Aedes aegypti OX513A

Draft Environmental Assessment for Investigational Use of Aedes aegypti OX513A

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1 Contents

2 List of Figures

3 List of Tables

4 List of acronyms, abbreviations and technical terms

ACL	Arthropod Containment Level
AMCA	American Mosquito Control Association
ASTMH	American Society of Tropical Medicine and Hygiene
BLAST	Basic Local Alignment Search Tools
Bs	*Bacillus sphaericus*
Bt	*Bacillus thuringinensis*
Bti	*Bacillus thuringinensis israeliensis*
CFR	Code of Federal Regulations
CFSAN	Center for Food Safety and Applied Nutrition (FDA)
CDC	Centers for Disease Control and Prevention
CVM	Center for Veterinary Medicine (FDA)
DDT	dichloro-diphenyl-trichloroethane
DNA	deoxyribonucleic acid
DSP	Daily Survival Probability
DsRed2	fluorescent marker gene from *Discosoma* species
EA	Environmental Assessment
EST	Expressed Sequence Tag
FAO	Food and Agriculture Organizaton
FARRP	Food Allergy Research and Resource Program
FASTA	Fast-ALL (DNA and protein sequence format)
FDA	US Food and Drug Administration
FEMA	Federal Emergency Management Agency
FL	Florida
FL DOH	Florida Department of Health
Ft	feet
KH	Key Haven
FKAA	Florida Keys Aquifer Authority
FKMCD	Florida Keys Mosquito Control District

GE	genetically engineered
HRU	Hatching and rearing unit
HSE	Health and Safety Executive, UK
HSV	Herpes simplex virus
HVAC	Heating, Ventilation and Air Conditioning
IAEA	International Atomic Energy Authority
IBC	Institutional Biosafety Committee
IPM	Integrated Pest Management
INAD	Investigational New Animal Drug
INSP	Instituto Nacional Salud Publica México
L1	1st instar larva
L4	4th instar larva
LPS	larval pupal sorter
LSTM	Liverpool School of Tropical Medicine, UK
LOER	Lowest Observable Effect Rate
Kdr	knockdown resistance
mRNA	messenger RNA
NA	Native Area
NAS	National Academy of Sciences, USA
NCBI	National Center for Biotechnology Information
NEPA	National Environmental Policy Act
NLAA	Not likely to adversely affect
NOAA	National Oceanic and Atmospheric Administration
NOER	No Observable Effect Rate
NRC	National Research Council USA
NST	Non sexual transfer
NWR	National Wildlife Reserve
OSTP	Office of Science and Technology Policy, US Government
PCR	polymerase chain reaction
PCT	Patent Cooperation Treaty Countries (148 countries worldwide)

RNA	ribonucleic acid
piRNA	PIWI- interacting ribonucleic acid
QC	Quality Control
RNA	ribonucleic acid
RIDL	Release of Insects with Dominant Lethal
rDNA	recombinant Deoxyribonucleic acid
RO	reverse osmosis
SEM	Standard Error of Mean
SI	Stock Island, Florida Keys
SIT	Sterile Insect Technique
SSSI	Site of Special Scientific Interest
SE	South Eastern
TA	Treated Area
tTA	tetracycline-transcriptional activator
tRE	tetracycline response element
tTAV	tetracycline-transcriptional activator variant
TetO	tetracycline operon
TetR	tetracycline repressor
UCA	Untreated Control Area
UK	United Kingdom
ULV	Ultra-low volume
USDA	United States Department of Agriculture
USFWS	United States Fish and Wildlife Service
U.S	United States of America
VP16	Activator sequence from Herpes simplex virus
v/v	volume/volume
WT	wild-type
WHO	World Health Organization
WWTF	Waste water treatment facility
WWTP	Waste water treatment plant

5 List of definitions

Term	Definition
Conditional lethal	Survival is dependent on the absence of the dietary antidote; absence of the dietary antidote is the condition that results in lethality. Also known as self-limiting
Diploid	an organism having two complete sets of chromosomes
Eclosion	the emergence of an adult insect from a pupal case
Fitness	the extent to which an organism is adapted to or able to survive and reproduce in a particular environment for which the organism is selectively adapted
Gene	Part of a chromosome that controls the expressions of certain biological characteristics of an organism; a portion of DNA that directs the synthesis of a protein
Gene construct	in this case, the recombinant DNA introduced into the organism to alter its phenotype
Genotype	an organism's heredity information, even if not expressed
#OX513	Gene construct used in the genetic engineering of the OX513A strain
Plasmid	DNA employed in the molecular cloning of DNA fragments
Penetrance	the proportion of individuals of a given genotype that exhibit the phenotype typical of that genotype.
Protein coding sequence	DNA sequence of a gene that is transcribed into mRNA and subsequently translated into protein
PIWI interacting RNA	PIWI-interacting RNAs (piRNAs) are endogenous small noncoding RNAs
Regulatory sequence	DNA sequence that is not translated into protein (non-protein coding) and acts to control the expression of a gene.
rDNA construct	The regulated article that is composed of regulatory and coding sequences introduced into an organism to alter its structure and function.
Sialome	the set of messages and proteins expressed in saliva glands

6 List of Appendices

APPENDIX A	Adequate response letter; Centers for Disease Control and Prevention.
APPENDIX B	Threatened and endangered species analysis for Monroe County, FL.
APPENDIX C	CBI-deleted Copy of PH-2013-4-v3a Investigating the tetracycline dose response of *Aedes aegypti* OX513A
APPENDIX D	CBI-deleted Copy of PH-2013-5-V3a Investigating the effects of larval rearing temperature on the phenotype of *Aedes aegypti* OX513A
APPENDIX E	Report ITF/2011/001 Liverpool Insect Testing Establishment study on insecticide resistance.
APPENDIX F	CBI-deleted Copy of PH-2013-3-V3a Assessment of heterozygous OX513A individuals surviving without provision of tetracycline in the diet: Longevity and Fecundity.
APPENDIX G	CBI-deleted Copy of PH-2013-2-v2 Ingestion of tetracycline by adult female *Aedes aegypti* does not affect penetrance of the OX513A transgenic phenotype.
APPENDIX H	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.
APPENDIX I	Expert opinion on toxicity and allergenicity of introduced proteins (2015).
APPENDIX J	Bioinformatics analysis for risks of allergenicity and toxicity of proteins encoded by the two genes introduced into GE mosquitoes (*Ae.aegypti*) strain OX513A for production of sterile males to reduce vector transmission of important human diseases (2013).
APPENDIX K	CBI-deleted Copy of Study Report SR-00004 ED 2.b Western blot analysis of *Aedes aegypti* OX513A saliva for the detection of tTAV and DsRed2 proteins.

7 Executive Summary

Oxitec Ltd. ("Oxitec") has developed a mosquito control program which is an adaptation of the Sterile Insect Technique (SIT), a methodology that has successfully controlled several insect species in different countries over the last 50 years using radiation based sterilization. The Oxitec mosquito control program involves the repeated controlled release of genetically engineered (GE) male *Aedes aegypti* mosquitoes (strain OX513A), expressing a conditional lethality trait and a fluorescent marker. The strain was first constructed in 2002, and a publication about it in a peer-reviewed scientific journal in 2007 (Phuc *et al*., 2007). It has been characterized for over 10 years. Male OX513A mosquitoes mate with the wild females of their own species only, leading to a reduction in the population of the local population of *Ae.aegypti*. Male mosquitoes do not bite humans or animals and therefore are unable to transmit or vector viruses or other saliva constituents. Oxitec mosquitoes can be used in two ways:

- To reduce the *Ae.aegypti* population in an area,
- And/or to prevent its recurrence once control in the area has been achieved.

Released adult OX513A mosquitoes are homozygous for a recombinant DNA (rDNA) construct that confers both late-acting lethality to the strain in the absence of tetracycline as a dietary supplement, and a gene that encodes a fluorescent marker (*DsRed2*), stably integrated at a specific site in a specific line of the *Ae.aegypti* mosquito. Penetrance[1] of expression of the lethality trait is > 95% (i.e., 95% of the GE mosquitoes contain the lethality trait). Eggs are produced in the UK for shipment to the Hatching and Rearing Unit (HRU) located in Marathon, Florida. Once introduced into the secured HRU, the mosquitoes are hatched and reared to pupae, which are sorted mechanically to >99.9% efficacy (Carvalho *et al*., 2014; 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.

