read 655 publications for evidence of allergenicity, an additional term, "tetracycline" was used to refine the source since tTAV is a tetracycline repressible transactivator. Only one publication was found then, Nishihara et al., 1998, describing the design of a transgenic bacterium that included regulatory expression of a cedar pollen allergen, Cry j 2 in *E. coli*. The results suggest that there are no natural allergens in *E. coli*. In order to ensure that the addition of the third term was not too restrictive, an additional strategy was used. Since, the sequence of the tTAV is identical to, or nearly identical to tetracycline repressible transactivator proteins that have been cloned from a few species of bacteria including *Salmonella sp.*, *Shigella sp.*, *Acinetobacter sp.*, and of the taxonomic family Enterobacteriaceae, a search was performed using "*Acinetobacter*" AND "allergen" as alternative search terms. Five publications were identified. Jadhav et al. (2013) found *Acinetobacter sp.* associated with isolates of nosocomial infections in a number of patients in hospitals, along with many other microbes, without any connection to allergy. Qiu et al. (2011) published data from a study demonstrating that an intentional lung infection with *Acinetobacter baumannii* reversed the Th2 response of eosinophilia and "allergic response" to ovalbumin in a mouse model. Skorska et al. (2007) tested skin prick tests and IgE responses against a number of gram negative and gram positive microbes commonly found in organic dust, using subjects who work in a poultry hatchery in Poland. The brief report did not detail proteins or specific data, only a trend that more workers showed positive precipitin reactions extracts (typically an IgG antibody complex reaction, indicating Th1 response) to *Escherichia coli* and *Acinetobacter baumannii* along with other fungi and bacteria than control subjects. Valerio et al. (2005) demonstrated by PCR of 16S ribosomal DNA, that a few bacterial species were present in cultures and culture medium of the allergenic house dust mite (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), including *Acinetobacter sp.*, however the prevalence was low and no proteins were identified or relationship to allergenicity in looking for the source of endotoxin in the allergenic mite cultures. Dutkiewicz et al. (2002) measured IgE levels to extracts of a number of bacterial species using sera from workers in a potato processing facility to evaluate potential causes of work-related asthma. Although *Acinetobacter calcoaceticus* was mentioned, there was no reference to it as an important target for IgE, whereas some other bacteria were implicated. Interestingly there is little evidence in the literature for microbial proteins causing allergic reactions, so the importance of the few reports on bacterial precipitin antibody binding reactions to bacterial proteins is not obvious (RE Goodman, personal observations). A search with "Acinetobacter" AND "allergy" listed 48 references. An attempt to focus on the protein was performed by inclusion of a third term "tetracycline repressor protein", and did not identify any publications. An evaluation of the entire list of 48 publications demonstrated that most reported identification of *Acinetobacter spp* .and other microbes in a study group, often from an institute of allergy, or in some cases (e.g. Renz et al., 2011), an animal

model was used to demonstrate that an infection with *Acinetobacter spp.* may suppress allergic responses. Other research points to the conclusion that exposure to *Acinetobacter spp.* protects individuals against allergy, possibly due to the lipopolysaccharide content that skews reactions toward a Th1 response (Debarry et al., 2010).

The terms "Herpes" AND "VP16" AND "Allergy" were used to evaluate the source of the transactivating portion of tTAV, with nine articles found. However all nine were only listed as they are from investigators at the National Institute of Allergy and Infectious Diseases. They did not demonstrate a relationship between the Herpes simplex VP16 and allergy. A search with "Herpes" AND "VP16" AND "allergen" yielded no publications.

Thus, a search of the literature for publications linking the source organisms, *Discosoma sp.*, *Escherichi coli, the* surrogate source (*Acinetobacter sp.*), and *Herpes simplex* did not uncover evidence that would implicate the sources or the proteins used in OX513A as likely allergens.

4.1.2 Toxicity. A search of PubMed using the taxonomic organism names for the taxonomic sources of the DsRed2 and tTAV along with the terms "toxin" or "toxicity" AND the protein ("DsRed2" or "tTAV") were reviewed.

For "Discosoma" AND "toxin", no references were found. For "Discosoma" AND "toxicity" two publications were found. One by Long et al. (2005) presented information on a new, mutated form of the red fluorescent protein from *Discosoma sp.*, and suggested that the DsRed2 protein might be toxic if expressed in transgenic mice, referring to an earlier paper by Hadjantonakis and Papaioannou (2004). However, after a careful read of the 2004 paper by Hadjantonakis and Papaioannou, I believe that their study did not demonstrate toxicity to DsRed2, rather it showed a failure to maintain highly expressing red-fluorescent stem cell lines, which might be due to position effects or a number of other technical issues. The second paper found in this search was by Murata et al. (2011), in which they indicate using DsRed2 as a marker in transgenic mice did not demonstrate any toxicity problems in the transgenic mice. A further search with "DsRed2" found 217 publications and rapid review did not indicate toxicity. Instead the publications demonstrate that the DsRed2 protein can be expressed in various cells of transgenic mice and transfected mouse cells that have been used for various physiological or toxicological studies without apparent toxicity. For example, a study by Ryu et al. (2013) reported successful transformation and use of transgenic mice with the DsRed2 reporter in a chimeric situation with maintenance of polyclonal tissues having cells that are from EGFP transformed or DsRed2 transformed mice, with no reported toxicity. These were generated by mating two lines of transgenic mice, one with DsRed2 and one with EGFP. The mice were surviving and healthy. In addition, Nordin et al. (2013) demonstrated that the OX513A mosquito larvae, which do express both the DsRed2 and tTAV

proteins can be consumed by two species of fly larvae (*Toxorhynchites spp.*) without apparent toxicity. From this literature search, there does not appear to be published evidence that the DsRed2 protein is toxic to microbes, fungi, insects, plants or mammals.

The tTAV protein is a fusion of two proteins. The tetracycline repressible transactivator protein is from *E. coli.* The second of the fusion protein is from the *Herpes* simplex virus. The first search with "Escherichia coli" AND "toxin" returned over 26,890 publications. Adding the term "tetracycline" reduced that to 229 publications, still a large number. Scanning titles of the 229 reveals that most are related to specific virulence factors or outbreaks of disease in livestock. Addition of the term "transactivator" removed all but one of the 229 publications. Baur et al., (1997) described tetracycline regulated expression of the diphtheria toxin A gene in human glioma cells using a rTA element. The toxicity was related to the toxicity of the toxin A gene. These results demonstrate that while the specific bacterium is associated with toxic responses, there is no obvious indication that the tetracycline activator protein is associated with toxicity.

As in the search for allergens, an additional search was performed using the *Acinetobacter sp.* as a search term since the proteins are 99% identical and the species are from the same family of bacteria. The intent was again to verify whether other researchers who study this species might have uncovered toxicity associated with the protein. A search with "Acinetobacter" AND "toxin" found 210 publications. There is ample evidence that the organism, *Acinetobacter sp.* is an opportunistic pathogen and that it does produce toxins such as lipopolysaccharide. However, limiting the search by including AND "tetracycline" reduced the publication list to two papers related to antibiotic resistance (Wieczorek et al., 2008 and Loeffelholz et al., 1987). Addition of "transactivator" reduced the list to zero. Finally, "Acinetobacter" AND "tetracycline repressor protein" identified only one publication, Thompson et al. (2007), which did not identify toxicity associated with the protein. This search did not identify evidence of any toxicity associated with the tetracycline repressor protein of *Acinetobacter sp.*

The VP16 protein of *Herpes simplex* virus is a transcriptional regulator that functions by binding to specific DNA sequences (TAATGARAT consensus sequence) present in virus genes that are up-regulated in the early cycle of viral infection (Simmen et al. 1997). It functions within the cell and is not expected to be taken up by eukaryotic cells or be active in cells that do not express the protein. In order to consider possible toxicity the terms "Herpes" AND "toxicity" as well as "Herpes" AND "toxin" were search in PubMed, returning 1381 references and 326 references respectively. Adding "VP16" as a search term reduced the number to 7 and 6 references respectively. The publications were searched for evidence of toxicity related to VP16 and no direct evidence was found. The

studies generally relate to attempts to use *Herpes simplex* virus as a transfection vector for efficient gene transfer to mice for mechanistic studies or possibly to humans to treat disease. The vectors have caused toxicity, thus a small number of publications are identified in this search. The conclusion of reading those publications that seemed most relevant was negative, that is, no direct toxicity associated with the VP16 protein.

The combined search information failed to identify any evidence that the proteins fused to provide the complete tTAV protein or the DsRed2 proteins have any known toxicity.

4.2 Sequence comparison of the DsRed2 and tTAV proteins in OX513A to allergens. The amino acid sequences of the DsRed2 protein and the tTAV protein (Table 1) were compared to known allergens using both a full-length FASTA alignment search and a sliding window of 80 comparisons against AllergenOnline.org, version 13. Additionally, a BLASTP search was performed against the NCBI database using keyword search limits of "allergen" and "toxin".

4.2.1 Full length FASTA3 vs. AllergenOnline. Results of the full length FASTA3 searches of the DsRed2 protein against AllergenOnline version 13 did not identify any significant alignment with an allergen. Scoring results for the DsRed2 protein showing alignments with *E* scores less than 1 are shown in Table 3 and demonstrate no significant matches with any allergen. The low-level alignment with various sequences of the same carrot PR-10 protein are insignificant matches. Their identities (%) are markedly below the level that is likely to indicate cross-reactivity (< 50% identity, Aalberse, 2000) and it is also below the 35% identity level suggested by Codex (2003) as a match that may possibly be cross-reactive. Thus, there is no scientific basis for assuming the DsRed2 protein is sufficiently similar to any allergen to suspect cross-reactivity and there is no rationale for performing serum IgE tests based on overall alignment, the most predictive bioinformatics comparison.

Table 2. Overall FASTA3 search of AllergenOnline.org database with the DsRed2 protein (225 amino acids). Only proteins identified with matches to allergens or putative allergens in AllergenOnline.org version 13, having an *E* score smaller than 1 are shown. None of the results were significant using the criteria of >35% identity over alignments of at least 80 amino acids.

Sequence GI #	Organism	Description	Length aa	*E* score	% Identity	aa Alignment length
302379155	*Daucus carota* carrot	Pathogenesis Related protein (Bet v 1 like)	154	0.23	23.2	125
302379157	*Daucus carota* carrot	Pathogenesis Related protein (Bet v 1 like)	154	0.36	23.2	125
302379159	*Daucus carota* carrot	Pathogenesis Related protein (Bet v 1 like)	154	0.36	23.2	125
19912791	*Daucus carota* carrot	Pathogenesis Related protein (Bet v 1 like)	154	0.42	23.2	125
302379151	*Daucus carota* carrot	Pathogenesis Related protein (Bet v 1 like)	154	0.42	23.2	125
302379153	*Daucus carota* carrot	Pathogenesis Related protein (Bet v 1 like)	154	0.58	23.2	125

Similarly, the tTAV protein amino acid sequence does not show any significant FASTA alignment to any known allergen in the AllergenOnline.org database (Table 3). The only alignment was to tropomyosin of the sea snail or whelk (Neptunea polycostata) and at only 22.1% identity with an E score of 0.053, which is considered irrelevant for potential cross-reactivity.

Table 3. Overall FASTA3 search of AllergenOnline.org database with the tTAV protein (338 amino acids). Only proteins identified with matches to allergens or putative allergens in AllergenOnline.org version 13, having an *E* score smaller than 1 are shown. None of the results were significant using the criteria of >35% identity over alignments of at least 80 amino acids. In addition to testing the complete 338 aa tTAV protein, the 208 aa tetracycline repressor protein and the VP16 127 aa proteins were tested independently and only the N-terminal 208 aa segment of the intact tTAV aligned with tropomyosin of sea snail. The complete tTAV alignment is shown here.

Sequence GI #	Organism	Description	Length aa	*E* score	% Identity	aa Alignment length
219806590	*Neptunea polycostata* Sea snail (whelk)	Tropomyosin	284	0.053	22.1	181

4.2.2 Sliding 80-amino acid window FASTA3 vs. AllergenOnline.org database. Results of the comparisons of the DsRed2 and tTAV protein sequences were tested against all of the sequences in Allergenonline.org version 13. The comparisons did not identify any possible match of > 35% identity with any known allergen in the database. Thus the risk of cross-reactions for allergic individuals is very low, and the data indicate there is no reason to perform serum IgE testing as there is not a target allergen to suspect cross-reactivity. Tables 5 and 6 give an indication of the results obtained for DsRed2 and tTAV proteins respectively.

Table 4. Scanning 80-mer Sliding Window Search Results for DsRed2 protein

80mer Sliding Window Search Results

Database	AllergenOnline Database v13 (February 12, 2013)
Input Query	>DsRed2 MARMASSENVIIEFMRFKVRMEGTVNGHEFEIEGEGEGRPYEGHNTVKLKVTKGGPLPFA WDILSPQFQYGSKVYVKHPADIPDYKKLSFPEGFKWERVMNFEDGGVATVTQDSSLQDGC FIYKVKFIGVNFPSDGPVMQKKTMGWEASTERLYPRDGVLKGEIHKALKLKDGGHYLVEF KSIYMAKKPVQLPGYYYVDAKLDITSHNEDYTIVEQYERTEGRHHLFL
Length	228
Number of 80 mers	149
Number of Sequences with hits	0

No Matches of Greater than 35% Identity Found

AllergenOnline Database v13 (February 12, 2013)

Table 5. Scanning 80-mer Sliding Window Search Results for tTAV protein of OX513A

80mer Sliding Window Search Results

Database	AllergenOnline Database v13 (February 12, 2013)
Input Query	>query MGSRLDKSKVINSALELLNEVGIEGLTTRKLAQKLGVEQPTLYWHVKNKRALLDALAIEM LDRHHTHFCPLEGESWQDFLRNNAKSFRCALLSHRDGAKVHLGTRPTEKQYETLENQLAF LCQQGFSLENALYALSAVGHFTLGCVLEDQEHQVAKEERETPTTDSMPPLLRQAIELFDH QGAEPAFLFGLELIICGLEKQLKCESGSGPAYSRARTKNNYGSTIEGLLDLPDDDAPEEA GLAAPRLSFLPAGHTRRLSTAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDS PGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDEYGG
Length	338
Number of 80 mers	259
Number of Sequences with hits	0

No Matches of Greater than 35% Identity Found

AllergenOnline Database v13 (February 12, 2013)

4.2.3 Eight amino acid match. Because some countries still require a search for any exact match of 8 or more contiguous amino acids between the GM protein and any known allergen, that comparison was performed using AllergenOnline.org database, version 13. Both the DsRed2 and the tTAV full length sequences were copied into the AllergenOnline.org search query box and tested. The results of these searches were negative.

4.2.4 BLASTP of NCBI Entrez using "allergen". The full-length amino acid sequences of the DsRed2 and tTAV proteins were compared to sequences in NCBI-Entrez, which were designated as "allergen" in the NCBI database on 14 August, 2013.

The top two aligned matches to DsRed2 have (Tables 6) have significantly small *E* scores, suggesting some evolutionary homology. However, the identity matches are low (25% in a 212 amino acid alignment to a recombinant pollen allergen Cry j 1 fused to a green fluorescent protein and 24% in a 212 amino acid alignment to cockroach allergen Bla g 1 fused to a green fluorescent protein). The low identity match is not considered a likely indication of allergic cross-reactivity (Aalberse, 2000). But importantly, the two matched sequences are synthetic constructs that include a green fluorescent marker protein that was originally derived from *Aequorea victoria* (GI:634009) described by Tsien (1998). The alignments of DsRed2 to those two synthetic constructs were only in the region of the green fluorescent protein, which is not known to cause allergies. The other alignments of DsRed2 were not significant as judged by the very large *E* score values (>0.001) and low identity matches (25% to 57%) with very short-partial protein alignments. The aligned proteins would not be considered homologues of the DsRed2.