The purpose of this investigational field trial is to evaluate the mating ability of released OX513A mosquitoes with local wild type *Ae. aegypti* females, to assess the survival of the resultant progeny in order to estimate mortality related to inheritance of the #OX513 rDNA construct, and to determine the efficacy of sustained releases of OX513A mosquitoes for the supression of a local population of *Ae.aegypti* in the defined release area in Florida Keys, specifically an area known as Key Haven, which is based in Monroe County, which is under the remit of the Florida Keys Mosquito Control District for mosquito control.

A risk assessment, which is performed to determine the potential for significant environmental impact (risk) employs the paradigm of "likelihood of exposure x consequence" or to put it in plain language "could it happen multiplied by what effect would it have if it did?", has been conducted to address the following questions:

[1] Penetrance is the extent to which the conferred trait is present in the resulting population. 95% penetrance means that 95% of the population expresses the introduced trait.

- Can OX513A *Ae.aegypti* escape the confined conditions in which it is reared?
- What is the likelihood that OX513A *Ae.aegypti* will survive and disperse once released into the environment?
- What is the likelihood that OX513A *Ae.aegypti* can reproduce and establish in the environment into which they are released?
- What are the potential impacts of OX513A *Ae.aegypti* in the environment, including on humans?
- What are the likely consequences for the surrounding environment, should OX513A survive and establish in the environment

In risk assessment, risk is estimated by estimating the likelihood of exposure as a function of consequence. If either one of the parameters is determined to be negligible (close to zero), then the likelihood of a significant impact is likely to be negligible as well, because the outcome is the two probabilities multiplied by each other. Data and information presented in this draft EA to address these risk questions are based on semi-field and field studies, laboratory studies, and published literature.

The likelihood of escape, survival, and establishment of OX513A is highly unlikely due to a combination of physical, geophysical, geographic, and biological measures that are in place during egg production, transport, local rearing, and release. Physical measures include premises that conform with the Arthropod Containment Guidelines[2] to prevent escape; use of screens, filters, traps, and multiple levels of containment; devices for transport that have multiple layers of containment; as well as use of trained personnel to ensure containment is appropriately implemented. Geographic containment is provided by the siting of the egg production unit in the UK, which is beyond the isothermal range of the mosquito (i.e., it is too cold for *Ae.aegypti* to survive outside the climate controlled environment of the laboratory). Geophysical containment is provided by the island location of the release site, where the site is predominantly surrounded by ocean, and the mosquito in any life stage cannot survive due to the high salinity of the waters. Biological containment is afforded by the introduction of the conditional lethality trait into the OX513A *Ae.aegypti* line, where on mating with the local females of the same species, >95% of the progeny will die in the absence of tetracycline (Harris et al., 2011)[3], leading to the overall reduction in the population of *Ae.aegypti* at a given site.

The consequences of escape, survival, and establishment of OX513A in the environment have been extensively studied: data and information from those studies indicates that there are unlikely to be any adverse effects on non-target species, including humans. There are also unlikely to be any adverse effects on foreign countries or the global commons. Risk of establishment or spread has been determined to be negligible. The trial is short in duration and any unanticipated adverse effects are unlikely to be widespread or persistent in the environment. Most importantly, the status of the environment is restored

[2] The Arthropod Containment Guidelines have been developed by the American Committee on Medical Entomology and American Society for Tropical Medicine and Hygiene to provide risk-based guidelines for arthropod containment and to safeguard individuals coming into contact with arthropods. They have been adopted by most institutions working with arthropods as the operating standard for containment, and can be found online at http://www.astmh.org/AM/Template.cfm?Section=ACME&Template=/CM/ContentDisplay.cfm&ContentID=1444

[3] Harris et al. 2011. Field Performance of engineered male mosquitoes. *Nature Biotechnology* 29: 1034-1037.

when releases are stopped (i.e., the released mosquitoes all die, and the environment reverts to the pre-trial status). Overall, the environmental assessment concludes that the production, rearing, and short term release of the *Ae.aegypti* strain OX513A for investigational use in Key Haven, Florida is unlikely to result in adverse effects on the environment or human health.

8 Purpose and Need

The U.S. Food and Drug Administration (FDA)'s Center for Veterinary Medicine (CVM) has received a proposal for Oxitec's proposed field trial of genetically engineered (GE) male *Ae.aegypti* mosquitoes of the strain OX513A in Key Haven, Monroe County, Florida under an investigational new animal drug (INAD) exemption (21 CFR 511.1(b)). *Ae.aegypti* is a known vector for the human diseases; Zika virus, dengue fever, chikungunya. OX513A have been genetically engineered to express a gene that encodes a conditional or repressible lethality trait (see below for discussion of how this function operates, also known as self-limiting) and a red fluorescent marker protein to aid in the identification of GE mosquitoes. The field trial will be carried out in conjunction with the Florida Keys Mosquito Control District (FKMCD) to evaluate the use of male *Ae.aegypti* OX513A strain to reduce the population of local *Ae.aegypti*.

This draft Environmental Assessment (draft EA) has been prepared by Oxitec as part of the regulatory consideration for FDA review of the field trial of Oxitec's OX513A. This current draft EA has been prepared to fulfil the sponsor's obligations as described in 21 CFR 511.1(b) (10).

Oxitec Ltd. intends to ship the OX513A line of *Ae.aegypti* mosquitos for a study in Key Haven, Monroe County, FL. In conjunction with the FKMCD, Oxitec is planning to conduct an open field release trial for the OX513A *Ae.aegypti* male mosquitos to determine whether such releases can reduce the population of local *Ae.aegypti*. Data collected during this study may be used in support of the New Animal Drug Application for this product.

Local transmission of dengue fever, a viral disease transmitted by the mosquito vector *Ae.aegypti* was reported in the Florida Keys in 2009 and 2010, with 22 people diagnosed in 2009 and a further 66 people in 2010, with other cases in Miami-Dade and Broward counties (CDC, 2010, Radke *et al*., 2012). Case counts for locally-acquired dengue and those imported from other countries can be found in the weekly surveillance report of the Florida Department of Health[4]. A CDC report issued in 2010 (CDC, 2010) estimated that nearly 1,000 people in the Florida Keys had been exposed to the virus (approximately 5% of the population). 2009 saw the first occurrence of locally-acquired dengue in the Keys since the 1930s; no locally acquired cases were reported in 2011, although in September 2012, one case of local transmission was recorded in Miami-Dade County (FL DOH, 2012). In 2013, further cases of locally acquired dengue were reported in Martin County, Florida, where a total of 28 individuals were identified as infected (FL DOH, 2013). In 2014, the Florida Department of Health confirmed locally acquired cases of chikungunya fever in Miami-Dade, Lucie, and Palm Beach Counties as well as 4 cases of locally acquired dengue. Frequent air travel to dengue endemic countries, transport of goods and trade, along with the continued presence of the vector species and human behaviors that facilitate mosquito bites means that

[4] http://www.doh.state.fl.us/Environment/medicine/arboviral/surveillance.htm

dengue and chikungunya virus transmission is therefore a consistent public health threat in this area (Teets, 2013).

Control of the *Ae.aegypti* mosquito, also known as vector control, is currently the most effective way of reducing the incidence of dengue[5]. Vector control is currently carried out by a variety of means including chemical control, source reduction such as removal of mosquito breeding sites, and use of trapping methods, and combinations thereof, known as integrated pest management (IPM). Even a well-organized mosquito control program, using integrated mosquito management measures, cannot always be effective against the mosquitoes as it is not possible to access all of the breeding sites with the current control measures. The constant threat of locally-acquired dengue and chikungunya in the Florida Keys with its potential spread to the suburban and urban environs of Miami and beyond, along with reduced effectiveness of chemicals, and pressure on vector control resources call for integration of reliable and new cost-effective tools into the mosquito management programs.

The FKMCD is interested in assessing the utility of new tools to manage *Ae.aegypti* populations. Based on promising results elsewhere (Cayman Islands (Harris *et al*., 2012; Harris *et al*., 2011); and Brazil (Carvalho *et al* 2015) including an approval for commercial scale use in Brazil[6], FKMCD is seeking to assess the utility of OX513A *Ae.aegypti* for *Aedes aegypti* vector control in Monroe County.

Oxitec Ltd. as the Sponsor will conduct the trial in collaboration with FKMCD[7]. This document constitutes the draft Environmental Assessment (EA) prepared by Oxitec Ltd that considers the potential consequences that such an investigational field trial may have on the environment and human and animal health.

8.1 Alternative action

Under the National Environmental Policy Act (NEPA), 42 U.S.C. § 4321 et seq., and its implementing regulations, all EAs should include a brief discussion of alternatives to the proposed action as well as environmental impacts of these alternatives. This section focuses on the "No Action" alternative and discusses its potential impact on the quality of the human environment in the United States.

A "No Action" alternative in this case would be for Oxitec not to carry out the field trial in Key Haven, Florida. The plausible outcomes of this decision are that Oxitec could continue development and commercialization of the product at locations outside of the United States with no intent to market the product in the United States, or select another location in the United States to conduct the field trials. With respect to the former, Oxitec may seek regulatory approval from other countries interested in its product. For example, Oxitec has performed several open field release trials in various countries including the Cayman Islands, Malaysia, Panama, and Brazil. Recently, the National Technical Commission for Biosecurity, the collegiate body responsible for approval and regulation of GE organisms in Brazil,

[5] Currently there are several clinical trials of vaccines against dengue, but the results have not indicated effective immunity against all strains of dengue (Swaminathan *et al*, 2013, Halsted *et al*, 2012).

[6] http://www.ctnbio.gov.br/index.php/content/view/19522.html[accessed 14 Jan 2015]

[7] FKMCD's role in the trial is as a collaborator. They are supplying resources and facilities to Oxitec for the conduct of investigational use. The collaboration has been approved by the publically appointed FKMCD Board.

approved commercialization of OX513A mosquitos for control of wild *Ae.aegypti* in Brazil (see footnote 5 below). Should Oxitec wish to select another location in the United States to conduct a field trial, it would prepare an environmental assessment for that investigational release.

An additional outcome for the "No Action" alternative is that the FKMCD would continue to use its existing control measures for the *Ae.aegypti* mosquitoes. Currently, FKMCD utilizes integrated mosquito management practices, which involve a variety of methods to reduce *Ae.aegypti* mosquitoes including adulticides, larvicides, source reduction, and biological controls.

The primary method of control of the *Ae.aegypti* mosquito is source reduction, involving domestic inspectors throughout the Florida Keys, and aerial larviciding (by helicopter) primarily in Key West. The inspectors' primary responsibility is to find and eliminate domestic breeding habitats. Where this is not possible, inspectors treat containers by hand. The larvicide utilized is largely dependent upon the species, juvenile life stage (instar) of the mosquito, and container size and type in which the mosquito larvae are found. Larvicides include *Bacillus thuringiensis israelensis* (Bti), *Bacillus sphaericus* (Bs), methoprene, temephos, pyrethoids and Spinosad, or oil dispersants such as Kontrol or CocoBear. These products are rotated to avoid prolonged exposure of mosquito larvae to a particular larvicides mode of action. Standard treatment of larval *Ae.aegypti* is Bti if the larvae are 1st through 3rd instar. The mosquitofish, *Gambusia affinis*, is also used as a larvicide in permanent water bodies such as cisterns, abandoned pools, and ornamental ponds.

The main delivery method of these larvicides is by helicopter, in the form of small droplets. However, backpack sprayers and direct treatments by hand; using granules, pellets, and tablets can also be utilized to treat smaller areas. The main larvicides utilized by inspectors by hand are methoprene and spinosad due to the residual properties of these products. Methoprene is an insect growth regulator that inhibits mosquito larvae from developing into viable adults. Spinosad causes excitation of the mosquito's nervous system leading to paralysis and death. Backpack sprayers are employed in the treatment of tire piles and large groups of breeding containers with temephos. Temephos is an organophosphate larvicide used for control of *Ae.aegypti* larvae. Larval control is by far the most efficient means of *Ae.aegypti* control; however, FKMCD also uses adult control methods when population numbers are high and disease is present.

Adult control of *Ae.aegypt*i is extremely difficult due to the behavior of the species; therefore, adulticide treatments are not regularly employed. The most common and effective treatment for adult *Ae.aegypti* is the use of handheld ultra low volume (ULV) sprayers. These are utilized by inspectors when *Ae.aegypti* are present during domestic inspections. The product used is a combination of sumithrin and prallethrin, which are classified as pyrethroids. In some instances, FKMCD uses the chemical Naled to control adult mosquitoes in an aerial program. The FKMCD is constantly monitoring for resistance of *Ae.aegypti* to all of these products to aid in the control of *Ae.aegypti*, the most effective means of control is source reduction and larviciding which is FKMCD's main emphasis. Even with these efforts, control of *Ae.aegypti* is at best 50% effective and there is increasing resistance developing to these insecticides (FKMCD, personal communication, 2015; Ranson *et al*, 2010).

9 Overview of the rDNA construct in the Ae.aegypti mosquito

9.1 Description of the product

The working product definition is

"The single integrated copy of the OX513 rDNA construct, located at the OX513 site, directing expression of an insect-optimized tetracycline repressible transactivator protein (tTAV), intended to produce conditional lethality and decreased survival of resulting progeny and a red fluorescent protein (DsRed2), to aid detection of these mosquitoes, contained within a specific homozygous diploid line (OX513A) of mosquito, *Aedes aegypti*."

The *Ae.aegypti* mosquito has been engineered to express two traits: the overexpression of a synthetic protein leading to lethality of the mosquito under the control of a tetracycline repressible promoter, and a fluorescent marker protein to aid detection. The conditional lethality trait or "self-limiting" trait prevents progeny inheriting the OX513A gene from surviving to adulthood in the absence of tetracycline. This is a similar concept as making insects sterile with irradiation (known as SIT), but avoids radiation damage to insects, the need for a radioactive source, and decreases the costs of the overall process. The sterile males compete with the wild males for female insects. If a female mates with a sterile male then it will have no offspring, reducing the next generation's population. Repeated release of insects can reduce the insect population to very low levels. SIT has been widely used as a successful control tool in plant pest species for over 50 years, but has been largely unsuitable for mosquitoes as the dose required to achieve sterility was too damaging to the fitness of the mosquito (Munhenga *et al*, 2011; Oliva *et al*, 2013). The fluorescent marker can be used to identify the GE mosquitoes as larvae and pupae in the laboratory and the field.

9.1.1 Putative mechanism by which tTAV causes developmental failure in Ae.aegypti

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 (Gossen and Bujard, 1992), thus preventing the transcription of the gene regulated by that promoter.

Therefore, tTAV acts as a tetracycline regulated switch. High level expression of tTAV is deleterious to cells as it represses normal transcriptional function. Transcription is the process in the cell by which RNA is produced (the transcript), and the transcript is "translated" to make a protein. Developmental failure occurs when the cells cannot make the proteins they require to function normally which then causes cell death. This is known as transcriptional squelching and may be independent of the DNA binding action (Lin *et al*., 2007) of the transcriptional activator. tTA and its variants, such as tTAV, have been used in fungi, rodents, plants, and mammalian cultures with no known non-target adverse effects on the environment

or human health[8]. Its wide use is due to the observation that it is well tolerated in eukaryotic systems (Schönig *et al.*, 2013, Naidoo and Young, 2012, Steiger *et al*., 2009, Munoz *et al.*, 2005, Zhu *et al*., 2002).

9.2 rDNA construct used for transformation

Genetic transformation of insects involves the stable integration of exogenous DNA into the genome of the insect. This requires a suitable method to get the DNA to insert itself into the genome. This is brought about by the use of non-autonomous transposons, which are genetic elements that will transpose, or move from one place to another in the genome, when an external source of an enzyme, referred to as a transposase is used. The non-autonomous transposons are incorporated into a gene construct along with the other genetic elements required to change the insect phenotype and are used for the transformation of the insect.