Table 6. BLASTP of NCBI Entrez with DsRed2 using the keyword "allergen". The scoring alignments with *E* scores below 10 are shown for this DsRed2 protein vs. all proteins labeled with the keyword "allergen" in the NCBI Entrez database on 14 August, 2013, using BLASTP. The sequence identities are low and / or the length of alignments are very short, indicting unlikely homology and that the overall structure is unlikely to be similar. Thus even if the NCBI sequence is a proven allergen, there is very little likelihood of cross-reactivity by the DsRed2 protein.

Sequence GI#	Organism	Description	Length aa	*E* score	% identity	aa Alignment length
223005744	*Synthetic construct* Cryptomera japonica AND GFP origin *Aequorea victoria*	Synthetic construct of T cell epitopes of the Cry j 1 *Cryptomera japonica)* and green fluorescent protein	412	1e-16	25	212
529482053	*Synthetic construct* *Blatella germanica* AND GFP origin *Aequorea*	Synthetic construct of cockroach allergen Bla g 1 and green fluorescent protein	416	1e-14	24	212

	victoria					
116333554	*Lactobacillus brevis* bacteria	Hypothetical protein LVIS_0955	321	0.34	36	50
156370878	*Nematostella vectensis* Sea anemone	Predicted protein sea anemone MD-2 like protein	299	3.1	32	38
493609361	*Oscillochloris trichoides* bacteria	Allergen V5/Tpx-1 family protein	495	4.0	29	77
403416894	*Fibroporia radiculosa* Brown rot fungus	Predicted protein MD-2 like protein	524	4.1	57	30

The tTAV protein only showed one very minor alignment by BLASTP limited by "allergen" (Table 7). This alignment is insignificant and does not represent an indication of possible cross-reactivity as described by Aalberse (2000) and Goodman et al. (2008).

Table 7. BLASTP of NCBI Entrez with tTAVusing the keyword "allergen". The only identified scoring alignments with *E* scores below 10 is shown for this tTAV. The sequence identities are low and / or the length of alignments are very short, indicting unlikely homology and that the overall structure is unlikely to be similar. Thus even if the NCBI sequence is a proven allergen, there is very little likelihood of cross-reactivity by the tTAV protein.

Sequence GI#	Organism	Description	Length aa	E score	% identity	aa Alignment length
493199135	*Treponema vincentii* Spirochette	Hypothetical protein, SCP like protein	221	1.7	55	20

4.2.5 BLASTP of NCBI without keyword limit. The full-length of the DsRed2 and tTAV proteins were compared to all sequences in NCBI-Entrez database on 14 August, 2013. The DsRed2 protein has 100 alignments of over 80% identity, mostly with synthetic constructs of transfection vectors as it is a marker gene/protein. Similarly, tTAV has many alignments of near-100% identity with synthetic constructs with partial alignments to tetracycline repressor proteins and with *Herpes simplex* 1 VP16. Because so many entries in NCBI show synthetic constructs that researchers are using for transfecting various organisms, it is difficult to trace out the origin of the proteins. The original literature on constructs is necessary to evaluate the origins (see the Introduction and Section 4.1 for references).

4.3 BLASTP of NCBI Entrez with "toxin". The full-length sequences of the DsRed2 and tTAV proteins were compared to sequences in NCBI-Entrez, which were designated as "toxin" in the NCBI database on 14 August, 2013. The top aligned proteins with E scores smaller than 10 are shown for each of the two proteins (Tables 8– 9).

DsRed2. The best scoring alignment with DsRed2 was to synthetic constructs. For the best aligned protein, the primary alignment of 98% identity is to a 223 amino acid portion of the synthetic protein construct that is from a red fluorescent protein (DsRed1) that is in the Green Fluorescent protein family, with a secondary poor alignment of 24% identity over 210 amino acids to the green fluorescent protein (Liu et al. 2011). The second best aligned sequence was a similar construct by the same authors (Liu et al., 2011), using slightly different order and sequences. The construct was used to transform cells for tests of physiological function. There is no evidence that the red fluorescent portion of the protein (with nearly 100% identity to DsRed2) is toxic. The third sequence with the next best alignments is a similar synthetic construct for testing cell physiology using a botulinum toxin substrate, by a different group, but with two green fluorescent proteins rather than one green and one red (Itoh et al., 2002). The fourth protein sequence with an alignment is another cloning construct with a green fluorescent protein and with a botulinum toxin in the same construct (Band et al. 2010). The fifth protein, phytoene dehydrogenase from the toxic bacterium Corynebacterium ulcerans is the highest scoring protein that is not a fluorescent marker protein (Sekizuka et al., 2012). However, the alignment is poor and the sequence is merely one of the sequences discovered by whole genome sequencing of the bacterium. The protein sequence (GI:397655072) was then compared to all of NCBI by BLASTP and it turns out to be one of the highly conserved enzymes related to phytoene desaturases, that are rather ubiquitous. No published evidence was found that this protein is toxic and furthermore, the sequence alignment to DsRed2 is weak. The final aligned protein is a transcription regulator from *Closteridium botulinum*, a toxic organism. A search of PubMed did not identify any publications that describe toxicity associated with the transcriptional regulators of *C. botulinum*. In general transcriptional regulators are only functional if they are expressed inside the cell of the organism containing the gene that is being regulated. I found no evidence that this protein could be taken in by the cells of other, non-bacterial organisms and cause gene expression changes.

Table 8. BLASTP of NCBI Entrez "toxin" with DsRed2 from OX513A. The best scoring alignments to putative toxins shown in the NCBI Entrez database on 14 August, 2013, were identified by BLASTP with the full-length sequence of the DsRed2 protein from OX513A.

Sequence GI#	Organism	Description	Length aa	*E* score	% identity	aa Alignment length
172054575	Synthetic construct Primary alignment	EGFP-Pak1-Rac1-dsRed1-CAAX fusion with Rac related to a botulinum toxin substrate Alignment only to red fluorescnt protein peptide	798	2e-145	98	223
172054575	Synthetic construct Secondary alignment	EGFP-Pak1-Rac1-dsRed1-CAAX fusion with Rac related to a botulinum toxin substrate Alignment only to green fluorescnt protein peptide	798	1e-14	24	210
16796513	Synthetic construct Primary alignment	dsRed1/Pak1/Rac1/ECFP fusion protein Alignment to red fluorescent protein	775	1e-144	98	222
16796513	Synthetic construct Secondary alignment	dsRed1/Pak1/Rac1/ECFP fusion protein Alignment to green fluorescent protein	775	4e-14	30	145
23095931	Synthetic construct Primary alignment	Raichu-1011x, rac and cdc42 Alignment to green fluorescent protein	763	1e-15	26	203
23095931	Synthetic construct Secondary alignment	dsRed1/Pak1/Rac1/ECFP fusion protein Alignment to green fluorescent protein	763	2e-14	26	209
259490938	Synthetic construct	deltaLC-GFP-BoNT/A rev	1230	2e-15	25	212
397655072	Corynebacterium ulcerans bacteria	Phytoene dehydrogenase	544	0.17	21	95
182674319	Corynebacterium botulinum bacteria	Transcriptional regulator AraC family	395	8.4	45	33

tTAV. The best scoring alignment with tTAV was to a tetracycline repressor protein TetR from *Escherichia fergussonii* (Table 9). The alignment is very significant, however the bacteria is considered a source of toxicity. But the TetR protein has very high identity with the TetR proteins of many species of bacteria. It is also a regulatory protein expressed in the bacteria, which acts on the DNA of the bacteria to suppress or promote expression of bacterial proteins. It is not known to be taken up by cells of other organisms and cause any toxic effects. The next alignment is almost identical to the first and from another species of the genus. In fact it is 100% identical to a shorter (196 aa) segment of the protein of tTAV. Running a BLAST comparison of these two proteins demonstrates very high identity matches with many cloned gene/proteins as the activity is useful for tetracycline dependent gene regulation, as used in OX513A. There were quite a few alignments of much lower length and identity, all beginning near the N-terminus of the tTAV protein. However, there was also a very poor alignment to a segment of tTAV beginning at amino acid 224, but that was also a transcriptional regulator. No evidence was found of homology of the tTAV protein to a true toxin.

Table 9. BLASTP of NCBI Entrez "toxin" with tTAV protein from OX513A. The best scoring alignments to putative toxins shown in the NCBI Entrez database on 14 August, 2013, were identified by BLASTP with the full-length sequence of the tTAV protein from OX513A. There were 93 alignments with *E* scores less than 10 using this sequence and the term "toxin" by BLASTP. However, in many cases it is clear that the sequence is "identified" as a toxin due to the toxicity of the source organism (from a number of toxic bacteria). Importantly, only a few sequences had high identity matches over any extensive length of sequence. Those were evaluated further here.

Sequence GI#	Organism	Description	Length aa	*E* score	% identity	aa Alignment length
218561676	*Escherichia fergusonii* bacteria	Tetracycline repressor protein TetR	208	3e-150	99	208
388377844	*Escherichia coli* bacteria	Tetracycline repressor protein TetR	197	4e-142	100	196
394430501	*Escherichia coli* bacteria	Tetracycline repressor protein TetR	141	1e-81	90	136
190903672	*Escherichia coli* bacteria	Tetracycline repressor protein class A from transposon 1721	219	2e-65	53	202
388363196	*Escherichia coli* bacteria	Tetracycline repressor protein from E. coli strain o111:H8, TetR C	225	2e-64	51	210

310286451	*Escherichia coli* bacteria	Tetracycline repressor protein Class A from E. coli	217	1e-63	50	209
397654352	*Corynebacterium ulcerans* bacteria	TetR family transcriptional regulator	203	1e-12	33	147
300850578	*Enterococcus faecalis* bacteria	TetR family transcriptional regulator	220	2e-11	31	159
292642929	*Enterococcus faecalis* bacteria	TetR family transcriptional regulator	222	2e-08	26	155
429514288	*Enterococcus faecalis* bacteria	TetR/AcrR family transcriptional regulator	189	1e-5	44	62

4.4 Bioinformatics summary for the DsRed2 and tTAV proteins of OX513A. Although the results of literature searches to the sources of the genes transferred into OX513A were challenging due to the some extensive annotations that suggest allergy or toxicity associated with the source organisms, careful evaluation of the abstracts and publications as well as refined searches did not identify publications with sufficient evidence to suspect the DsRed2 or tTAV proteins represent risks of allergy or toxicity.

None of the results from the bioinformatics searches of the DsRed2 or tTAV protein amino acid sequences indicate that these proteins represent a risk of allergy or toxicity that is greater than a typical dietary protein. There were no matches of either protein to known allergens with more than 50% identity over the full-length. There were no matches of >35% identity over 80 or more amino acid segments compared to known or putative allergens. There were no identical matches of 8 or more contiguous amino acid segments. These highly conservative comparisons did not identify sequence similarities that would suggest the proteins are allergens or are sufficiently similar to an allergen to cause cross-reactions. They did not identify matches to toxins to suggest they may be toxic.

5.0 Conclusions

No convincing evidence was found to suggest that the DsRed2 protein or the tTAV protein expressed in the OX513A mosquitos represent risks of allergy or toxicity to humans (or other mammals). Based on the guidelines of the Codex Alimentarius Commission (2003 and 2009), and on common practices for evaluation of potential risks of allergy or toxicity from GMO (plants, animals or microbes), there is no reason to perform additional tests to evaluate potential risks of allergy or toxicity for these proteins. Although the guidelines are intended primarily evaluating potential food safety concerns regarding potential risks from genetically engineered organisms, the same safety evaluation process is scientifically sound as an approach for evaluating other potential routes of exposure, namely via airway (inhalation of insect body parts) or through insect bites (e.g. mosquito saliva). There is no evidence that these proteins pose any risk of eliciting allergic or toxic reactions.

6.0 References

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7.0 Appendix 1, AllergenOnline Database, version 13, February, 2013 (see attached PDF).

Appendix 3

OX513A Part A - Supplement to Bioinformatics -Other genetic elements literature search

Appendix 3- Supplement to *OX513A Technical Dossier- Part A - Appendix 2*.

Bioinfomatics - Other genetic elements literature search

Literature searches conducted for genetic elements of pOX513A not included in the bioinformatics analysis report for tTAV and DsRed2 proteins from the Food Allergy Research and Resource Program at the University of Nebraska U.S.A.

To address the risk question as to whether the GE animal contains sequences that are likely to pose potential hazards to the animal, to humans or animals consuming food from that animal, or the environment, scientific literature searches were conducted in the PubMed (NCBI) database (http://www.ncbi.nlm.nih.gov/pubmed) maintained by the US National Library of Medicine.

The PubMed database was used as the primary data source for scientific literature on allergy and toxicity and pathogenicity of donor organism sequences. The primary question researched on the literature database was whether the source of the gene or sequence used in the construct is a common cause of allergy or toxicity or is linked to pathogenicity. The data (authors, publication, date and abstracts) from searches were saved to files for review. All publication abstracts were manually reviewed and any likely relevant publications suggesting adverse health risks were investigated further by reading the journal articles.

Information from such searches for the inserted genes, DsRed2 and tTAV are presented in the bioinformatics report titled *Bioinformatics analysis for risks of allergenicity and toxicity of proteins encoded by the two genes introduced into genetically engineered mosquitoes Aedes aegypti strain OX513A for production of sterile males to reduce vector transmission of important human diseases,* by the Food Allergy Research and Resource Program at the University of Nebraska U.S.A.

This section looks at the potential toxicity, allergenicity and pathogenicity of the other genetic elements in the pOX513 construct using the same search methodology.

Search for toxicity. Search terms “gene source” AND “toxin” OR “toxicity” were used.

Search for allergenicity. Search terms “gene source” AND “allergen” OR “allergy” were used.

The analysis of the potential for the other genetic sequences from *Trichoplusia ni* and *Drosophila melanogaster* to be toxins or allergens are reported below:

Toxicity: The search terms used “*Trichoplusia ni*” and “toxin” or “toxicity” AND “*piggybac*”, returned no references when conducted on 2 Sept 2013. The search terms “*Drosophila melanogaster*” and “ toxin” or “toxicity” AND each of the following search terms in turn “*hsp70*”, “K10 terminator”, “*adh* intron”,”Drosomycin untranslated region” on 2 Sept 2013 . Table 3 below shows the results of the search for toxicity for the various genetic elements in pOX513 from *Drosophila melanogaster.*

Table 1: PubMed search results for "toxicity" for *Drosophila melanogaster* genetic elements in pOX513

Search term	Results returned	Refined search	Results returned
hsp70	1293 references	AND " minipromoter" search term added	0 references
K10 terminator	0 references	N/A	
adh intron	1 reference Alcohol Clin Exp Res. 1996 Feb;20(1 Suppl):33A-35A. Analysis of CA repeats in first intron of class I ADH gene in Long-Evans Cinnamon rats developing fatal intoxication after ethanol intake. Katsuki S, Kato J, Nakajima M, Inui N, Sasaki K, Kohgo Y, Niitsu Y.	N/A	
Drosomycin 3'UTR	0 references	N/A	

Only the elements *hsp70* and *adh* intron returned results in the PubMed database search, with the *hsp70* term listing 1293 references. In an attempt to focus the search further the additional search term "minipromoter" was added to the *hsp70* search string, which did not identify any publications. The one *adh* intron publication identified in the search used an animal model to identify abnormal alcohol dehydrogenase metabolism in a mutant rat strain (Katsuki et al, 1996), and was not related to toxicity of an *adh* intron region from *Drosophila* species.