#OX513[9] is a recombinant deoxyribonucleic acid (rDNA) construct consisting of regulatory sequences from *Ae.aegypti* and *Drosophila melanogaster* and protein coding sequences from tetracycline transcriptional activator variant known as tTAV (synthetic source; see Table 1) and DsRed2 (sourced from the *Discosoma* species of marine coral) and non-autonomous transposon inverted terminal repeat sequences from the *Trichoplusia ni piggyBac* transposable element. A full list of the genetic elements in #OX513, their originating donor organisms and primary literature reference, is provided in Table 1. DNA sequences are not taken directly from the donor organism but from sequence databases and then optimized for expression in insects. Sequencing analysis, conducted by Oxitec, has confirmed the plasmid sequence is as expected.

Table 1: Genetic elements, their donor organisms, and function in #OX513

Genetic Element	Location (bp) in plasmid pOX513	Size (bp)	Originating Donor Organism and Common name	Reference	Function
3' Inverted Terminal Repeat (ITR)	8508-8570	63	*Trichoplusia ni* (Cabbage looper moth)		Short related sequences in reverse orientation at the end of the *piggybac* transposon. Transposases recognize these to integrate the DNA into the chromosome.
***piggyBac* 3'**	7524-8507	984	*Trichoplusia ni* (Cabbage looper moth)	(Cary et al., 1989, Thibault *et al*., 1999)	DNA transposable element with sequence deletions to prevent mobility.
Non-coding	7484-7523	40			
Actin5C	4833-7483	2651	*Drosophila melanogaster* (Vinegar fly)		Promoter element driving the expression of the marker gene.

[8] http://www.tetsystems.com/science-technology/highlighted-publications/ [Accessed 5 Feb 2015]

[9] #OX513 is the designation Oxitec uses to name the rDNA construct introduced into *Ae.aegypti*; OX513A refers to the resulting GE *Ae.aegypti* mosquito line.

Non-coding	4818-4832	15			
DsRed2	4134-4817	684	*Discosoma* (Coral)	(Lukyanov *et al*., 2000, Matz *et al*., 1999)	Red fluorescent protein marker gene.
Non-coding	4126-4133	8			
Drosomycin 3' UTR	3340-4125	786	*Drosophila melanogaster* (Vinegar fly)		Terminator region (polyadenylation signal).
Non-coding	3301-3339	39			
***tetO*x7**	3005-3300	296	*Escherichia coli* (bacteria)	(Gossen and Bujard, 1992)	Non-coding binding site for tTAV.
Non-coding	3000-3004	5			
***hsp70* minpro**	2870-2999	130	*Drosophila sp.* (Vinegar fly)		Promoter element driving tTAV .expression
Non-coding	2858-2869	12			
***adh* intron**	2788-2857	70	*Drosophila sp.* (Vinegar fly)		Enhances gene expression.
Non-coding	2780-2787	8			
tTAV	1766-2779	1014	Synthetic DNA based on a fusion of sequences from *E. coli* (tetR - tetracycline repressor) and HSV-1 (VP16 transcriptional activator)	(Gossen and Bujard,1992, Gong *et al*., 2005)	Tetracycline repressible transcriptional activator.
Non-coding	1716-1765	50			
K10 terminator	934-1715	782	*Drosophila sp*. (Vinegar fly)		Terminator region (polyadenylation signal).
Non-coding	830-933	103			
***piggyBac* 5'**	192-829	638	*Trichoplusia ni* (Cabbage looper moth)	(Cary *et al*., 1989)	DNA transposable element with sequence deletions to prevent mobility.
5' ITR	157-191	35	*Trichoplusia ni* (Cabbage looper moth)		Short related sequences in reverse orientation at the end of the *piggybac* transposon. Transposases recognize these to integrate the DNA into the chromosome.

9.2.1 Potential for transposon mediated remobilization

The *piggyBac* transposable element is a non-autonomous transposon isolated from the cabbage looper moth *Trichoplusia ni*, which has been well studied and used to transform a wide range of insect taxa: Diptera, Lepidopteran, Coleoptera (Handler, 2002; Jasinskiene *et al*., 1998; Koukidou *et al*., 2006; Kuwayama *et al*., 2006; Labbé *et al*., 2010; Tamura *et al*., 2000). A non-autonomous transposon, which has integrated into the genome, is prevented from moving within or outside the genome of its host because it does not encode or produce the associated transposase enzyme that is necessary for such movement. 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 the inserted *piggyBac* elements are completely stable and unable to remobilize (O'Brochta *et al*., 2003; Sethauraman *et al*., 2007; Palavesam *et al*., 2013). Arensburger (2011) has proposed that the stability of the transposons in *Ae.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 100 generation equivalents (see Section 9.2.3).

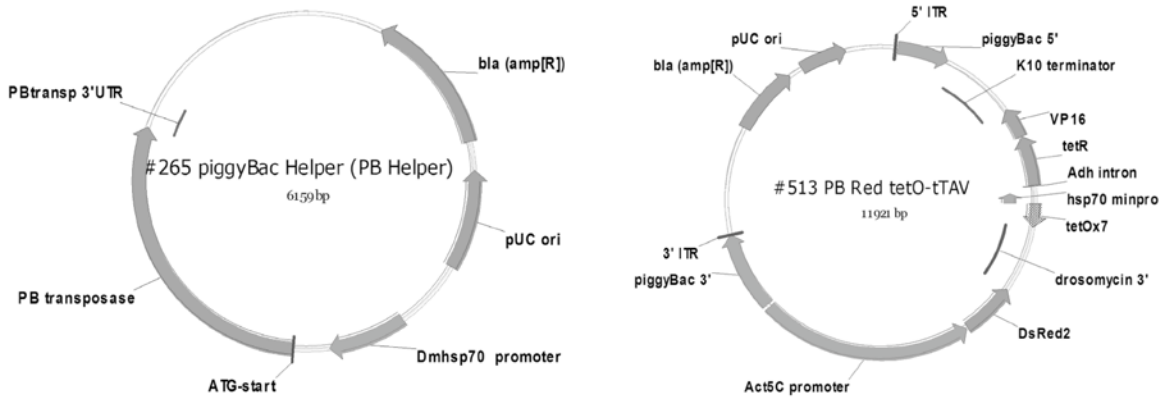
9.2.2 Assessment of the introduced genetic elements for their likelihood to pose potential hazards

The potential for the inserted genetic elements to pose potential risks to humans, non-target animals, or the environment has been evaluated in Section 13. In addition to the analysis reported in that Section, further scientific literature searches in the PubMed (NCBI) database maintained by the U. S. National Library of Medicine were conducted to address the issue of whether the introduction of these mosquitoes could likely have a direct or indirect impact on human health. The database was queried as to whether the source of the gene or sequence used in the construct, #OX513, is a common cause of allergy or toxicity or is linked to pathogenicity. The scientific literature review determined that there were no sequences in the construct that are directly or indirectly likely to be toxic, allergenic, or pathogenic to humans, animals, or the environment. The release will use >99.9% male OX513A mosquitoes which cannot bite humans. However to assess the potential risk of a bite from a female OX513A mosquito, Oxitec performed a study to determine whether the synthetic proteins tTAV and DsRed2 are detectable in the female OX513A mosquito saliva (see Section 13.6.4).

9.2.3 Production of strain OX513A

Strain OX513A was produced in 2002 (Phuc *et al*., 2007) by microinjecting the #OX513 rDNA construct with a transposase helper plasmid (#265) into individual embryos of *Ae .aegypti* from a Rockefeller strain background (Figure 1). The transposase helper plasmid provides a source of *piggyBac* transposase, to allow the rDNA construct to be integrated into the germline of *Ae.aegypti.* The non- autonomous transposon has no endogenous source of transposase in mosquitoes and has had no further translocation.

Figure 1 Map of the vector plasmid pOX513 and the helper plasmid #265



Survivors from the microinjection (G_0) were back-crossed to wild-type *Ae.aegypti* and the females were allowed to lay eggs (G_1). Hatched G_1 larvae were screened for the fluorescent marker gene. Two independent GE strains were recovered from approximately 200 fertile G_0 back crosses. The strain designated LA513A in the paper describing transformation (Phuc *et al*., 2007) and subsequently renamed as OX513A, was selected for further development due to the strong expression of the fluorescent maker gene and the high penetrance (>95%) of the lethality trait when reared in the absence of tetracycline. This strain has been maintained in culture at Oxitec Ltd. since that time, often in pooled rearing, where eggs are collected at particular time points allowing egg storage for extended periods. *Ae.aegypti* development time varies with temperature, so along with the egg storage, this leads to a time-based estimate of the rate of progress through generations rather than a discrete, generation-based rearing. Consequently, generations are referred to as "generational equivalents" based on time rather than discrete generations.