Allergenicity: Similar searches were conducted in the PubMed literature database with the terms "allergen" or "allergy", for the genetic elements that are not included in the report in Appendix X, namely "*Trichoplusia ni*" and *Drosophila melanogaster* "*hsp70*", "*K10 terminator"*, "*adh* intron", "*Drosomycin untranslated region*". The results are listed below in Table 4:

Table 2: PubMed search results for "allergen" or "allergy" for *Drosophila melanogaster* genetic elements in pOX513

Search term	Results returned	Refined search	Results returned
Trichoplusia ni	48614	AND "*piggybac*"	0 references
Drosophila melanogaster hsp70	25	AND "minipromoter" search term added	0 References
Drosophila melanogaster K10 terminator	0 (search performed 2 Dec 2013)	Not conducted	Not applicable
Drosophila melanogaster *adh* intron	5 (search performed 2 Dec 2013)	Not conducted	Not applicable
Drosophila melanogaster Drosomycin 3'UTR	1 Imler JL and Bulet P (2005) Antimicrobial peptides in Drosophila: structures, activities and gene regulation. Chem Immunol Allergy 86 1-21	Not conducted	Not applicable

Search for *hsp70* identified 25 papers. *hsp70* is a highly conserved region in many organisms and there is evidence from the 25 references returned that the *hsp70*-expressed proteins can have allergenic epitopes. When the additional search term "minipromoter" was added to the search, no references were returned. This refinement added confidence to the results of the search, that the mini-promoter region from *hsp70* is not directly reported to have allergenic epitopes.

The search for "*Drosophila melanogaster"* with the search terms "K10 terminator", "*adh* intron" and "drosomycin 3'UTR" returned 0, 5 and 1 papers respectively. None of these papers from a review of the abstract indicated that there was any direct association with the gene sequence and allergy.

Pathogenicity: A PubMed search was also conducted using the search terms "*Drosophila melanogaster"* AND "pathogenicity" on 2 Dec 2013 and the searches saved in PubMed. A total of 377 citations were returned, all of which used the *Drosophila* as a model system for the study of intracellular pathogens and the host response, none describing any innate pathogenicity of the organism.

A similar search was conducted on the same day using the search terms "pathogen*" AND

a)"*Trichoplusia ni*"

This search returned 111 references. Some of these described the use of *T. ni* as a permissive host for assessment of baculoviruses, nuclear polyhedrosis viruses and interactions with *Bacillus thuringinensis*, or the use of *piggyBac* sequences for insect transgenesis. No direct pathogenicity of *Trichoplusia ni* was identified from examination of the abstracts.

b) "*Escherichia coli*" AND "CSH26" and "RR1"

The search terms "CSH26" and "RR1" was used as examples of the laboratory strains of *E. coli* cited in Altschmied et al.(1988). This search returned 0 references when conducted on 2 Dec 2013. We therefore conclude there is unlikely to be any direct pathogenicity from the sequences.

c) *Discosoma*

Eight references were returned when conducted on 2 Dec 2013. All abstracts referred to the use of the DsRed gene and its variants as a marker for explorations of toxicity and pathogenicity in other species and were devoid of direct pathogenicity as a result of the use of *Discosoma* fluorescent proteins.

d) Herpes simplex virus (HSV) VP16 transcriptional activator

Six papers were returned with the search. VP16 is a virion phosphoprotein of HSV and a transcriptional activator of viral *immediate-early* (IE) genes and requires an acidic transcriptional domain, which if absent then the VP16 is impaired in its capacity to support the infectious cycle. VP16 also requires transport to the nuclear membrane and binding to various co-factors in the nucleus for activation. For activation to occur the co-factors must be present. In pOX513, VP16 is used in a fusion protein with domains from *E. coli* and known as tTAV. tTA and its variants (e.g. tTAV) have been widely used in a large number of eukaryotic systems without any pathogenesis being observed. (There are over 10,000 publications on its use in a wide range of systems[1]). It is for these reasons that it is highly unlikely that the use of the VP16 sequence in *Aedes aegypti* could be the cause of pathogenesis in humans or animals.

Conclusions

A review of the literature in the PubMed database for toxicity, allergenicity and pathogenicity of donor organisms has been conducted. Furthermore, bioinformatic analysis of the gene construct sequences (tTAV and DsRed2) for allergenicity and toxicity has been conducted by the Food Allergy Research and Resource Program at the University of Nebraska U.S.A. (**OX513A Technical Dossier- Part A - Appendix 2**)

These analyses determined that there are no sequences in the construct that are directly or indirectly likely to be toxic or allergenic or pathogenic or in other ways harmful to humans,

[1] http://www.tetsystems.com/science-technology/highlighted-publications/ [accessed 31/08/2016)]

animals or the environment. However, although not directly or indirectly toxic, it is the specific and intended effect of the genetic modification that the expression of tTAV confers conditional lethality in both sexes to the progeny of matings of *Aedes aegypti* OX513A males with wild females, in the absence of tetracyclines.

Appendix 3.1

OX513A Part A - Expert opinion 2015 Transgenic protein tTAV - assessment of allergenic risk

TRANSGENIC PROTEIN tTAV: Assessment of allergenic risk

Background

tTAV is a recombinant tetracycline repressible activator protein.

Genetically modified, transgene homozygous, mosquitos (*Aedes aegypti)* have been developed to control and limit mosquito population growth and vector transmission.

The transgene codes for a protein (tTAV) that inhibits cellular function. The dominant lethal transgene is carried in genetically modified male mosquitos that are released to breed with wild-type females. The trait prevents the resulting progeny that carry the gene from reaching maturity in the absence of tetracycline.

The tTAV gene is expressed in a number of transgenic insect tissues and it is probable, therefore, that the gene will be transcribed in the salivary glands of transgenic mosquitos.

The concern that has been raised is that if tTAV protein has inherent allergenic properties, and if this protein is indeed in the saliva, then the protein could potentially induce allergic sensitisation in those bitten by female mosquitos (male mosquitos do not bite). There is a case to answer because allergic reactions due to sensitisation to normal mosquito salivary proteins have been described (Kulthanan et al., 2010). Alternatively/additionally, a related concern is that tTAV might have a level of homology with a known protein allergen sufficient to elicit an allergic reaction in those already sensitised to the cross-reactive protein allergen.

The two issues addressed here are: (a) whether there is an inherent allergenic hazard, and (b) whether there are possible human health risks with respect to allergic sensitisation.

Inherent allergenic hazard

The primary approach adopted to evaluate the inherent allergenic potential of tTAV has been to use a suite of bioinformatic tools to examine whether tTAV displays sequence homology with, or structural similarity to, known protein allergens. This approach was developed originally for the purposes of determining whether transgenes introduced into crop plants had the potential to cause allergic sensitisation and food allergy in future consumers. However, it must be appreciated that the factors that confer on proteins allergenic activity are independent of the route through which encounter with/exposure to protein occurs. That is, the properties that confer on proteins an ability to cause food allergy are the same as those that will enable a protein to cause allergic sensitisation of the respiratory tract. Thus, for instance, ovalbumin from hens' eggs can cause food allergy and also respiratory allergy among those working in egg processing plants (James and Crespo, 2007). Moreover, there is now growing evidence that allergic sensitisation to peanut proteins can occur via skin contact in addition to dietary exposure (Kimber et al., 2014). It is therefore legitimate to use this well-established and well-validated bioinformatics approach to evaluate whether proteins have intrinsic allergenic hazard irrespective of the route(s) through which exposure may occur.

In the first series of bioinformatics analyses it was reported, using standard assessment criteria, that tTAV lacked sequence homology with known allergens (or toxins) (Goodman, 2011). This was subsequently confirmed in a second updated analysis in which it was again established that tTAV lacked significant homology with any known allergens. In the same series of investigations it was also reported that a second transgene product, DsRed2, a red fluorescent marker protein derived from coral and sea anemone species, also lacked homology with any known allergens (Goodman, 2013).

The conclusion drawn from that second series of bioinformatics analyses was that tTAV (and DsRed2) lacks allergenic potential and does not display cross-reactivity with any known protein allergens (Goodman, 2013).

On the basis of these data it can be stated that tTAV protein does not have the inherent potential to induce allergic sensitisation. The tTAV protein also lacks cross-reactivity with known human allergens and will therefore fail to elicit allergic reactions in subjects sensitised to other proteins.

The conclusion is that neither tTAV, nor DsRed2, represent an allergenic hazard.

Human allergy health risks

It can be argued that if tTAV (and DsRed2) lack inherent allergenic properties (either the ability to cause the acquisition of sensitisation, or the ability to elicit allergic reactions in subjects sensitised to cross-reactive proteins), then there are no health risks irrespective of the route of exposure.

However, for the purposes of completeness it is important to emphasise that even if there did exist an allergenic hazard then the likelihood that that would translate into a human health risk is very low.

In this instance exposure would be associated solely with bites by female mosquitos resulting in the intradermal delivery of salivary proteins. Although there is a precedent for the acquisition of sensitisation to proteins constitutively borne in mosquito saliva, the amount of transgene product that would be encountered via this route would be exceedingly small, if present at all, and unlikely to elicit an immune response.

Conclusions

- The available evidence indicates that tTAV (and DsRed2) lacks the inherent potential to induce allergic sensitisation.
- In addition, neither tTAV, nor DsRed2, display a level of homology with known human allergens that would be required for the elicitation of cross-reactive allergic reactions.
- Levels of exposure to tTAV (and DeRed2) via mosquito bite will be extremely low, if present at all, and unlikely to initiate an immune response.
- The transgene proteins to not pose human health risks with regard to allergy or allergic sensitisation.

References

Goodman RE (2011) Bioinformatics evaluation of transgenic protein tTAV from mosquito. Report to Oxitec Ltd (unpublished).

Goodman RE (2013) Bioinformatics analysis for risks of allergenicity and toxicity of proteins encoded by the two genes introduced into genetically engineered mosquitos (*Aedes aegypti*), strain OX513A for production of sterile males to reduce vector transmission of important human diseases. Report to Oxitec Ltd (unpublished).

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Kimber I, Griffiths CEM, Basketter DA, McFadden JP, Dearman RJ (2014) Epicutaneous exposure to proteins and skin immune function. Eur. J. Dermatol. 24, 10-14.

Kulthanan K, Wongkamchai S, Triwongwaranat D (2010) Mosquito allergy: clinical features and natural course. J. Dermatol. 37, 1045-1031.

Ian Kimber, January 2015

Appendix 4

OX513A Part A - OX513A insecticide resistance test-2011

Do Not Photocopy This Document COPY NO.: 2

LSTM
LIVERPOOL SCHOOL OF TROPICAL MEDICINE

INSECTICIDE TESTING FACILITY

Final Study Report

Reference Number: ITF/2011/001

Study Title: Evaluation of insecticide susceptibility status of RIDL strain of *Aedes aegypti.*

Test organism: *Aedes aegypti* Strain OX513A RIDL

Distribution: (1) Master study File, (2) Oxitec Ltd. (3) Head of Insecticide Testing Facility, (4) Head of Vector Group,

Author:	**Study Director:**
Print Name: John Gilmour	**Print Name:** Hilary Ranson
Date: 5th April 2011	**Date:** 5-4-11

Revision History:

Revision	Change Description	Author	Approved by	Date
01	Original version	J. Gilmour		5/4/11

Test Facility: Insecticide Testing Facility, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA

Client: Oxitec Limited, 71 Milton Park, Oxford OX14 4RX

Brief Summary: The *Aedes aegypti* Strain OX513A RIDL is fully susceptible to WHO discriminating doses of 4 of the 5 insecticides tested. 53% survival after bendiocarb exposure was observed. (Note bendiocarb 'resistance' has been detected in several alternative laboratory reference strains and this result suggests, rather than indicating resistance, that the bendiocarb discriminating dose is not appropriate for *Aedes aegypti*). Neither of the knock-down resistance (kdr) mutations, associated with resistance to pyrethroids and DDT (1016 and 1534) was present in the *Aedes aegypti* OX513A RIDL strain.

1.0 Responsible Personnel

All personnel involved in the completion of this study are listed below:

1.1 Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA:

(i) Author: John Gilmour B.Sc, MBA, Head of ITF.

(ii) Study Director: Hilary Ranson PhD Head of Vector Group

(iii) Ms Lori Flood B.Sc: Laboratory Technician

(iv) Ms Grace Matthews B.Sc: Insectary Technician

1.2 Oxitec Limited, 71 Milton Park, Abingdon, Oxford OX14 4RX:

(i) Andrew McKemey (PhD) Technical Development Manager

2.0 Laboratory QA Statement

All investigations were carried out to standard operating procedures at the Insecticide Testing Facility (ITF), Liverpool School of Tropical Medicine (LSTM), Pembroke Place, Liverpool L3 5QA. This facility is not currently GLP accredited. All laboratory equipment used has been verified to function properly and has been calibrated either internally or externally as appropriate. All study staff have been appropriately trained in performance of the required experimental procedures. Experimental data and the final report are reviewed by the Head of ITF for accuracy, completeness and clarity and then approved by the Head of the Vector Group.

3.0 Introduction

Oxitec Ltd is a British company using modern biotechnology to develop transgenic insect strains that can be used to control pests of both public health and agriculture. A key part of this development work is the characterization of these transgenic insect species for insecticide resistance. LSTM has appropriate expertise and experience in insecticide testing and genetic characterization. LSTM offers a suitable testing service which includes genotyping as well as bioassays to clients such as Oxitec Ltd.

Viable eggs from *Aedes aegypti* Transgenic Strain OX513A RIDL were provided by Oxitec Ltd. A susceptible laboratory strain (*Aedes aegypti* New Orleans) was provided by LSTM. LSTM has agreed to characterise the transgenic insect strain provided both with respect to bioassay against five insecticides and genotyping for the presence of two mutations associated with insecticide resistance.

4.0 Aim

The aim of this study was to characterise *Aedes aegypti* Strain OX513A RIDL with respect to

(i) Resistance against the following insecticides: temephos, permethrin, deltamethrin, bendiocarb and malathion.

(ii) Genotyping for 2 knock-down resistance (kdr) mutations associated with resistance to insecticides: 1016, and 1534.

5.0 Study Schedule

All work on this study was carried out between 4th January 2011 and 5th April 2011 at the LSTM facilities in Liverpool.

6.0 Test Strain / Reference Strain

6.1 Test strain: *Aedes aegypti* Strain OX513A RIDL

6.2 Reference strain: *Aedes aegypti* Strain New Orleans.

7.0 Test Systems

7.1 Bioassays:

(i) Larval bioassay was carried out with temephos as follows: 100 larvae from *Aedes aegypti* Strain OX513A RIDL were set up as 4 x25 mosquitoes in 250 ml water containing 0.012mg/l temephos and left overnight. Mortality was scored after 24 hours.

(ii) Adult mosquito bioassays were performed using the WHO cone test as described in WHO bulletin (2006).

7.2 Genotyping Assays:

Genotyping for the two kdr mutations was carried out using

(i) HOLA (1016 mutation), method according to A. Lynd *et al* (2005)

(ii) Tetraplex PCR (1534 mutation), method according to A. F. Harris *et al* (2010)

8.0 Experimental Procedures

8.1 Bioassays

Eggs provided were reared by ITF at LSTM and the following bioassays were carried out both with the transgenic line and with the susceptible strain:

(i) 4th instar larvae were tested using a 24 hour exposure to a discriminating dose of temephos (0.012mg/l)

(ii) 2-3 day old female adults were tested using a 1 hour exposure to the following insecticides
- 0.75% permethrin
- 0.05% deltamethrin
- 0.1% bendiocarb
- 0.8% malathion

All bioassays were performed on a minimum of 100 individuals from the RIDL OX513A strain and 100 from the susceptible New Orleans strain (for bendiocarb and pyrethroids only).
All assays were performed according to standard WHO procedures.
Mortality was recorded 24 hours after exposure. Control bioassays in which mosquitoes were exposed to the carrier only were performed simultaneously. In any cases where control mortality exceeded 5% the results of the days assays were discarded.