The strain was made homozygous by repeated back-crossing and then the insert was introgressed into an *Ae.aegypti* Latin strain background from Instituto Nacional de Salud Publica (INSP), Mexico. The strain has been maintained by Oxitec Ltd. in a continuously cycling insect colony for the equivalent of over 100 generations.

9.2.4 Molecular characterization and genetic stability of OX513A

Inverse PCR has been used to identify the genomic sequence adjacent to the insertion site of OX513A according to the method of Handler *et al*., 1998. Briefly, restriction enzymes were chosen that cut in the *Ae.aegypti* genome approximately every 500 bp -5 kb. The fragments were circularized and amplified using primer sequences in opposite orientation within the *piggyBac* restriction site and terminus for each junction (5' and 3'). The products were gel purified, cloned, and sequenced. PCR products were compared to *piggyBac* terminal sequences by DNA alignment and BLAST analysis to identify genomic insertion sites. The results revealed the expected *piggyBac* inverted terminal repeats sequences immediately adjacent to a TTAA tetranucleotide sequence characteristic of all *piggyBac* integrations and flanking sequences of 307 bp and 315 bp at either side of the insertion site. The combined flanking sequence was compared with the relatively poorly annotated *Ae.aegypti* genome sequence (publically available via Vectorbase https://www.vectorbase.org/organisms/aedes-aegypti), transcript and EST databases using the BLAST tool.

The sequence was compared in both orientations at the nucleotide level and translated sequence in all six reading frames, to deposited amino acid sequences. The flanking sequence shows 94.6% identity across its length to a single genome sequence contig (1.859), giving an unambiguous match. No new open reading frames were found in all six possible reading frames, inferring that no genes appear to be disrupted by the #OX513 rDNA construct insertion and no new genes are created.

9.2.5 Confirmation of a single insertion site

Southern blot analysis was used to detect the number of insertion sites. The Southern blot was conducted on genomic DNA extracted from individuals of the OX513A line from the generational equivalent 96.

Three restriction enzymes (AgeI, BglII, and SalI) were chosen such that they cleaved the DNA only once in area of the rDNA construct recognized by the chosen probes (A5C+DsR and TetR) as shown in Figure 2.

Figure 2 Schematic of Restriction Enzyme Strategy for the Southern Blot

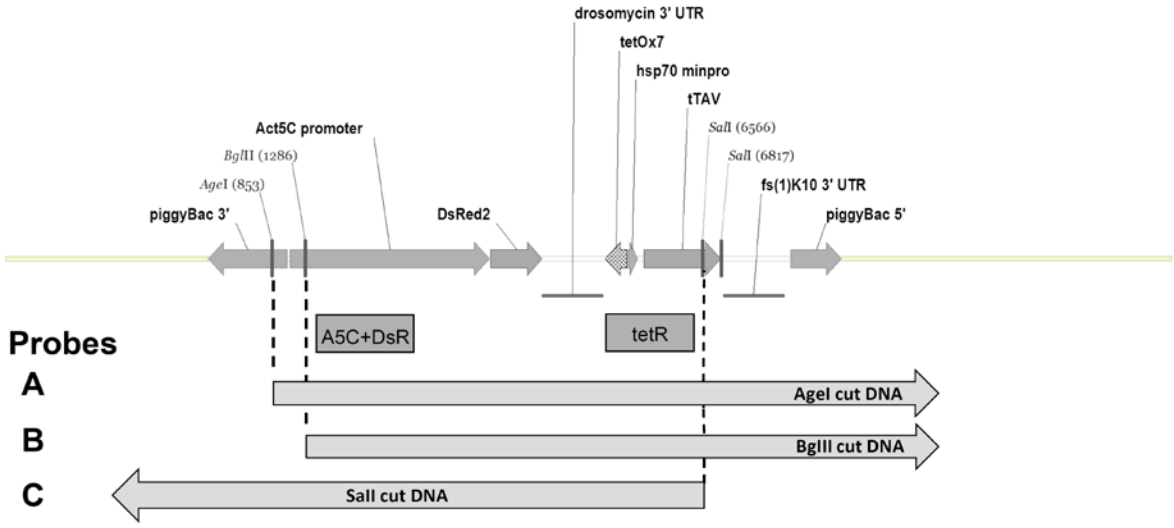


Figure 2 is illustrative only providing a representation of the restriction enzyme sites

AgeI cleaves within the *piggyBac* 3' of the rDNA construct at 853 bp and further downstream in the genomic DNA to produce a band expected to be more than 7565 bp. BglII cleaves within the Act5C promoter sequence at 1286 bp and so is expected to produce a fragment of more than 7131 bp. SalI cleaves within the tTAV sequence at 6566 bp and 6817 bp to produce a band expected to be more than 6566 bp on the Southern Blot. Following gel electrophoresis and probing of the membrane with the specific probes identified by the green boxes in Figure 2, bands of the expected sizes were obtained.

The entire integrated #OX513 rDNA construct insertion in the insect has been sequenced and compared to the sequence of the injected plasmid rDNA construct. There was 100% identity between the sequenced fragments and the #OX513 vector plasmid and genomic flanking sequences indicating no re-arrangements have occurred.

Both of these tests confirm that there is a single, complete copy of the rDNA construct in OX513A *Ae.aegypti* at a discrete integration site.

9.2.6 Detecting the absence of plasmid backbone in OX513A Ae.aegypti

The backbone sequence of the #OX513 plasmid comprises an ampicillin resistance gene and a bacterial origin of replication to allow growth in *E.coli*. Sequencing of the flanking genomic DNA showed no evidence of the plasmid backbone at the site of the rDNA construct insertion.

9.2.7 Conclusion

The molecular characterization of the OX513A line has shown that the sequence of the insert in the GE insect is as intended without re-arrangements. Based on flanking sequence analysis, the insert does not interrupt any genes and, based on flanking sequence analysis, no additional proteins apart from the intended ones are likely to be produced. The GE insect does not contain plasmid backbone sequences as verified by PCR analysis. 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. Additionally, the insert has been shown to be stable and a complete single copy insertion. Genotyping of generational equivalents at $G_{60\text{-}64}$ and G_{100} showed that the genotype has been consistent across 36 generational equivalents. No sequences have been inserted that encode for pathogens, toxins, or allergens as evidenced by both literature searches and bioinformatics studies (see Section 13.5).

Therefore, there are unlikely to be potential risks to the animal (OX513A *Ae.aegypti*) from the genetic engineering, apart from the intended effect of lethality in the absence of tetracycline.

10 Product

10.1 Product identity

Oxitec is currently operating under the following working product definition:

"The single integrated copy of the #OX513 rDNA construct, located at the OX513 site, directing the expression of an insect-optimized tetracycline-repressible transactivator protein (tTAV), intended to produce conditional lethality and decreased survival of resulting progeny, and a red fluorescent protein (DsRed2), to aid detection of these mosquitoes, contained with a specific homozygous diploid line (OX513A) of mosquito, *Aedes aegypti*."

10.2 Proposed Product Claim

A working claim, against which this investigational use will be assessed, in order to validate the proposed claim has been determined as:

"OX513A males mate with local wild type, non-GE female *Aedes aegypti* in a population so that the resulting progeny carry a copy of the #OX513 rDNA construct and produce at least a 2-fold increase in mortality of these #OX513 rDNA construct-bearing progeny relative to local non-GE progeny before they reach functional adulthood."

As this is a working claim, and it is the purpose of the investigational use proposed to test the claim, it is subject to change.

10.3 Conditions for use

This investigational use includes all processes regarding the import, rearing, and field release of OX513A *Ae.aegypti* for the conduct of the trial. OX513A eggs will be produced at Oxitec Ltd., UK and shipped by air in multiple shipments to the USA[10] for rearing to adults in a specialized facility, known as the Hatching and Rearing Unit (HRU), located in Marathon, FL. Adult male mosquitoes will be released up to three times per week over a time period of up to 22 months for the evaluation of the efficacy of the control of local populations of *Ae.aegypti* at the specific site identified in Key Haven, Monroe County, FL, although the trial may be concluded earlier if the operational objectives have been met.

[10] See Section 10.4.1.6 for a more complete description of import permits.

10.4 Product sources

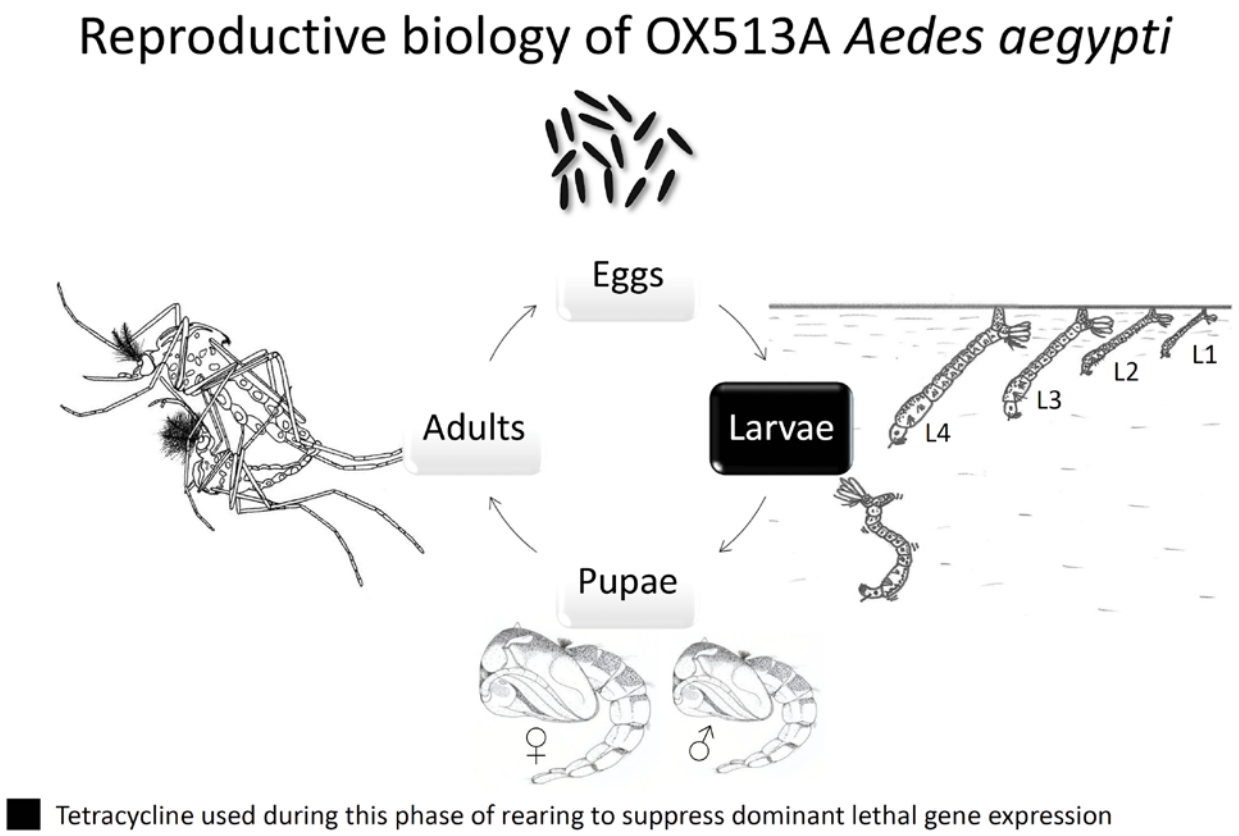
10.4.1 General overview of Ae.aegypti OX513A production

A general overview of *Ae.aegypti* lifecycle and the methods used in the productions of *Ae.aegypti* OX513A is given below.

10.4.1.1 Mosquito life cycle

Ae.aegypti undergoes complete metamorphosis, i.e., the juvenile form is anatomically different from the adults. Juveniles live in a different habitat, eat different foods, and pass through both a larval and pupal stage. Transformation to the adult form takes place during the pupal stage. The larval and pupal stages are aquatic, where the adult phase is land-based. Eggs are laid by females on the water surface, or close to the water-line where they will be flooded. The lifecycle is described in Figure 3 below:

Figure 3 General overview of the lifecycle of *Ae.aegypti* OX513A.



The eggs can remain viable as 'dried' eggs (not submerged in water) for several months. The eggs of *Ae.aegypti* hatch when submerged in water, the larvae then go through 4 molts (L1-L4), growing between each molt. As pupae they metamorphose into adults and emerge onto the water's surface after about 48 hours. Male and females mate and the females take a blood meal to get nutrients to develop eggs. When rearing OX513A from egg to adult tetracycline is added to the water during the larval phase to suppress the conditional lethal gene expression. In adults, the OX513 gene is inherited by all the offspring creating a true breeding line for the OX513 gene.

10.4.1.2 Mosquito breeding and husbandry

General environmental conditions: OX513A mosquitoes are reared in temperature and humidity controlled facilities. For eggs and larvae, temperature generally has the greatest effect on survival and development rate. Insectary conditions vary slightly depending on location but generally have a light:dark cycle of 12:12 hours and a temperature of 27°C +/-4°C and a high relative humidity.

Mosquito eggs: OX513A mosquito eggs require approximately 48 hours to complete embryogenesis and become fully developed un-hatched larvae, although if a water source is present they can hatch immediately. After they have matured, the eggs can remain viable as 'dried' eggs for several months. Storage of eggs is accomplished by maturing for at least five days after being laid to ensure embryogenesis has completed and

the chorion of the egg has matured to prevent desiccation. After maturing, eggs are processed into batches and stored.

Hatching eggs: Eggs hatch most readily when oxygen levels in the water are low, and can be induced by applying a vacuum, which decreases oxygen concentration in the water.

Rearing Conditions

Larvae: Larvae are reared in water containing nutrients such as fish food and tetracycline to suppress the conditional lethal gene expression. Larvae can be reared in many different types of containers but generally a surface area in the range 400 to 800 cm^2 and minimum depth of 1 cm are required. The amount of daily nutrient to be fed to the larvae is calculated taking into account the density of the larvae, temperature, and water quality. Larvae go through four stages of molting over about 7-10 days: at each molt they grow in size but are essentially identical in morphology.

Pupae: Approximately two days after the fourth molt, larvae develop into pupae. The smaller male pupae develop faster than the larger female pupae, providing the underlying mechanism for sorting pupae by sex. Development times are mainly dependent on temperature, density of larvae, and dietary resources.

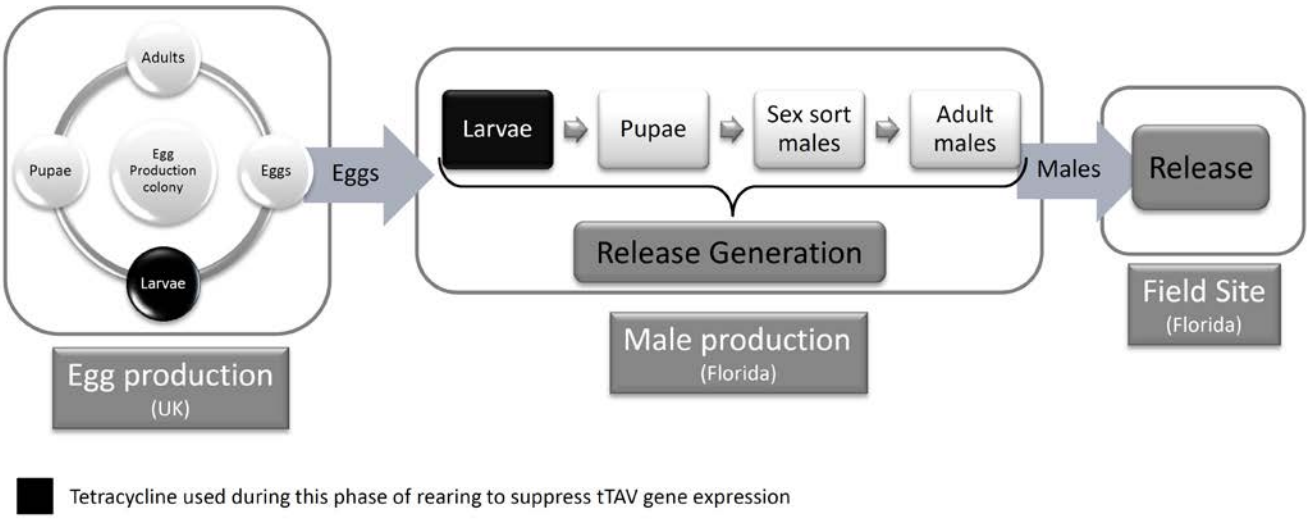
Adults: Pupae undergo metamorphosis into adults over a 48 hour period after which they emerge onto the water's surface by breaking out of the pupal casing. Adults are placed into cages that provide space for flying, mating, and resting, as well as sugar water (10% v/v sucrose) for energy, and where necessary, blood for females to feed on.