8.2 kdr genotyping:

10 Individuals from the *Aedes aegypti* Strain OX513A RIDL colony were genotyped for the two kdr mutations associated with pyrethroid and DDT resistance: 1016 and 1534 using either the HOLA method or tetraplex PCR method.

10 individuals were considered to be sufficient to detect any mutations present as the material provided was representative of a highly derived colony.

9.0 Results

Results obtained with the bioassays are presented in table 1 below and results obtained with the genotyping assays are given in table 2:

Table 1: Mosquito mortality recorded 24 hours after exposure to insecticide

Insecticide	No tested	No Alive	No Dead	% mortality		No tested	No Alive	No Dead	% mortality
	OX513A					New Orleans Strain			
Temephos	102	0	102	100		n/d	n/d	n/d	n/d
Permethrin	100	0	100	100		63	0	63	100
Deltamethrin	100	0	100	100		41	0	41	100
Bendiocarb	200	106	94	47		100	49	51	51
Malathion	100	0	100	100		n/d	n/d	n/d	n/d

Mortality recorded in all control bioassays was 0% and so all results were considered to be valid.

Table 2: Results of kdr genotype tests.

Sample name	Tetraplex (1534)	Hola (Val1016Ile)
RIDL 1	Wild type	Wild type
RIDL 2	Wild type	Wild type
RIDL 3	Wild type	Wild type
RIDL 4	Wild type	Wild type
RIDL 5	Wild type	Wild type
RIDL 6	Wild type	Wild type
RIDL 7	Wild type	Wild type
RIDL 8	Wild type	Wild type
RIDL 9	Wild type	Wild type
RIDL 10	Wild type	Wild type

10.0 Discussion

Results obtained with the WHO cone tests indicate susceptibility of the *Aedes aegypti* Strain OX513A RIDL to discriminating doses of temephos, permethrin, deltamethrin and malathion. Significant survival to 0.1% bendiocarb was however noted. However, high survival rates after 1 hour exposure to 0.1 % bendiocarb exposure to were also observed in the susceptible New Orleans strain. These results taken together indicate that the bendiocarb discriminating dose is not appropriate for *Aedes aegypti* rather than indicating actual resistance to bendiocarb in the OX513A strain.

Results obtained with genotyping against the knock-down resistance (kdr) mutations, associated with resistance to pyrethroids and DDT (1016 and 1534) indicated that neither of these mutations are present in the *Aedes aegypti* OX513A RIDL strain.

11.0 Conclusions

The *Aedes aegypti* OX513A RIDL strain is fully susceptible to WHO discriminating doses of temephos, permethrin, deltamethrin and malathion. However 53% survival after bendiocarb exposure was observed. It should however be noted that apparent bendiocarb 'resistance' has been detected in several alternative laboratory reference strains. It is concluded that these results suggest that the bendiocarb discriminating dose is not appropriate for *Aedes aegypti* and does not indicate actual resistance to bendiocarb. Neither of the tested kdr mutations (1016 or 1534) associated with resistance to pyrethroids and DDT was present in the Aedes aegypti OX513A RIDL strain.

12.0 Cross Referenced Methods

12.1 "A simplified high-throughput method for pyrethroid knock-down resistance (kdr) detection in *Anopheles gambiae*", A. Lynd *et al* Malaria Journal **4**, pp16-21 (2005).

12.2 "Pyrethroid Resistance in *Aedes aegypti* from Grand Cayman" Angela. F. Harris *et al.* Am. J. Trop. Med. Hyg. **83**(2), pp 277-284 (2010).

12.3 "Guidelines for testing Mosquito adulticides for Indoor residual spraying and treatment of Mosquito nets" WHO/CDS/NTD/WHOPES/GCDPP/2006.3 World Health Organization 20 Avenue Appia CH-1211 Geneva 27 Switzerland (2006).

13.0 Archiving

All DNA samples tested will be archived for a period of 1 year in secure storage at -70°C in LSTM. The Final Study Report and raw data will be archived for a period of 5 years in a secure location at LSTM.

14.0 Final Study Report Approvals

14.1 Author: 5/4/2011

John Gilmour B.Sc., MBA
Head of Insecticide Testing Facility
LSTM
Liverpool

14.2 Study Director

Hilary Ranson PhD
Head of Vector Group
LSTM
Liverpool

Appendix 5

OX513A Part A - Final Report 232SRFR12C1 Poecilia Aedes OX513A

FINAL REPORT

STUDY NUMBER: 232SRFR12C1 **TRIAL NUMBER:** SRFR12-001-232XC1

TITLE

A laboratory prolonged toxicity study to determine the effects of ingestion of larvae and pupae of the genetically modified sterile mosquito strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae); according to OECD No. 204 (1984) modified for oral route of exposure.

AUTHOR

E. YTHIER

GUIDELINE

OECD No. 204 (1984) modified for oral route of exposure

SYNTECH RESEARCH TEST FACILITY

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France

TEST SITE

SynTech Research France S.A.S.
1095 Chemin du Bachas
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France

SPONSOR

Oxitec Ltd
71, Milton Park
Abingdon, Oxfordshire, OX14 4RX
United Kingdom

Study Initiation Date: 05 OCT 2012

Study Completion Date: 11 MAR 2013 **Total number of pages:** 43

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GLP COMPLIANCE STATEMENT

A laboratory prolonged toxicity study to determine the effects of ingestion of larvae and pupae of the genetically modified sterile mosquito strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae); according to OECD No. 204 (1984) modified for oral route of exposure.

I confirm that I fulfilled the responsibilities of Study Director for the above non-clinical health and environmental safety (regulatory) study. I declare that the objectives laid down in the Study Plan were achieved and the data generated are valid.

Each phase of this study was conducted in accordance with the Principles of Good Laboratory Practices (GLP):
* The OECD Principles of Good Laboratory Practice, N°1, as revised in 1997 [ENV/MC/CHEM (98) 17].
* The application of the OECD Principles of GLP to the Organisation and Management of Multi-site Studies, N°13, 2002 [ENV/JM/MONO (2002) 9].
* The country-specific regulations embodying these principles where appropriate.

These phases were in compliance to GLP with the following exceptions: raw data related to the preparation of ISO reconstituted water. These data were not generated according to GLP principles. These exceptions are considered not to affect the GLP status of the study and the validity of the conclusions drawn.

In addition, I certify that Study Plan and Final Report are conformed to the OECD Principles of GLP and French GLP regulations (« Article Annexe II à l'Article D523-8 du Code de l'Environnement du 16 Octobre 2007 »).

E. YTHIER
Study Director
SynTech Research

11 MAR 2013
Study completion date

QUALITY ASSURANCE STATEMENT

A laboratory prolonged toxicity study to determine the effects of ingestion of larvae and pupae of the genetically modified sterile mosquito strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae); according to OECD No. 204 (1984) modified for oral route of exposure.

Accuracy of transcription from the raw data generated by SynTech Research to the Final Report was checked. The Final Report fully reflects the raw data generated during the study.
The following study specific audits have been carried out by the Quality Assurance personnel at SynTech Research France in accordance with SynTech Research policy and procedures for Good Laboratory Practice.

Table 1: Specific study inspection dates

Type of Inspection	Inspection date	Date of inspection report	Date of dispatch to*
Study Plan verification	04 OCT 2012	05 OCT 2012	05 OCT 2012
Lab-based inspections: Test system, application, assessment and Lab Note Book	25 OCT 2012	09 NOV 2012	09 NOV 2012
Reported data: Final Report, Lab Note Book and raw data	21 DEC 2012	11 JAN 2013	11 JAN 2013

* Study Director, Management and Principal Investigator if relevant

In addition, the following facility and procedure based inspections associated with this type of study have been carried out.

Table 2: Facility and procedure inspection dates

Type of Inspection	Inspection date	Date of inspection report	Date of dispatch to*
Facility: Nîmes (30) (organization, staff, facilities, equipment, documentation)	14 MAY 2012	25 MAY 2012	25 MAY 2012
Facility: La Chapelle de Guinchay (71) (organization, staff, facilities, equipment, documentation)	25-27 JUL 2012	22 AUG 2012	22 AUG 2012
Process (balance & masses calibration)	10 APR 2012	18 MAY 2012	18 MAY 2012
Process (weighing)	30 APR2012	18 MAY 2012	18 MAY 2012
Process (shipping)	22 MAY 2012	24 MAY 2012	24 MAY 2012
Process (archiving)	28 SEP 2012	28 SEP 2012	28 SEP 2012

*Management and relevant personals

Y.TACIK
Test Facility QA
SynTech Research

11 MAR 2013
Date

PRINCIPAL STUDY PERSONNEL

E. YTHIER	Study Director
L. MARTIN	Principal Investigator

REVIEWERS / SUPERVISORS

p.o.

P. ESCHENBRENNER
Test Facility Management
SynTech Research

11 March 2013

Date

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SUMMARY

Report: Ythier, E. (2012): A laboratory prolonged toxicity study to determine the effects of ingestion of larvae and pupae of the genetically modified sterile mosquito strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae); according to OECD No. 204 (1984) modified for oral route of exposure.
Source: SynTech Research France, 613 Route du Bois de Loyse, F-71570 La Chapelle de Guinchay, France
Report No: 232SRFR12C1, issued 11 March 2013
Guidelines: OECD No. 204 (1984) modified for oral route of exposure
Deviations: No deviation
GLP: Yes

Materials and methods:
Guppys *Poecilia reticulata* (Actinopterygii: Poeciliidae), measuring 20 to 26 mm at the start of the test, were orally exposed to mixed larvae and pupae of the genetically modified sterile strain *Aedes aegypti* OX513A over a period of 14 days, in laboratory semi-static conditions.
During the study period, the fish were fed with the transgenic mosquitoes once daily, by incorporating freshly defrosted larvae and pupae into the fish diet, at the rate of 700 g mosquitoes/kg diet. The rate to be tested was determined following a 96-hour non-GLP compliant range-finding study conducted with 20, 100, 300, 500 and 700 g mosquitoes/kg diet, in order to evaluate the maximum rate of insects (mosquitoes) the guppy can ingest (in natural conditions the mean ratio of insects ingested by this species is usually about 50% w/w, i.e. 500 g insects/kg food). Each tested rate was entirely consumed and no adverse effect was observed during the 96-hour range-finding study. The quantity of diet administered daily did not exceed the amount ingested immediately by the fish and was kept constant during the study duration, i.e. 4 per cent of the initial fish weight. A control (non-genetically modified mosquitoes of the same background strain, incorporated to the fish diet at the same rate of 700 g mosquitoes/kg diet) was included to assess the natural mortality rate, appearance, size and behaviour of the test organisms. A toxic reference item (potassium dichromate, applied at a concentration of 100 mg a.s./L) was included to indicate the relative susceptibility of the test organisms and the test system.
Acute and sublethal (appearance, size and behaviour) effects were observed daily during the test period. Data were analysed for significant differences compared to the control group using ANOVA ($p \leq 0.05$) and to determine values for the LR_{50}, ER_{50}, LOER and NOER.

Dates of work: 15 October 2012 - 28 October 2012

Findings (Table 3): Summary of *P. reticulata* mortality, length and weight after 14-day oral exposure to *Aedes aegypti* OX513A

Test item	Genetically modified sterile strain *Aedes aegypti* OX513A		
Test organism	*Poecilia reticulata*		
Test medium	ISO reconstituted water		
Exposure	Daily oral exposure		
Endpoint	14-day mortality [%]	14-day length [mm]	14-day weight [mg]
Control (700 g non-GM mosquitoes/kg diet)	10	22.44	198.3
OX513A (700 g GM mosquitoes/kg diet)	0	23.20	212.9
LR_{50} / ER_{50} [g GM mosquitoes/kg diet]	> 700		
LOER [g GM mosquitoes/kg diet]	> 700		
NOER [g GM mosquitoes/kg diet]	700		

GM = genetically modified

Conclusions:

The study is valid since mean mortality in the control did not exceed 10% during the test period (actual value: 10%), dissolved oxygen concentration was over 60% of the air saturation value throughout the test (actual minimum value: 73.5%) and environmental conditions (T°, pH) remained constant throughout the test.

The potential acute and sublethal effects of ingestion of mosquitoes of the genetically modified sterile strain *Aedes aegypti* OX513A on the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae) were investigated during 14 days in laboratory semi-static conditions.

There was no significant difference between mortality, fish length, weight, appearance and behaviour in the control and the test item, after 14 days. Hence the NOER was found to be 700 g GM mosquitoes/kg diet and the LOER and LR_{50}/ER_{50} were estimated to be > 700 g GM mosquitoes/kg diet.

INTRODUCTION

The test item is a mixture of larval and pupal life stages (collected 7-8 days post hatching) of heterozygous *Aedes aegypti* mosquito strain OX513A from an Asian background expressing a repressible lethality trait (based on the tet-off system (Gossen and Bujard 1992) and DsRed2 fluorescent marker gene).

The objective of the study was to determine potential acute and sublethal effects of ingestion of mosquitoes of the genetically modified sterile strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae) in laboratory semi-static conditions, following oral exposure to larval and pupal life stages of *Aedes aegypti* OX513A over a period of 14 days.

During the study period, the fish were fed with the transgenic mosquitoes once daily, by incorporating freshly defrosted larvae and pupae into the fish diet, at the rate of 700 g mosquitoes/kg diet. The quantity of diet administered daily did not exceed the amount ingested immediately by the fish and was kept constant during the study duration, i.e. 4 per cent of the initial fish weight.

The rate to be tested was determined following a 96-hour non-GLP compliant range-finding study conducted with 20, 100, 300, 500 and 700 g mosquitoes/kg diet, in order to evaluate the maximum rate of insects (mosquitoes) the guppy can ingest (in natural conditions the mean ratio of insects ingested by this species is usually about 50% w/w, i.e. 500 g insects/kg food). Each tested rate was entirely consumed and no adverse effect was observed during the 96-hour range-finding study.

A control (non-genetically modified mosquitoes of the same background strain as the test substance, incorporated to the fish diet at the same rate of 700 g mosquitoes/kg diet) was included to assess the natural mortality rate, appearance, size and behaviour of the test organisms. A toxic reference item (potassium dichromate, applied at a concentration of 100 mg a.s./L) was included to indicate the relative susceptibility of the test organisms and the test system.

Acute and sublethal (appearance, size and behaviour) effects were observed once a day during 14 days. As no adverse effect was observed in the fish group fed with OX513A mosquitoes between 7 and 14 days, whilst control mortality remained at an accepted level (10%), the study duration was not extended.

The study was conducted in accordance with the OECD guideline No. 204 (1984) modified for oral route of exposure. The experimental phase of the study was performed at the test site of SynTech Research France SAS, 1095 chemin du Bachas, 30000 Nîmes, France.

All aspects of the study were carried out according to international Good Laboratory Practice (GLP) guidelines and were based on the international codes of GLP (see References on p.19).

The study encompassed the objectives of Regulation (EC) No. 1107/2009 and was designed to comply with the FAO Guidelines on Producing Pesticide Residue Data from supervised trials, Rome 1990 and “Commission Working Document 7029/VI/95 - Rev. 5, July 1997”.

The study was conducted in accordance with French GLP regulations (“Article Annexe II à l'Article D523-8 du Code de l'Environnement du 16 octobre 2007”). This study is referred to GLP area of expertise No.4: “Environmental toxicity studies on aquatic or terrestrial organisms”.