Oogenesis (egg production): Females feed on the blood provided, which enables development and laying of eggs. No blood feeding will be conducted in the HRU in Florida as eggs are not produced; only rearing of the eggs to adults occurs at this facility. Therefore any potential or hypothetical risks that might be associated with blood feeding the mosquitoes in the laboratory have not been addressed in this draft EA.

10.4.1.3 Mosquito production for investigational use

There are two production sites: a UK-based egg production site to produce *Ae.aegypti* OX513A eggs and a local facility (the HRU) in Marathon, FL, US, rearing eggs to adults for release. In the UK egg production facility, eggs are continually produced from a cycling colony of homozygous OX513A parent mosquitoes. The eggs will be shipped in multiple shipments throughout the course of the investigation to the HRU facility near the trial site where they will be reared through to pupae, sex sorted to select male pupae, the males matured to adults, and then released at the pre-designated trial site (summarized in Figure 4; the associated process flows for egg production and production of males for release are shown, respectively, in Figure 5 and Figure 6.)

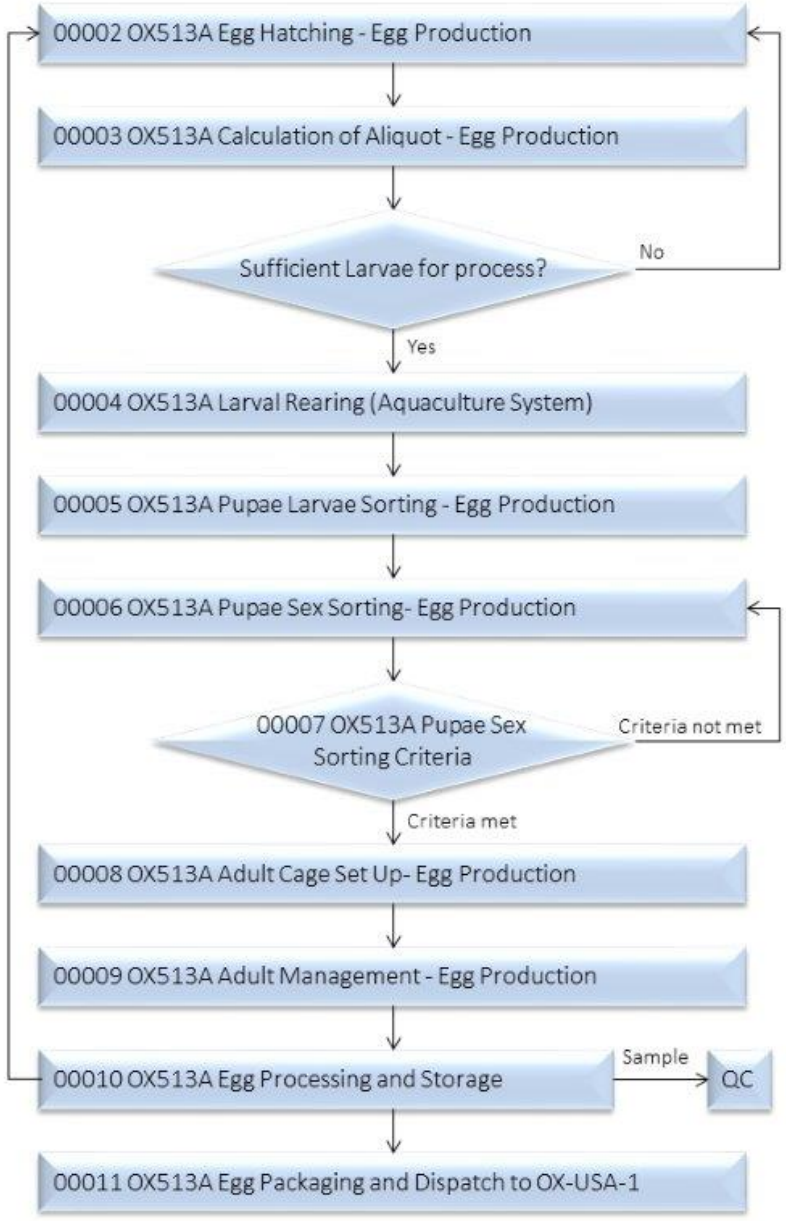
Figure 4: A Schematic of the Production Processes for Producing Males for Release.



The following sections of the draft EA describe the main production processes for each of these facilities in the UK and the US.

The process used to produce eggs in the UK is summarized in Figure 5.

Figure 5 Process Flow for UK Egg Production.



Oxitec Ltd. has dedicated rearing production facilities for its insects in the UK. The facility is licensed by the UK Health and Safety Executive (HSE) for the holding of GE organisms in contained use, under the UK Genetically Modified Organisms (Contained Use) Regulations (2014). The facility is inspected by the HSE for compliance with these regulations. Based on a verbal close-out meeting with the agency at the last inspection conducted in 2013, some minor deficiencies were noted, which were subsequently corrected satisfactorily with no further action required on the part of Oxitec Ltd.

10.4.1.4 Egg production

In the egg production facility, 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, therefore, require a blood meal between each laying cycle. They are fed twice a week for 4-6 weeks to have the necessary dietary resources to produce eggs. Approximately three days after blood feeding, female mosquitoes develop a batch of eggs and are ready to oviposit (lay eggs). A damp substrate (e.g., seed germination paper in a container half-filled with water) is provided for the females to lay eggs. The eggs take about five days to mature, at which time they can be dried and stored under insectary conditions. Insectary conditions are generally maintained at temperatures of 27^{o}C+/-4^{o}C and a high relative humidity.

10.4.1.5 Blood feeding females for 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^{o}C. Female mosquitoes readily feed through the mesh of the cage and engorge on blood. 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. The host range of *Aedes aegypti* and *Aedes albopictus* does not extend to the UK[11] 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.

10.4.1.6 Shipment of eggs to the United States

Shipping from the UK will be conducted in accordance with requirements of US Federal Regulations 7 CFR Part 340, 42 CFR Part 71.54, 9 CFR Part 122 and 21 CFR Part 511. Oxitec and/or FKMCD will obtain all necessary permits and make required notifications prior to shipment. Eggs from the UK production facility will be packed in at least two levels of shatterproof containment (e.g., sealed plastic bags/polystyrene container/cardboard boxes) and with all the relevant permits and permit stickers attached to outer shipment containers, as required by the Federal Regulations cited above. Boxes will be shipped through a courier service that has a tracking facility to ensure the whereabouts of the shipment is known at all times. Shipping from the UK to the USA will need to occur regularly (probably weekly) prior to and during the investigational use. Shipments are labelled as to be kept above 10^{o}C and to only be opened by

[11] Kraemer, M.U.G., et al., (2015). The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. albopictus*. eLife, 4:e08347. DOI: 10.7554/eLife.08347