TEST ITEM *AEDES AEGYPTI* OX513A

Table 4:

Test item code	*Aedes aegypti* OX513A
Physical state, appearance	Mixture of larval and pupal life stages in distilled water
Quantity received / Date of receipt	327.33 g on 31 August 2012
Storage requirement	In its original container, tightly closed, in frozen conditions.
Test item supply	Oxitec Ltd, 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX, United Kingdom

Three batches of the test item were received on 31 AUG 2012 at the test site of Nîmes and identified as SynTech Research No. NI12-303, NI12-304 and NI12-305. The test item was stored deep frozen between -18.4°C and -38.3°C between its receipt and its last use.

CONTROL ITEM *AEDES AEGYPTI* WILD TYPE

Table 5:

Test item code	*Aedes aegypti* Wild Type
Physical state, appearance	Mixture of larval and pupal life stages in distilled water
Quantity received / Date of receipt	203.01 g on 31 August 2012
Storage requirement	In its original container, tightly closed, in frozen conditions.
Test item supply	Oxitec Ltd, 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX, United Kingdom

Two batches of the control item were received on 31 AUG 2012 at the test site of Nîmes and identified as SynTech Research No. NI12-306 and No. NI12-307. The control item was stored deep frozen between -18.4°C and -38.3°C between its receipt and its last use.

REFERENCE ITEM POTASSIUM DICHROMATE

Table 6:

Reference item	Potassium dichromate
Batch No.	102403H
Reference item (nominal conc.)	Potassium dichromate (1000 mg/kg)
Reference item (actual conc.)	Potassium dichromate (999.7 mg/kg)
CAS No.	7778-50-9
Formulation density [g/ml]	1 (solid)
Physical appearance	Orange solid crystals
Storage requirement	Dry, cool and well-ventilated area
Product supply	Merck KGaA, 64271 Darmstadt, Germany

The reference item was received on 03 DEC 2010 at the test facility of La Chapelle de Guinchay (identified as SynTech Research No. CG10-349) and transferred to the test site of Nîmes on 23 MAR 2011 (identified as SynTech Research No. NI11-302). The reference item was stored between 12.6°C and 24.9°C between its receipt and its last use. The material safety data sheet was available on 03 DEC 2010. A retained sample of formulated product used as reference item is kept by SynTech Research (No. CG10-349A).

EXPERIMENTAL PHASE

Study Plan Amendments and Deviations

No Study Plan Amendment and Deviation.

Study organisation

Table 7:

Study Sponsor:	Oxitec Ltd 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX, United Kingdom	
Study Monitor and Sponsor's Representative:	Camilla BEECH	Tel: 44 (0)1235 433549 e-mail: Camilla.Beech@oxitec.com
Test Facility:	SynTech Research France SAS 613 route du Bois de Loyse, 71570 La Chapelle de Guinchay, France	Tel: +33 (0)3 85 36 82 36 Fax: +33 (0)3 85 36 78 97
Management: Study Director: Lead Quality Assurance:	Pierre ESCHENBRENNER Eric YTHIER Yannick TACIK	e-mail: peschenbrenner@syntechresearch.com e-mail: eythier@syntechresearch.com e-mail: ytacik@syntechresearch.com
Test site for experimental phase:	SynTech Research France SAS Aquatoxicology Laboratory 1095 chemin du Bachas F-30000 Nîmes, France	Tel: +33 (0)4 66 70 98 65
Principal Investigator:	Lucie MARTIN	e-mail : lmartin@syntechresearch.com
Item supply:	Oxitec Ltd Merck KGaA	71, Milton Park, Abingdon, Oxfordshire, OX14 4RX, United Kingdom 64271 Darmstadt, Germany

Archiving

After completion of the final report, the Study Director will transfer the following data generated in the study to:

Camilla BEECH
Oxitec Ltd
71, Milton Park, Abingdon,
Oxfordshire, OX14 4RX,
United Kingdom

Data to be transferred will include, but not be limited to:
1. The original study plan, amendments, and deviations
2. The original final report
3. Test item characterisation and certification documentation
4. The original raw data package

Copies of the study plan, raw data, amendments, deviations and final report, as well as all non-study specific data (e.g. log books describing equipment maintenance and calibration) will be stored in the archives of SynTech Research France SAS for ten years. No data will be discarded without the Sponsor's prior written consent.

Test system

The experimental phase of the study was conducted at the Aquatoxicology laboratory of SynTech Research France SAS, 1095 chemin du Bachas, 30000 Nîmes, France.

The fish used for this study were the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae; source: La Grande Rivière, France). The fish were obtained and held in the laboratory for 12 days before they were used for testing. In order to adapt the fish to the test conditions, a fish culture in the test medium was prepared 12 days before start of the test under the following conditions:

- light: 16 hours photoperiod daily
- temperature: 21°C to 25°C
- oxygen concentration: at least 80% of air saturation value
- feeding (diet without mosquitoes - see diet composition below): once daily until 24 hours before the study start

Following a 48-hour settling-in period, mortality was recorded to be < 5% (actual value: 0%) and the batch was accepted to be used for the study.

All organisms used for the study were originated from cultures established from the same healthy stock of fish. At the start of the test, the animals were 20 mm ± 10 and the loading was < 1 g fish/L test medium (actual size values 20 to 26 mm / mean 22.5 mm; actual loading value 0.698 g fish/L; based on 10 organisms randomly sampled in the fish culture the day before the start of the test; see Appendix 2). They were in good health and free from any apparent malformation. The fish were not fed from 24 hours before the test start and during the test period.

Test vessels (= test units) consisted of 4 L glass jars containing 3 L of test medium. During the test period, test units were capped to reduce the loss of water due to evaporation and to avoid the entry of dust into solutions. Each test unit was labelled with the study number and a unique test unit number.

The ISO test medium was used. The composition of the test medium is described in Annex 3 of OECD guideline No. 203. The test medium was made at the test site, using distilled water. The test medium was aerated until oxygen saturation and then stored for 2 days prior to use. The test medium was aerated during the study. The test medium was renewed twice weekly and at the time of each renewal

the test medium temperature, dissolved oxygen and pH were recorded (see Appendix 3). At each renewal, a second series of test vessels were prepared and the test organisms were transferred to them.

The study comprised a control, a toxic reference item and one rate of the test item: 700 g mosquitoes/kg diet. There was one test unit with 10 replicates (= 10 fish) for each test item, control and toxic reference item.

A control (non-genetically modified mosquitoes of the same background strain, incorporated to the fish diet at the rate of 700 g mosquitoes/kg diet) was included to assess the natural mortality rate, appearance, size and behaviour of the test organisms. A toxic reference item was included in the study to demonstrate the susceptibility of the test organism and the sensitivity of the test system. The toxic reference item was potassium dichromate applied at an application concentration of 100 mg a.s./L (the toxic reference group was fed with diet incorporated with non-genetically modified mosquitoes at the rate of 700 g mosquitoes/kg diet).

The items comprised:
- Test item: genetically modified mosquitoes *A. aegypti* OX513A at 700 g mosquitoes/kg diet
- Control: non-genetically modified mosquitoes *A. aegypti* at 700 g mosquitoes/kg diet
- Toxic reference item: potassium dichromate at 100 mg a.s./L (fed with non-genetically mosquitoes *A. aegypti* at 700 g mosquitoes/kg diet)

The diet was administered daily, at the dose of 4% of the initial fish weight. Quantity of administered diet was calculated each day according to the number of living fish. The sequence of feeding was as follows: control group, followed by the test item group and finally the toxic reference item group.
The diet (TetraMin®, used during both holding and exposure periods) consisted of:
- fish and fish derivatives,
- cereals,
- yeasts,
- vegetable protein extracts,
- molluscs and crustaceans,
- oils and fats,
- algae,
- sugars,
- mineral substances.
- components: protein 47%, fat 10%, fiber 3%, vitamins D3 and A, elements Mn, Zn, Fe and Co.

During the holding phase (12 days before fish were used for testing), the diet was administered daily, except during the 24 hours before the study start (exposure phase). The diet was administered without mosquitoes during the holding phase.
During the exposure phase, genetically modified (OX513A) or non-genetically modified (control and reference item) mosquitoes were incorporated to the fish diet at the rate of 700 g mosquitoes/kg diet and the new diet was administered daily.
In both holding and exposure phases, the quantity of diet administered daily did not exceed the amount ingested immediately by the fish and was kept constant during the study duration, i.e. 4 per cent of the initial fish weight. Quantity of administered diet was calculated each day according to the number of living fish.

Twice a week, the temperature, dissolved oxygen and the pH were recorded (see Appendix 3). Test units were kept in controlled environment conditions between 21ºC to 25°C (constant within the range of ± 2ºC; actual values: 20.5-22.4°C) and received 16 hours light (1120-1340 lux) and 8 hours dark cycle. Item groups were placed on separated shelves in the laboratory.

Table 8: Test system summary

Experimental phase location:	Aquatoxicology Laboratory SynTech Research France SAS 1095 Chemin du Bachas 30000 Nîmes, France
Test organism (species):	Guppy, *Poecilia reticulata* Peters (Actinopterygii: Poeciliidae)
Test system:	Items: 3 (1 test item, 1 toxic reference item, 1 control). Test unit: 4 L capped glass jar (one per test item). Test organisms (= replicates): 10 *Poecilia reticulata* in each test unit ; 20 to 26 mm (mean 22.5 mm) and loading 0.698 g fish/L at the start of the test. Test medium: ISO reconstituted water.
Items:	- Test item: genetically modified mosquitoes *A. aegypti* OX513A at 700 g mosquitoes/kg diet - Control item: non-genetically modified mosquitoes *A. aegypti* at 700 g mosquitoes/kg diet - Toxic reference item: potassium dichromate at 100 mg a.s./L (fed with non-genetically mosquitoes *A. aegypti* at 700 g mosquitoes/kg diet)
Number of applications:	1 toxic reference application
Number of feeding:	The fish were fed once daily with prepared diet (4% of the initial fish weight).
Number of renewals:	The test medium was renewed twice weekly.
Replicates:	10 replicates (= fish) for each test item, control and reference item.
Item details:	Item groups were separated from each other in the culturing chamber to avoid contamination between treated/control test units and between treated test units.
Test duration	The duration of the test was 14 days.
Test organism destruction:	At the end of the study, the remaining test organisms were destroyed according to SynTech SOPs.
Test conditions:	Monitoring of environmental conditions was carried out throughout the study, at regular intervals, using calibrated equipment. Organisms were maintained at temperature of 20.5-22.4°C and in 16 hours light cycle (1120-1340 lux).
Guideline:	The study was conducted in accordance with the OECD guideline No. 204 modified for oral route of exposure.

Exposure details

Table 9: item applied concentrations

Item ID	Item	a.s. concentration / L test medium	f.p. concentration / L test medium*	Mosquitoes / kg diet
C101	Control	NA	NA	700 g non-GM mosquitoes / kg diet
T102	OX513A	NA	NA	700 g GM mosquitoes / kg diet
R103	Potassium dichromate	100 mg a.s./L	100.03 mg f.p./L	700 g non-GM mosquitoes / kg diet

* Based on the actual a.s. content of the toxic reference item.
NA = not applicable; a.s. = active substance; f.p. = formulated product; GM = genetically modified.

Assessment details

The test endpoint is acute toxicity. Fish were considered as dead if there is no visible movement and if touching of the caudal peduncle produces no reaction.

Sublethal effects were also recorded. These include all effects observed on the appearance, size and behaviour of the fish that make them clearly distinguishable from the control animals, e.g. different swimming behaviour, different reaction to external stimuli, changes in appearance of the fish, reduction or cessation of food intake, changes in length or body weight.

Food intake was evaluated by verifying if the entire administrated diet quantity was consumed or if remaining diet was found in the test unit 1 hour after its administration.

Each test unit was inspected daily during the exposure period.

Representative samples of the test population were weighed and measured before the test starts. All survivors were weighed and measured at the termination of the test.

The mortality was determined according to the following expression:
Mean mortality (%) = 100 x [(T-L)/T]
L = number of living organisms, T = total number of organisms

The results were corrected for control mortality according to Abbott (1925):

$$M\% = \left(\frac{Mt - Mc}{100 - Mc}\right) \times 100$$

where M% = corrected mortality
Mt = % mortality in the test or toxic reference item group
Mc = % mortality in the control

The statistical evaluation (NOEC/LOEC determination) was conducted with the software Minitab® Release 14.

Table 10: Assessments details and dates

Study Plan timing	Actual date	Action
Day before exposure	14 OCT 2012	Length / weight of representative samples of the test population.
First day of exposure	15 OCT 2012	Application of the reference item and first oral exposure (feeding). Assessment (O_2 / temperature / pH).
Once daily during exposure period	15 to 28 OCT 2012	Assessment (mortality / sublethal effects / food intake).
Twice weekly during exposure period	18, 22, 25 OCT 2012	Test medium renewal. Assessment (O_2 / temperature / pH) on the fresh and aged test medium.
Last day of exposure	28 OCT 2012	Length / weight of all surviving test organisms.

RESULTS

Validity criteria:

The experimental phase of this study is valid, because:
- Mean mortality in the control did not exceed 10% during the test period (actual value: 10%).
- dissolved oxygen concentration was over 60% of the air saturation value throughout the test (actual minimum value: 73.5%) and environmental conditions (T°, pH) remained constant throughout the test (see Appendix 3).

A summary of the results is given below and the individual data are shown in Appendix 2.

Mortality:

Table 11: *P. reticulata* 14-day mean mortality

Item ID	Item	14-day mean mortality [%]
C101	Control (700 g non-GM mosquitoes/kg diet)	10
T102	OX513A (700 g GM mosquitoes/kg diet)	0
R103	Potassium dichromate (100 mg a.s./L)	100*

* Item group significantly different from the control (ANOVA plus Dunnett's after Log transformation, see Appendix 2).
a.s. = active substance; GM = genetically modified.

The mean mortality was 10% in the control and 100% in the toxic reference item. There was no significant difference between mortality in the control and the test item, after 14 days (ANOVA plus Dunnett's, 95% confidence level).

Abbott (1925) corrected mortality:

Table 12: *P. reticulata* 14-day Abbott corrected mean mortality

Item ID	Item	14-day Abbott corrected mean mortality [%]
C101	Control (700 g non-GM mosquitoes/kg diet)	0
T102	OX513A (700 g GM mosquitoes/kg diet)	- 11.1
R103	Potassium dichromate (100 mg a.s./L)	+ 100*

* Item group significantly different from the control (ANOVA plus Dunnett's after Log transformation, see Appendix 2).
a.s. = active substance; GM = genetically modified.

Corrected mortality in the reference item group was 100%. There was no significant difference between corrected mortality in the control and the test item, after 14 days (ANOVA plus Dunnett's, 95% confidence level).

The NOER was found to be 700 g GM mosquitoes/kg diet and both LOER and LR_{50} were estimated to be > 700 g GM mosquitoes/kg diet.

Food intake, body length and weight:

During the exposure period, the entire administrated diet quantity was consumed by the fish in both control and test item. No remaining diet was found in the test units 1 hour after administration.

The day before the start of the test, 10 representative samples of the test population were randomly sampled and were weighed and measured. The animals were 20 to 26 mm (mean 22.5 mm) and 95.5 to 371 mg (mean 206.8 mg; loading 0.698 g fish/L; see Appendix 2). All survivors in control and test item groups were weighed and measured at the termination of the test (see Table 13 below and Appendix 2).

Table 13: *P. reticulata* 14-day body length and weight

Item ID	Item	14-day mean length [mm]	14-day mean weight [mg]
C101	Control (700 g non-GM mosquitoes/kg diet)	22.44	198.3
T102	OX513A (700 g GM mosquitoes/kg diet)	23.20	212.9

GM = genetically modified.

There was no significant difference between fish length and weight in the control and the test item, after 14 days (ANOVA plus Dunnett's, 95% confidence level). Hence the NOER was found to be 700 g GM mosquitoes/kg diet and both LOER and ER_{50} were estimated to be > 700 g GM mosquitoes/kg diet.

Other observed biological effects:

No abnormal behaviour or appearance was observed among the fish in the test item, 14 days after exposure to the test item, in comparison to the control.

DISCUSSION AND CONCLUSION

The study evaluated potential acute and sublethal effects of ingestion of mosquitoes of the genetically modified sterile strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae) in laboratory semi-static conditions, following oral exposure to larval and pupal life stages of *Aedes aegypti* OX513A over a period of 14 days.

During the study period, the fish were fed with the transgenic mosquitoes once daily, by incorporating freshly defrosted larvae and pupae into the fish diet, at the rate of 700 g mosquitoes/kg diet. The quantity of diet administered daily did not exceed the amount ingested immediately by the fish and was kept constant during the study duration, i.e. 4 per cent of the initial fish weight.

Acute and sublethal (appearance, size and behaviour) effects were observed once a day during 14 days.

Table 14: Summary of *P. reticulata* mortality, length and weight after 14-day oral exposure to *Aedes aegypti* OX513A

Test item	Genetically modified sterile strain *Aedes aegypti* OX513A		
Test organism	*Poecilia reticulata*		
Test medium	ISO reconstituted water		
Exposure	Daily oral exposure		
Endpoint	14-day mortality [%]	14-day length [mm]	14-day weight [mg]
Control (700 g non-GM mosquitoes/kg diet)	10	22.44	198.3
OX513A (700 g GM mosquitoes/kg diet)	0	23.20	212.9
LR_{50} / ER_{50} [g GM mosquitoes/kg diet]	> 700		
LOER [g GM mosquitoes/kg diet]	> 700		
NOER [g GM mosquitoes/kg diet]	700		

GM = genetically modified

Conclusion

The potential acute and sublethal effects of ingestion of mosquitoes of the genetically modified sterile strain *Aedes aegypti* OX513A on the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae) were investigated during 14 days in laboratory semi-static conditions.

There was no significant difference between mortality, fish length, weight, appearance and behaviour in the control and the test item, after 14 days. Hence the NOER was found to be 700 g GM mosquitoes/kg diet and the LOER and LR_{50}/ER_{50} were estimated to be > 700 g GM mosquitoes/kg diet.

REFERENCES

Code de l'Environnement. Article Annexe II à l'Article D523-8 du Code de l'Environnement du 16 octobre 2007.

Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonization of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

OECD guideline No. 203. Fish, Acute Toxicity Test (Adopted 17 July 1992). 9 pp.

OECD guideline No. 204. Fish, Prolonged Toxicity Test: 14-day Study (Adopted 4 April 1984). 9 pp.

OECD Principles of Good Laboratory Practice (as revised in 1997). Series on Principles of GLP and Compliance Monitoring, No. 1. ENV/MC/CHEM(98)17 and No. 13. ENV/JM/MONO (2002)9. Organisation for Economic Co-operation and Development, Paris, France which are accepted by regulatory authorities throughout the European Community, the United States of America (FDA and EPA) and Japan (MHW, MAFF and MITI) on the basis of intergovernmental agreements.

Regulation (EC) No.1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC.

Appendix 1
Study Plan 232SRFR12C1

(16 pages)

SynTech Research

STUDY PLAN

STUDY NUMBER: 232SRFR12C1 **TRIAL NUMBER:** SRFR12-001-232XC1

STUDY TITLE:
A laboratory prolonged toxicity study to determine the effects of ingestion of larvae and pupae of the genetically modified sterile mosquito strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae); according to OECD No. 204 (1984) modified for oral route of exposure.

STUDY DIRECTOR:
Eric YTHIER

DATE:
05 October 2012

TEST FACILITY:
SynTech Research France SAS
613 route du Bois de Loyse
71570 La Chapelle de Guinchay, France

SPONSOR:
Oxitec Ltd
71, Milton Park
Abingdon, Oxfordshire, OX14 4RX
United Kingdom

APPROVED BY:

Study Director:
SynTech Research France
05 oct 2012
Eric YTHIER

Test Facility Management:
SynTech Research France
05 oct 2012
Pierre ESCHENBRENNER

Test Facility Lead Quality Assurance:
SynTech Research France
05 OCT 2012
Yannick TACIK

Study Monitor
Sponsor's Representative:
Oxitec Ltd
08 oct 2012
Camilla BEECH

Proposed study timetable

Experimental Starting Date (first exposure):	October 2012
Laboratory experimental completion date:	November 2012
Final Report Issue:	December 2012

Total number of pages: 16

TABLE OF CONTENTS

1. PURPOSE OF THE STUDY

The objective of the study is to determine potential acute and sublethal effects of ingestion of mosquitoes of the genetically modified sterile strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae) in laboratory semi-static conditions, following oral exposure to larval and pupal life stages of *Aedes aegypti* OX513A over a minimum period of 14 days.

During the study period, the fish will be fed with the transgenic mosquitoes once daily, by incorporating freshly defrosted larvae and pupae into the fish diet, at the rate of 700 g mosquitoes/kg diet. The quantity of diet administered daily will not exceed the amount ingested immediately by the fish and will be kept constant during the study duration, i.e. 4 per cent of the initial fish weight.

The rate to be tested was determined following a 96-hour non-GLP compliant range-finding study conducted with 20, 100, 300, 500 and 700 g mosquitoes/kg diet, in order to evaluate the maximum rate of insects (mosquitoes) the guppy can ingest (in natural conditions the mean ratio of insects ingested by this species is usually about 50% w/w, i.e. 500 g insects/kg food). Each tested rate was entirely consumed and no adverse effect was observed during the 96-hour range-finding study.

A control (non-genetically modified mosquitoes incorporated to the fish diet at the same rate of 700 g mosquitoes/kg diet) will be included to assess the natural mortality rate, appearance, size and behaviour of the test organisms. A toxic reference item (potassium dichromate, applied at a concentration of 100 mg a.s./L) will be included to indicate the relative susceptibility of the test organisms and the test system.

Acute and sublethal (appearance, size and behaviour) effects will be observed once a day during 14 days. If adverse effects in the fish group fed with OX513A mosquitoes increase between 7 and 14 days, whilst control mortality remains at an accepted level (i.e. ≤10%), the study duration will be extended to 21 or 28 days (depending on effects between 14 and 21 days) maximum.

The biological part of the study will be performed in the Aquatoxicology laboratory of SynTech Research France SAS and the method will be based on the OECD guideline n°204 modified for oral route of exposure. All aspects of the study will be carried out according to international Good Laboratory Practice (GLP) guidelines, and will be based on the following guidelines and international codes of GLP:

- OECD guideline n°204. Fish, Prolonged Toxicity Test: 14-day Study (Adopted 4 April 1984). 9 pp.

- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonization of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

- OECD Principles of Good Laboratory Practice (as revised in 1997). Series on Principles of GLP and Compliance Monitoring, No. 1. ENV/MC/CHEM(98)17 and No. 13. ENV/JM/MONO (2002)9. Organisation for Economic Co-operation and Development, Paris, France which are accepted by regulatory authorities throughout the European Community, the United States of America (FDA and EPA) and Japan (MHW, MAFF and MITI) on the basis of intergovernmental agreements.

The study will encompass the objectives of Regulation (EC) No 1107/2009 and will be designed to comply with the FAO Guidelines on Producing Pesticide Residue Data from supervised trials, Rome 1990 and "Commission Working Document 7029/VI/95 - Rev. 5, July 1997".

The experimental phase of this study will be conducted in accordance with French GLP regulations ("Article Annexe II à l'Article D523-8 du Code de l'Environnement du 16 octobre 2007"). This study will be referred to GLP area of expertise n°4: "*Environmental toxicity studies on aquatic or terrestrial organisms*".

2. STUDY ORGANISATION

Study Sponsor:	Oxitec Ltd 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX, United Kingdom	
Study Monitor and Sponsor's Representative:	Camilla BEECH	Tel: 44 (0)1235 433549 e-mail: Camilla.Beech@oxitec.com
Test Facility:	SynTech Research France SAS 613 route du Bois de Loyse, 71570 La Chapelle de Guinchay, France	Tel: +33 (0)3 85 36 82 36 Fax: +33 (0)3 85 36 78 97
Management: Study Director: Lead Quality Assurance:	Pierre ESCHENBRENNER Eric YTHIER Yannick TACIK	e-mail: peschenbrenner@syntechresearch.com e-mail: eythier@syntechresearch.com e-mail: ytacik@syntechresearch.com
Test site for experimental phase:	SynTech Research France SAS Aquatoxicology Laboratory 1095 chemin du Bachas F-30000 Nîmes, France	Tel: +33 (0)4 66 70 98 65
Principal Investigator:	Lucie MARTIN	e-mail : lmartin@syntechresearch.com
Item supply:	Oxitec Ltd Merck KGaA	71, Milton Park, Abingdon, Oxfordshire, OX14 4RX, United Kingdom 64271 Darmstadt, Germany

3. GLP PRINCIPLES

3.1. GLP compliance

This study will be performed according to the procedures described in this study plan and in accordance with OECD Principles of GLP and Compliance Monitoring No.1 revised [(ENV/MC/CHEM(98)17] and Monitoring No.13 [(ENV/JM/MONO(2002)9]. The Study Director will be responsible for compliance with the relevant national GLP regulations.

3.2. Quality Assurance

Inspection of laboratory phase of the study, including the report, will be the responsibility of the Test Facility Quality Assurance. Study plan and one critical laboratory phase will be inspected (minimum). All inspection and audit findings will be reported to the Study Director and line management as appropriate, on completion of each audit. The final report will be audited to determine that it reflects the procedures adopted and the raw data generated and that it meets GLP requirements.

3.3. Standard Operating Procedures

Study procedures will follow the applicable SOPs of the respective test facilities/sites, unless they conflict with study plan requirements, which always override standard procedures.

4. ITEM DESCRIPTION

It is the responsibility of the Study Director to request timely dispatch of the relevant test and reference items, Material Safety Data Sheets and Certificate(s) of Analysis to the appropriate study personnel. These are ordered from the Sample Dispatch Co-ordinator.

4.1 Test item

The test item is a mixture of larval and pupal life stages (collected 7-8 days post hatching) of heterozygous *Aedes aegypti* mosquito strain OX513A from an Asian background expressing a repressible lethality trait (based on the tet-off system (Gossen and Bujard 1992) and DsRed2 fluorescent marker gene).
The test item will be supplied frozen (-15°C) in distilled water. Test Item not used will be returned to the Sponsor or discarded by the contract test site following local regulations and after agreement with the Sponsor.

Test item code	*Aedes aegypti* OX513A
Physical state, appearance	Mixture of larval and pupal life stages in distilled water
Quantity received / Date of receipt	327.33 g on 31 August 2012
Storage requirement	In its original container, tightly closed, in frozen conditions.
Test item supply	Oxitec Ltd, 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX, United Kingdom

4.2 Toxic reference item

The Material Safety Data Sheets will be provided by the product supplier to the Study Director before the laboratory phase is commenced. Toxic reference item not used in the study will be stored in the test facility until the use-by date stated on the container label.

Reference item	Potassium dichromate
Batch No.	102403H
Active substance (nominal conc.)	Potassium dichromate (1000 g/kg)
Active substance (actual conc.)	Potassium dichromate (999.7 g/kg)
Active substance CAS number	7778-50-9
Physical state, appearance	Solid, orange crystalline powder
Storage requirement	Cool, dry and well-ventilated place. In the original container.
Reference item supply	Merck KGaA, 64271 Darmstadt, Germany

5. TEST SYSTEM

5.1 Test organisms

The fish used for this study will be the guppy *Poecilia reticulata*. The source and the maintenance details of the fish before use in the study will be recorded in the raw data and included in the final report.

In order to adapt the fish to the test conditions, they will be held in the laboratory for at least 12 days before they will be used for testing. A fish culture in the test medium will be prepared at least 7 days before start of the test under the following conditions:

- light: 12 to 16 hours photoperiod daily
- temperature: 21°C to 25°C
- oxygen concentration: at least 80% of air saturation value
- Feeding (diet without mosquitoes - see diet composition below): once daily until 24 hours before the test will start

Following a 48-hour settling-in period, mortalities will be recorded and the following criteria will be applied:

- mortality > 10%: rejection of the entire batch
- mortality between 5% and 10%: acclimatisation continued for 7 additional days
- mortality < 5%: acceptance of the batch

At the start of the test, the animals will be 2 cm ± 1. They will be in good health and free from any apparent malformation. The fish will not be fed from 24 hours before the test is started.

Individuals for use in the study will be selected without conscious bias but any that are obviously unhealthy or damaged will be discarded. Procedures for the assignment of individual test organisms to test groups will be recorded in the raw data and described in the study report.

5.2 Test medium

The ISO test medium (reconstituted test water) will be used. The composition of the test medium is described in Annex 2 of OECD guideline n°203. The test medium will be made at the test site, using distilled water. The test medium will be aerated until oxygen saturation and then stored for 2 days prior to use. The total hardness, the pH and the conductivity will be recorded. The test medium will be renewed twice weekly. At each renewal, a second series of test vessels will be prepared and the test organisms will be transferred to them.

5.3 Test units

Test vessels (= test units) will consist of 4 L glass jars containing 3 L of test medium. Test units will be thoroughly cleaned before each use.
During the test period, test units will be capped to reduce the loss of water due to evaporation and to avoid the entry of dust into solutions. Aeration can be used.
Each test unit will be labelled with the study number and a unique test unit number.

5.4 Diet

The diet (TetraMin®, to be used during both holding and exposure periods) will consist of:

- fish and fish derivatives,
- cereals,
- yeasts,
- vegetable protein extracts,
- molluscs and crustaceans,
- oils and fats,
- algae,
- sugars,
- mineral substances.
- components: protein 47%, fat 10%, fiber 3%, vitamins D3 and A, elements Mn, Zn, Fe and Co.

During the holding phase (at least 12 days before fish are used for testing), the diet will be administered daily, except during the 24 hours before the test (exposure phase) is started. The diet will be administered without mosquitoes during the holding phase.

During the exposure phase, genetically modified (OX513A) or non-genetically modified (control and reference item) mosquitoes will be incorporated to the fish diet at the rate of 700 g mosquitoes/kg diet and the new diet will be administered daily.

In both holding and exposure phases, the quantity of diet administered daily will not exceed the amount ingested immediately by the fish and will be kept constant during the study duration, i.e. 4 per cent of the initial fish weight. Quantity of administered diet will be calculated each day according to the number of living fish.

5.5 Test groups

The study will comprise a control, a toxic reference item and one rate of the test item: 700 g mosquitoes/kg diet. There will be one test unit with 10 replicates (= 10 fish) for each test item, control and toxic reference item.

A control (non-genetically modified mosquitoes incorporated to the fish diet at the rate of 700 g mosquitoes/kg diet) will be included to assess the natural mortality rate, appearance, size and behaviour of the test organisms. A toxic reference item will be included in the study to demonstrate the susceptibility of the test organism and the sensitivity of the test system. The toxic reference item will be potassium dichromate applied at an application concentration of 100 mg a.s./L and should result in a cumulative 14-day mean mortality > 50% (the toxic reference group will be fed with diet incorporated with non-genetically modified mosquitoes at the rate of 700 g mosquitoes/kg diet).

The items will comprise:

- Test item: genetically modified mosquitoes *A. aegypti* OX513A at 700 g mosquitoes/kg diet
- Control: non-genetically modified mosquitoes *A. aegypti* at 700 g mosquitoes/kg diet
- Toxic reference item: potassium dichromate at 100 mg a.s./L

The diet will be administered daily, at the dose of 4% of the initial fish weight. Quantity of administered diet will be calculated each day according to the number of living fish.

The sequence of feeding will be as follows: control group, followed by the test item group and finally the toxic reference item group. Twice a week, the temperature, dissolved oxygen and the pH will be recorded.

6. EXPERIMENTAL PHASE LOCATION AND CONDITIONS

The experimental phase of the study will be carried out at the Aquatoxicology laboratory of SynTech Research France SAS, 1095 chemin du Bachas, 30000 Nîmes, France. Test units will be maintained under controlled environment conditions during the tests. Conditions will be recorded and all environmental data will be included in the report.

7. EXPERIMENTAL PROCEDURES

Study number:	232SRFR12C1	**Trial number:**	SRFR12-001-232XC1
Experimental phase location:	Aquatoxicology laboratory SynTech Research France SAS 1095 Chemin du Bachas 30000 Nîmes, France		
Test organism (species):	Guppy *Poecilia reticulata* Peters (Actinopterygii: Poecillidae)		
Test system:	Test medium: ISO reconstituted water. Items: 3 (1 test item, 1 toxic reference item, 1 control). Test unit: 4 L capped glass jar. Test organisms (= replicates): 10 *Poecilia reticulata* (2 cm ± 1) in each test unit (maximum loading: 1 g fish /L of solution).		
Items:	- Test item: genetically modified mosquitoes *A. aegypti* OX513A at 700 g mosquitoes/kg diet - Control item: non-genetically modified mosquitoes *A. aegypti* at 700 g mosquitoes/kg diet - Toxic reference item: potassium dichromate at 100 mg a.s./L (fed with non-genetically mosquitoes *A. aegypti* at 700 g mosquitoes/kg diet)		
Number of application:	1 toxic reference application.		
Number of feeding:	The fish will be fed once daily with prepared diet (4% of the initial fish weight).		
Number of renewals:	The test medium will be renewed twice weekly.		
Replicates:	10 replicates for each test item, control and toxic reference item.		
Study duration:	14 to 28 days, depending on effects after 14 and 21 days.		
Test organism destruction:	At the end of the study, the remaining test organisms will be destroyed according to SynTech SOPs.		
Test conditions:	Monitoring of environmental conditions will be carried out throughout the trial, either at regular intervals or continuously, using calibrated equipment. Organisms will be maintained between 21°C to 25°C (constant within the range of ± 2°C), in 12 hours to 16 hours light cycle.		
Guideline:	The study will be conducted in accordance with the OECD guideline n°204 modified for oral route of exposure.		

Exposure details:

The actual concentration of toxic reference item potassium dichromate (999.7 g a.s./kg) will be used when preparing the solution and when calculating the deviation percentage. Full details of dose preparation procedures will be recorded in the raw data and presented in the study report.

Item ID	Item	a.s. concentration / L	f.p. concentration / L*	Mosquitoes / kg diet
C101	Control	NA	NA	700 g non-GM mosquitoes / kg diet
T102	OX513A	NA	NA	700 g GM mosquitoes / kg diet
R103	Potassium dichromate	100 mg a.s./L	100.03 mg f.p./L	700 g non-GM mosquitoes / kg diet

* Based on the actual concentration of the toxic reference items. a.s. = active substance; f.p. = formulated product; NA = not applicable; GM = genetically modified. A variation of ±10% is acceptable.

Test conditions:

Test units will be maintained under controlled environment conditions during the test: a culturing chamber will be used, in which the test medium temperature will be maintained between 21°C to 25°C and constant within the range of ± 2°C. The test units will receive 12 hours to 16 hours light cycle. Oxygen concentration will be maintained over 60% of the air saturation value. Aeration can be used.
The study will be carried out without adjustment of pH. Conditions will be recorded and all environmental data will be included in the report.
Short-term deviations from these temperature and light conditions when handling the test units (less than two hours) are not expected to have an adverse effect on results and will not be reported as deviations from the study plan, according to SynTech SOPs.
pH of the solutions will be measured twice weekly. The pH should be in the range of 6 to 8.5.

Validity criteria:

The study will be invalid and will be repeated if:
- the mortality in the control is more than 10% during the test period.
- the conditions are not maintained constant throughout the test.
- the dissolved oxygen concentration falls under 60% of the air saturation value throughout the test.

Assessment details:

The test endpoint is acute toxicity. Fish will be considered as dead if there is no visible movement and if touching of the caudal peduncle produces no reaction.

Sublethal effects will be also recorded. These include all effects observed on the appearance, size and behaviour of the fish that make them clearly distinguishable from the control animals, e.g. different swimming behaviour, different reaction to external stimuli, changes in appearance of the fish, reduction or cessation of food intake, changes in length or body weight.

Food intake will be evaluated by verifying if the entire administrated diet quantity is consumed or if remaining diet is found in the test unit 1 hour after its administration.

Each test unit will be inspected daily during the exposure period.

Representative samples of the test population will be weighed and measured before the test starts. All survivors will be weighed and measured at the termination of the test.

Study schedule:

Timing*	Action
Day before exposure	Length / weight of representative samples of the test population.
First day of exposure	Application of the reference item and first oral exposure (feeding). Assessment (O_2 / temperature / pH).
Once daily during exposure period	Assessment (mortality / sublethal effects / food intake).
Twice weekly during exposure period	Test medium renewal. Assessment (O_2 / temperature / pH) on the fresh and aged test medium.
Last day of exposure	Length / weight of all surviving test organisms.

* Acceptable tolerance: ± 1 day.

8. BIOLOGICAL DATA ANALYSIS

Results will be analysed with the statistical software Minitab® Release 14 (ANOVA test plus Dunnett's) to determine any significant differences.

Results will be corrected for control using an adaptation of Abbott's formula (1925) as follows:

$$M\% = \left(\frac{Mt - Mc}{100 - Mc} \right) \times 100$$

where Mt = % mortality in the test or toxic reference item
Mc = % mortality in the control

9. AMENDMENTS TO THE STUDY PLAN

The Study Director must approve all amendments to this study plan before implementation. The Study Monitor will be notified before the implementation of the amendment. The Sponsor will sign the amendment. Amendments will contain the following information:

1. A detailed description of the amendment.
2. The reasons for the amendment.
3. The signatures of the Study Director, Management, Sponsor and Lead Quality Assurance.
4. Impact of the amendment on the study.
5. The date upon which the amendment was signed.

10. STUDY PLAN DEVIATIONS

Any deviation from the study plan will be identified in writing and communicated to the Study Director and Study Monitor as soon as possible. The Study Monitor will receive the draft deviation statement before signature. The final deviation statement will then be signed by the Study Director. Any statement regarding a study plan deviation will include a description of the deviation, the reason for the deviation, the date of occurrence and its anticipated effect on the outcome of the study.

11. DATA REPORTING

The draft report will be sent to the Study Monitor for review. The report will be in the standard SynTech Research format and will include but not be limited to the following:

1. Study title and number
2. Name and address of the Test Facility and study initiation and termination dates
3. Name of Study Director, Study Monitor and all personnel involved in the study
4. Objectives and procedures stated in the study plan, including amendments and deviations to the study plan
5. Quality Assurance Statement listing procedures audited, data and reports reviewed, the respective inspection dates, and the dates the findings were reported to the Study Director and Study Director's management
6. Study Director's signature
7. Good Laboratory Practice Compliance Statement signed by the Study Director
8. Complete identification of test item identified by name, source, lot or batch number, characteristics (purity etc.) as provided by the Sponsor
9. Description of test site, including location, etc
10. Description of the experimental design and all procedures used during the conduct of the study, including test item preparation, administration to the test system, environmental parameter monitoring and data collection
11. Description of testing conditions, including temperature and test item rate
12. Description of any statistical procedures conducted (e.g. analysis of variance)
13. An exact description of any adverse effects of the test item on the test system

14. A description or presentation of all transformations, calculations, or operations performed on the data, along with a summary of the statistical analyses and a statement of the conclusions drawn from the analyses and calculations
15. A description of all circumstances that may have adversely affected the quality or integrity of the data.
16. Location where the final report and raw data are to be archived
17. A copy of the GLP compliance certificate for the Testing Facility during the study
18. A copy of the original Study Plan and any amendments
19. Information on the test organisms

12. RECORDS TO BE MAINTAINED

Records to be maintained and provided in the raw data by the Study Director include, but are not limited to the following:

1. The original study plan, any amendments and deviations
2. A list of all study participants and their signatures and initials
3. A list of equipment used in the study
4. A list of SOPs followed
5. SOP deviations, if any, and their impact on the study
6. Test item Material Safety Data Sheet and Certificate of Analysis
7. Test and reference item receipt and use records
8. Items preparation and application records
9. Test organism receipt details, where applicable
10. Environmental data collected during study
11. All original data collection sheets
12. Written correspondence between the Sponsor and Test Facility

13. RETENTION OF RECORDS

After completion of the final report, the Study Director will transfer the following data generated in the study to: Camilla BEECH
Oxitec Ltd
71, Milton Park, Abingdon,
Oxfordshire, OX14 4RX,
United Kingdom

Data to be transferred will include, but not be limited to:

1. The original study plan, amendments, and deviations
2. The original final report
3. Test item characterisation and certification documentation
4. The original raw data package

Copies of the study plan, raw data, amendments, deviations and final report, as well as all non-study specific data (e.g. log books describing equipment maintenance and calibration) will be stored in the archives of SynTech Research France SAS for ten years.

14. ARCHIVING

An aliquot of the test item will be retained by the test facility until at least the expiry date of the batch used in this study. An aliquot of toxic reference item will be archived at SynTech Research France SAS until expiry date of the product. Test items not used in the study will be returned to the Sponsor or discarded by SynTech Research following local regulations. No data will be discarded without the Sponsor's prior written consent.

15. STUDY PLAN DISTRIBUTION LIST

Study Monitor and Sponsor's Representative	C. BEECH
Study Director	E. YTHIER (original)
Test Facility Lead Quality Assurance	Y. TACIK
Test Facility Management	P. ESCHENBRENNER
Experimental phase Principal Investigator	L. MARTIN

Either a paper copy or an electronic copy (pdf-file) is acceptable.
The Study Director is responsible for forwarding a copy to his QA unit.

16. REFERENCES

- OECD guideline n°204. Fish, Prolonged Toxicity Test: 14-day Study (Adopted 4 April 1984). 9 pp.

- OECD guideline n°203. Fish, Acute Toxicity Test (Adopted 17 July 1992). 9 pp.

- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonization of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

- OECD Principles of Good Laboratory Practice (as revised in 1997). Series on Principles of GLP and Compliance Monitoring, No. 1. ENV/MC/CHEM(98)17 and No. 13. ENV/JM/MONO (2002)9. Organisation for Economic Co-operation and Development, Paris, France.

- Code de l'Environnement. Article Annexe II à l'Article D523-8 du Code de l'Environnement du 16 octobre 2007.

APPENDIX 1. ACKNOWLEDGEMENT OF STUDY PLAN

EXPERIMENTAL PHASE:

SynTech Research

Principal Investigator: Lucie MARTIN 09 OCT 2012 Date

Principal Investigator will be responsible for forwarding a copy to his QA unit.

APPENDIX 2. CERTIFICATE OF ANALYSIS

MERCK

Certificate of Analysis
CertiPUR® Reference Material

Merck Volumetric Standard
1.02403 Potassium dichromate
Batch No. 102403H

Secondary Reference Material for redox titrations
traceable to Standard Reference Material of NIST
(National Institute of Standards and Technology, USA).

Merck volumetric standards are used for the adjustment and control of volumetric solutions. They are manufactured under stringently controlled conditions in order to guarantee the highest quality standards. The general standard corresponds at least to the "GR" grade. It's suitability for use as a volumetric standard is based on a direct comparison with Standard Reference Material obtained from NIST.

The oxidimetric assay of this batch is

99.97%

- Measurement uncertainty: +/-0,05% (coverage factor *k=2 ; confidence level 95%*).
- The content indicated is based on a molecular mass M = 294.184 g/mol dried substance.
- Directly traceable of NIST SRM potassium dichromate batch 136e.
- Volumetric standard for standardisation of volumetric solutions in accordance to the chapter reagents of the Pharmacopoeia (USP).
- Standardisation was carried out using sodium thiosulfate solution as titration solution using a potentiometric procedure.
- Drying: When used as a volumetric standard, the potassium dichromate must be dried at 110°C for 2 hours.
- Storage: The volumetric standard should be stored at room temperature (+15 to +25°C) tightly closed (in the original container) and protected from light and moisture.
- The original unopened container may be used until: 31.07.2015

Date of release: 08.07.2010

Dr. S. Frey

Dr. Stefan Frey
(responsible laboratory manager quality control)

APPENDIX 3. GLP CERTIFICATE

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE
GROUPE INTERMINISTERIEL DES PRODUITS CHIMIQUES

CERTIFICAT DE CONFORMITE AUX BONNES PRATIQUES DE LABORATOIRE SELON LES DIRECTIVES 2004/9/CE ET 2004/10/CE
CERTIFICATE OF COMPLIANCE WITH GOOD LABORATORY PRATICES ACCORDING TO DIRECTIVES 2004/9/CE AND 2004/10/CE

Certificat n°: 2012/14

Société ou organisme : SYNTECH RESEARCH France - Le bois de Luyse
Company : 71570 LA CHAPELLE DE GUINCHAY

Installation d'essais : SYNTECH RESEARCH France - Le bois de Luyse
Test facilities : 71570 LA CHAPELLE DE GUINCHAY

Vu les articles D 523-8 et suivants du code de l'environnement relatifs au groupe interministériel des produits chimiques,
Having regard to the articles D.523-8 and onwards relating to the interministerial group of chemical products (GIPC),

Vu les résultats de l'inspection périodique réalisée par le Comité français d'accréditation (COFRAC) - Section Laboratoires – le : 29 et 30 juin 2011
Having regard to the results of the periodic inspection realised by the French Committee of accreditation (COFRAC) Laboratory Section – on the : *29 et 30 June 2011*

Vu l'avis du GIPC en date du : 29 novembre 2011
Having regard to the GIPC's opinion dated *29 November 2011*

La conformité aux principes des BPL de l'installation précitée est reconnue dans les domaines suivants :
Compliance with the principles of GLP is recognized for the facility above in the following areas:

4 - études écotoxicologiques sur les organismes aquatiques et terrestres
(environmental toxicity studies on aquatic and terrestrial organisms)
5 - études portant sur le comportement dans l'eau, dans le sol et dans l'air : bioaccumulation
(studies on behaviour in water, soil and air; bioaccumulation)
6 - études portant sur les résidus *(residue studies)*
7 - études portant sur les effets, sur les mécosystèmes et les écosystèmes naturels
(studies on effects on mesocosms and natural ecosystems)

Fait à Paris, le 16 janvier 2012

Le Président,

Jean-Pierre FALQUE-PIERROTIN

Secrétariat général du GIPC - DGAIn, Service de l'industrie, bureau de la chimie - 12, rue Vilhot - 75572 Paris cedex 12
Téléphone : 01 53 44 96 10 Télécopie : 01 53 44 91 72

MINISTÈRE DE L'ÉCOLOGIE
DES ÉNERGIES ET DU DÉVELOPPEMENT

Appendix 2
Individual Data and Statistical Analysis

(4 pages)

Study number : **232SRFR12C1**
Trial number : **SRFR12-001-232XC1**

Assessment timing	Acute and sublethal effects	Mortality ; Abnormal behaviour/appearance		
		C101	T102	R103*
Day 1	Number of dead	0	0	0
	Number of unusual behaviour	0	0	0
	Number of moribund	0	0	0
Day 2	Number of dead	0	0	2
	Number of unusual behaviour	0	0	1
	Number of moribund	0	0	0
Day 3	Number of dead	0	0	4
	Number of unusual behaviour	0	0	0
	Number of moribund	0	0	0
Day 4	Number of dead	0	0	4
	Number of unusual behaviour	0	0	1
	Number of moribund	0	0	0
Day 5	Number of dead	0	0	5
	Number of unusual behaviour	0	0	1
	Number of moribund	0	0	1
Day 6	Number of dead	0	0	6
	Number of unusual behaviour	0	0	1
	Number of moribund	0	0	1
Day 7	Number of dead	0	0	7
	Number of unusual behaviour	0	0	1
	Number of moribund	0	0	0
Day 8	Number of dead	1	0	7
	Number of unusual behaviour	0	0	0
	Number of moribund	0	0	1
Day 9	Number of dead	1	0	8
	Number of unusual behaviour	0	0	0
	Number of moribund	0	0	1
Day 10	Number of dead	1	0	9
	Number of unusual behaviour	0	0	0
	Number of moribund	0	0	0
Day 11	Number of dead	1	0	9
	Number of unusual behaviour	0	0	0
	Number of moribund	0	0	1
Day 12	Number of dead	1	0	10
	Number of unusual behaviour	0	0	NA
	Number of moribund	0	0	NA
Day 13	Number of dead	1	0	10
	Number of unusual behaviour	0	0	NA
	Number of moribund	0	0	NA
Day 14	Number of dead	1	0	10
	Number of unusual behaviour	0	0	NA
	Number of moribund	0	0	NA

* Item groups significantly different from control after 14 days
L = Living; D = Dead; NA = Not Applicable

Items	
C101	Control (70% w/w non-GM mosquitoes)
T102	Test item (70% w/w OX513A mosquitoes)
R103	Potassium dichromate (100 mg a.s./L)

14-day Exposure	
LR_{50}/ER_{50}	> 70% w/w OX513A mosquitoes
NOER	70% w/w OX513A mosquitoes
LOER	> 70% w/w OX513A mosquitoes

	Size and weight of the fish at the beginning of the test									Mean	Loading (mg/L)*
Length (mm)	26	22	25	21	22	24	23	20	19	23	22,50	689,4
Weight (mg)	371,0	105,4	243,4	98,7	114,5	401,4	167,7	95,5	183,1	287,6	206,8	

* Based on 10 organisms randomly sampled the day before the start of the test and on test units of 3L test medium for 10 fish

		Size and weight of the surviving fishes at the end of the test										Mean	Loading (mg/L)*
C101	Length (mm)	25	23	21	22	24	22	20	21	24	/	22,44	595,0
	Weight (mg)	299,8	275,6	101,0	125,1	387,6	108,6	85,4	100,7	301,2	/	198,3	
T102	Length (mm)	24	23	20	21	27	25	22	23	25	22	23,20	709,6
	Weight (mg)	238,7	187,9	124,3	98,6	412,2	398,4	132,1	157,6	257,0	121,9	212,9	
R103	Length (mm)	/	/	/	/	/	/	/	/	/	/	/	/
	Weight (mg)	/	/	/	/	/	/	/	/	/	/	/	

* Based on surviving test organisms at the end of the test and on test units of 3L test medium

One-way ANOVA: 14-day Mortality versus Item

```
Source     DF      SS      MS      F      P
Item   2  6,0667  3,0333  91,00  0,000
Error      27  0,9000  0,0333
Total      29  6,9667
S = 0,1826   R-Sq = 87,08%   R-Sq(adj) = 86,12%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean   StDev  ---+---------+---------+---------+------
C101   10  0,1000  0,3162    (---*--)
R103*  10  1,0000  0,0000                              (---*--)
T102   10  0,0000  0,0000  (--*--)
                          ---+---------+---------+---------+------
                           0,00      0,35      0,70      1,05

Pooled StDev = 0,1826

Dunnett's comparisons with a control

Family error rate = 0,05
Individual error rate = 0,0273
Critical value = 2,33

Control = level (C101) of Item
Intervals for Item mean minus control mean

Level    Lower   Center   Upper  --------+---------+---------+---------+-
R103*   0,7095   0,9000  1,0905                              (-----*----)
T102   -0,2905  -0,1000  0,0905  (----*-----)
                                 --------+---------+---------+---------+-
                                       0,00      0,35      0,70      1,05
```

One-way ANOVA: 14-day Length versus Item

```
Source     DF       SS       MS     F      P
Item   1  0,00084  0,00084  0,70  0,414
Error      17  0,02026  0,00119
Total      18  0,02110
S = 0,03452   R-Sq = 3,97%   R-Sq(adj) = 0,00%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean   StDev    +---------+---------+---------+---------
C101    9  1,3691  0,0309    (---------------*--------------)
T102   10  1,3824  0,0375              (-------------*-------------)
                            +---------+---------+---------+---------
                          1,344     1,360     1,376     1,392

Pooled StDev = 0,0345

Dunnett's comparisons with a control

Family error rate = 0,05
Individual error rate = 0,0500
Critical value = 2,11

Control = level (C101) of Item
Intervals for Item mean minus control mean

Level     Lower   Center    Upper     +---------+---------+---------+---------
T102   -0,02018  0,01329  0,04676     (----------------*---------------)
                                      +---------+---------+---------+---------
                                   -0,020     0,000     0,020     0,040
```

One-way ANOVA: 14-day Weight versus Item

```
Source     DF      SS      MS     F      P
Item   1  0,0110  0,0110  0,19  0,665
Error      17  0,9590  0,0564
Total      18  0,9700
S = 0,2375   R-Sq = 1,13%   R-Sq(adj) = 0,00%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean   StDev  ----+---------+---------+---------+-----
C101    9  2,2314  0,2588  (-----------------*----------------)
T102   10  2,2795  0,2168        (---------------*---------------)
                          ----+---------+---------+---------+-----
                            2,10      2,20      2,30      2,40

Pooled StDev = 0,2375

Dunnett's comparisons with a control

Family error rate = 0,05
Individual error rate = 0,0500
Critical value = 2,11

Control = level (C101) of Item
Intervals for Item mean minus control mean

Level    Lower  Center   Upper  -----+---------+---------+---------+----
T102   -0,1821  0,0481  0,2783  (------------------*------------------)
                                -----+---------+---------+---------+----
                                  -0,12      0,00      0,12      0,24
```

Appendix 3
Environmental Conditions Data

(1 page)

Study number : **232SRFR12C1**
Trial number : **SRFR12-001-232XC1**

Assessment timing	Parameters	Fresh Test Medium			Aged Test Medium		
		C 101	T 102	R 103	C 101	T 102	R 103
Day 1	pH	7,57	7,58	6,61	7,31	7,34	6,29
	T°C	21,8	21,8	21,8	21,6	21,4	21,6
	O2 (mg/L)	9,9	9,9	9,8	7,4	7,6	7,9
Day 4	pH	7,61	7,64	/	7,28	7,25	6,27
	T°C	21,6	21,6	/	21,8	21,6	21,6
	O2 (mg/L)	9,9	9,9	/	7,3	7,5	7,9
Day 8	pH	7,55	7,51	/	7,22	7,18	6,21
	T°C	21,8	21,8	/	21,6	21,8	21,6
	O2 (mg/L)	9,8	9,7	/	7,2	7,4	7,5
Day 11	pH	7,57	7,56	/	7,34	7,23	6,18
	T°C	21,8	21,6	/	21,6	21,6	21,6
	O2 (mg/L)	9,9	9,8	/	7,4	7,5	7,3
Day 1 to Day 14		Temperature (°C)			20,5 - 22,4		
		Light intensity (lux)			1120 - 1340		

Appendix 4
Software Verification

(1 page)

MINITAB:

MINITAB ONE-WAY ANOVA VERIFICATION

01/06/2012 17:24:03

```
Welcome to Minitab, press F1 for help.
```

One-way ANOVA: C2 versus C1

```
Source  DF     SS     MS     F      P
C1       3  341,9  114,0  9,01  0,000
Error   36  455,6   12,7
Total   39  797,5

S = 3,557   R-Sq = 42,87%   R-Sq(adj) = 38,11%

                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level       N    Mean  StDev  -+---------+---------+---------+--------
Sample 1   10  83,600  4,033                              (------*------)
Sample 2   10  79,400  2,503                    (------*------)
Sample 3   10  78,600  3,307                  (------*------)
Sample 4   10  75,400  4,142        (------*------)
                                    -+---------+---------+---------+--------
                                   73,5      77,0      80,5      84,0

Pooled StDev = 3,557
```

Published example of statistical analysis used:
"Procedure for computing one-way ANOVA". In "Fowler, J., Cohen, L., & Jarvis, P. (1998). Practical statistics for field biology. Second edition. John Wiley & Sons Ltd, Chichester, England". From pp181 to pp184.

L. DESLANDES

01 JUN 2012

On computers n° : 01279
01951
01813

The cited example was used to validate MINITAB software one-way ANOVA.

Appendix 5
Certificate of Analysis

(1 page)

MERCK

Certificate of Analysis
CertiPUR® Reference Material

Merck Volumetric Standard
1.02403 Potassium dichromate
Batch No. 102403H

Secondary Reference Material for redox titrations
traceable to Standard Reference Material of NIST
(National Institute of Standards and Technology, USA).

Merck volumetric standards are used for the adjustment and control of volumetric solutions. They are manufactured under stringently controlled conditions in order to guarantee the highest quality standards. The general standard corresponds at least to the "GR" grade. It's suitability for use as a volumetric standard is based on a direct comparison with Standard Reference Material obtained from NIST.

The oxidimetric assay of this batch is

99.97%

- Measurement uncertainty:+/-0,05% (coverage factor *k=2 ; confidence level 95%).*
- The content indicated is based on a molecular mass M = 294.184 g/mol dried substance.
- Directly traceable of NIST SRM potassium dichromate batch 136e.
- Volumetric standard for standardisation of volumetric solutions in accordance to the chapter reagents of the Pharmacopoeia (USP).
- Standardisation was carried out using sodium thiosulfate solution as titration solution using a potentiometric procedure.
- Drying: When used as a volumetric standard, the potassium dichromate must be dried at 110°C for 2 hours.
- Storage: The volumetric standard should be stored at room temperature (+15 to +25°C) tightly closed (in the original container) and protected from light and moisture.
- The original unopened container may be used until: 31.07.2015

Date of release: 08.07.2010

Dr. S. Frey

Dr. Stefan Frey
(responsible laboratory manager quality control)

99395

Appendix 6
GLP Certificate

(1 page)

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE

GROUPE INTERMINISTERIEL DES PRODUITS CHIMIQUES

CERTIFICAT DE CONFORMITE AUX BONNES PRATIQUES DE LABORATOIRE SELON LES DIRECTIVES 2004/9/CE ET 2004/10/CE

CERTIFICATE OF COMPLIANCE WITH GOOD LABORATORY PRATICES ACCORDING TO DIRECTIVES 2004/9/CE AND 2004/10/CE

Certificat n°: 2012/14

Société ou organisme : SYNTECH RESEARCH France - Le bois de Loyse
Company : 71570 LA CHAPELLE DE GUINCHAY

Installation d'essais : SYNTECH RESEARCH France - Le bois de Loyse
Test facilities : 71570 LA CHAPELLE DE GUINCHAY

Vu les articles D.523-8 et suivants du code de l'environnement relatifs au groupe interministériel des produits chimiques,
Having regard to the articles D.523-8 and onwards relating to the interministerial group of chemical products (GIPC),

Vu les résultats de l'inspection périodique réalisée par le Comité français d'accréditation (COFRAC) - Section Laboratoires – le : 29 et 30 juin 2011
Having regard to the results of the periodic inspection realised by the French Committee of accreditation (COFRAC) – Laboratory Section – on the : 29 et 30 June 2011

Vu l'avis du GIPC en date du : 29 novembre 2011
Having regard to the GIPC's opinion dated : 29 November 2011

La conformité aux principes des BPL de l'installation précitée est reconnue dans les domaines suivants :
Compliance with the principles of GLP is recognized for the facility above in the following areas:

4 - études écotoxicologiques sur les organismes aquatiques et terrestres
(environmental toxicity studies on aquatic and terrestrial organisms)
5 - études portant sur le comportement dans l'eau, dans le sol et dans l'air ; bioaccumulation
(studies on behaviour in water, soil and air; bioaccumulation)
6 - études portant sur les résidus (*residue studies)*
7 - études portant sur les effets, sur les mécososmes et les écosystèmes naturels
(studies on effects on mesocosms and natural ecosystems)

Fait à Paris, le 16 janvier 2012

Le Président,

Jean-Pierre FALQUE-PIERROTIN

Secrétariat général du GIPC - DGCIS, Service de l'industrie, bureau de la chimie - 12, rue Villiot - 75572 Paris cedex 12
Téléphone : 01 53 44 96 10 – Télécopie : 01 53 44 91 72

MINISTÈRE DE L'ÉCONOMIE
DES FINANCES ET DE L'INDUSTRIE

Appendix 6

OX513A Part B - A-100 Oxitec Lab Floor Plan Option-3-Layout2

A floor plan of the internal layout of a current mobile rearing unit configuration has been provided

Appendix 6.1

OX513A Part B - A landscape ecological vegetation map of Saba

Can be found at:

http://edepot.wur.nl/376259

Accessed 01/12/2016

Appendix 7

OX513A Part B -Hurricanes Tropical Storms

Can be found at:

http://www.meteo.cw/Data_www/pdf/pub/HurricanesTropicalStorms_DC.pdf

Accessed 01/12/2016

Appendix 8

OX513A Part B - Final Report of the Application stage of the Biological vector Control Project in Saba Island

Report provided 13 January, 2016 by:

Dr Koen Hulshof MD MPH
Saba Public Health Department
Email: koen.hulshof@sabagov.nl

Appendix 9

OX513A Part B - Introduced agricultural pests, plant and animals diseases and vectors in the Dutch Caribbean

Can be found at:

http://library.wur.nl/WebQuery
wurpubs/422603

Accessed 01/12/2016

Appendix 10

Saba Exotic Species Ordinance-AB2000

Can be found at:

http://dcnanature.org/wp-content/uploads/2012/09/B9-SabaExoticSpeciesOrdinance-AB2000.doc

Accessed 01/12/2016

Appendix 11

OX513A Part B -Biological Inventory Saba

Can be found at:

http://www.dcbd.nl/document/biological-inventory-saba

Accessed 01/12/2016

Appendix 12

OX513A Part B -AOAC Method for Tetracycline

Can be found at:

http://www.aoacofficialmethod.org/index.php?main_page=product_info&cPath=1&products_id=1072

Accessed 01/12/2016

Appendix 13

OX513A Part B - Reforming Sabas Agri-sector

Can be found at:

http://sabareach.com
downloads/2014.03.18%20-%20Final%
20Report%20D.%20Leutscher%20-%
20Reforming%20Sabas%20Agri-sector.pdf

Accessed 01/12/2016

Appendix 14

OX513A Part C - Invasive-Alien-Species-Strategy-for-Caribbean-Netherlands-SS-DB

Can be found at:

http://edepot.wur.nl/296484

Accessed 01/12/2016

Appendix 15

OX513A Part C - Towards a design for an improved drinking water supply

Can be found at:

http://repository.tudelft.nl/islandora/object/uuid:26074f78-f193-4367-9d45-55ff6f788370/datastream/OBJ/download

Accessed 01/12/2016