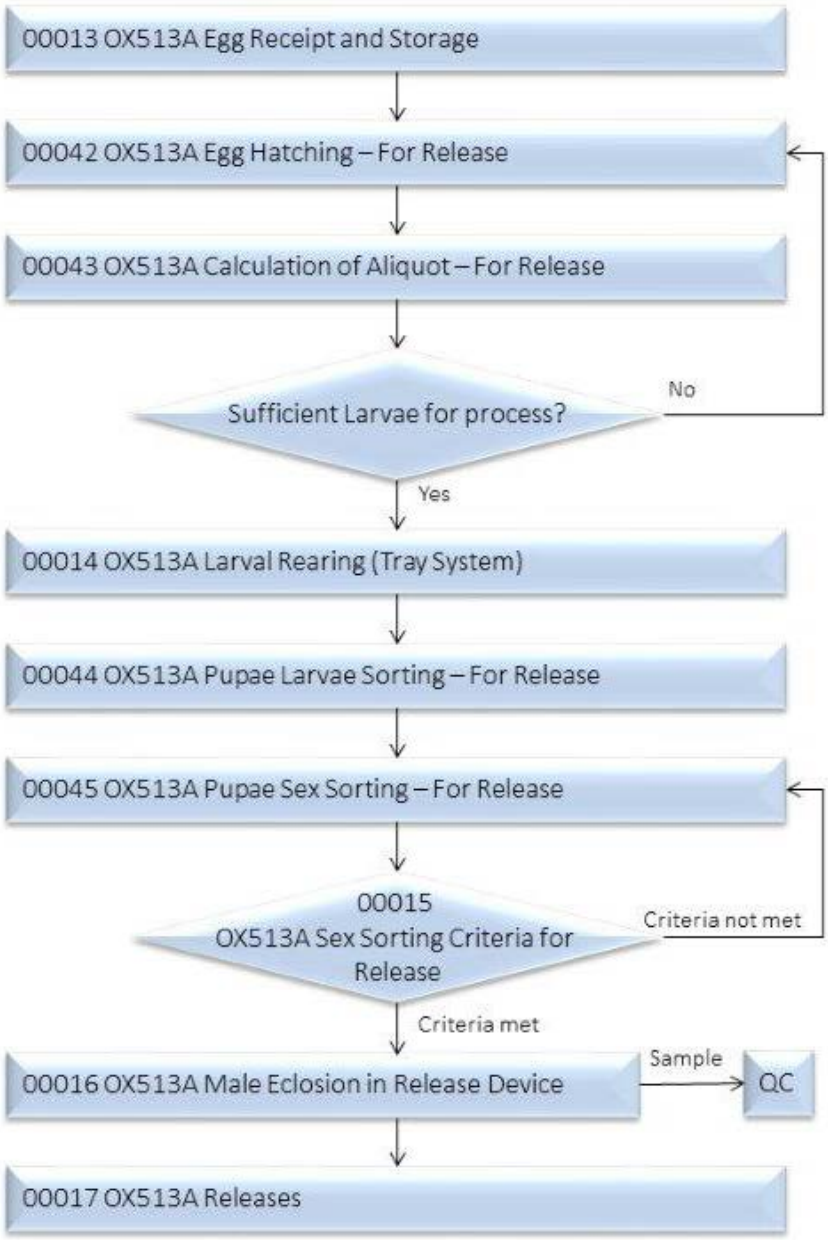
inspection officials or Oxitec and/or FKMCD staff to prevent inadvertent release. Eggs are a non-motile life stage of *Ae.aegypti* and under the correct conditions can remain viable for several months.

On receipt by Oxitec or FKMCD, shipments will only be opened by authorized staff and within the designated facility (the HRU). Rearing will be performed as described in Section 10.4.2 and the associated SOPs. Shipping materials will be disposed of by freezing at ≤ -15°C for at least 12 hours to kill any remaining eggs prior to disposal via incineration by an external contractor.

10.4.2 Activities based in the United States

The process used to produce mosquitoes for release is shown in Figure 6.

Figure 6 Process Flow for Male Production for Release



Production of adults in the US is proposed in an HRU. This is a dedicated, containment facility for the production of OX513A male adults for release. The HRU will be located in the FKMCD site in Marathon and will be accessible only to authorized FKMCD or Oxitec staff. The HRU has been inspected by CDC under 42

CFR 71.54, where some minor departures from recognized safety standards were noted. These have all been corrected and a letter of satisfactory response has been issued by CDC (*Appendix A*).

10.4.2.1 Production of adults

All egg production will take place in the UK, the HRU unit will rear eggs produced in the UK and shipped to the HRU to adulthood for release at the trial site. The following procedures will be employed:

Egg hatch

Eggs will be weighed, added to water, and hatched under vacuum. Vacuum hatching assists with synchronous hatching of the eggs, and eggs normally hatch within an hour under vacuum.

Larvae rearing

Following egg hatching, first instar larvae(L1 as shown in Figure 3) will be put into rearing trays containing water with tetracycline (30 µg/ml) to allow the insects to survive to adulthood as tetracycline switches off the repressible lethality system. To give a consistent density in each tray (of approximately 3000 larvae/liter) the L1 larvae will be counted and aliquoted volumetrically. The larval diet is added daily. Most of the male larvae will pupate at Days 7 and 8 post hatching.

Pupal processing

Pupae will be processed when the optimum numbers of male larvae have reached the pupal stage (~8-9 days). Pupae processing will consist of two steps; separation of larvae from pupae, followed by separation of male from female pupae.

Larvae separation from pupae

Pupae are separated from larvae using a proprietary sieve device (pending PCT Patent number PN798902WO) known as a Larval Pupal Sorter (LPS) that separates larvae from pupae based on size; the gap size can be adjusted so that larvae can pass through but pupae cannot.

Sex separation of male and female pupae

Mechanical size separation will be used to separate sexes as the majority of female pupae are larger than males (Ansari *et al*., 1977; Sharma *et al*., 1972). Using the proprietary method above, it is possible to separate males from females with a sorting accuracy of >99.9% (Figure 7) (Harris et al., 2012; Carvalho et al. 2014). Quality control processes are established to ensure accuracy of the sorting does not exceed a maximum of 0.2% females. Two samples of 500 pupae are taken for analysis and the number of female pupae counted by trained staff. The sample number is based on the probability to achieve releases with as close to 100% males as possible. If more than 0.2% of the sorted population is female the batch is re – sorted prior to release to ensure meeting the 0.2% criterion.

Figure 7 Average Presence of Females in Sorted Male Pupae from Cayman and Brazilian trials.

Study	Cayman	Brazil
% Sex sorting efficiency	99.93%	99.98%
Published reference	Harris *et al*., (2012) Nat. Biotech. 30:828-830	Carvalho *et al*., (2014) J. Vis. Exp. Jan 4:(83).

10.4.2.2 Disposal of female insects

In the male production facility, after the male and female pupae have been sorted the female pupae and the larvae are killed by freezing (≤ -15°C) for more than 12 hours and then disposed of by an external contractor by incineration.

10.4.2.3 Release devices

Male pupae are placed into release devices to emerge and mature before release. Release devices are containers in which the pupae can be placed in about 1-2 cm depth of water, have enough space for adults to survive at the required density for up to five days (including pupation) and a mesh lid through which sugar water can be provided and the males released. The appropriate number of male pupae is aliquoted into release devices volumetrically and water added to a depth of approximately 1 cm. Sugar is provided as a 10% solution through a suitable wick (i.e., cotton wool or cotton dental sticks). After two days under insectary conditions, the water is drained from the release device. Depending on the cycle of releases, the release devices can be maintained under insectary conditions for a further 1-3 days, and are provided with the sugar solution. The release devices are placed into a double-sealed container, labelled, and transported to the release site. At the appropriate release coordinates, a release device is removed from double containment and the lid is opened to release the mosquitoes. After release, individual release devices are returned to double containment for transportation back to the rearing facility where they are frozen (≤-15°C) for over 12 hours to kill any remaining adults.

10.4.2.4 Transport to release site

Transport from the HRU facility to the release site will be by vehicle driven by authorized staff from either FKMCD or Oxitec. Release devices for adult release will be packed in the vehicle. Insects will be double contained for transport to the field site for release. One level of containment will be the release device itself and another will be a suitable container, such as a polystyrene box or sealed bag around the release devices. If temperatures are high, cooling devices such as ice packs may be used with the insects in the transport containers. Oxitec has instituted a chain of custody protocol such that release devices are signed out of the facility, and signed for upon receipt by authorized personnel at the field site. Outer containers will be labelled “Genetically engineered mosquitoes – only to be opened by FKMCD/Oxitec staff”. For transport of release devices back from the field site they will be placed back into the container or bag and frozen (≤-15°C) when returned to kill any remaining adults. All life stages of OX513A mosquitoes not

required for analysis that have been previously frozen will be discarded by incineration via an external contractor.

10.4.2.5Field release

At the trial site, releases will occur up to three times a week. OX513A release devices will be opened and the adult mosquitoes released in a systematic manner from a pre-determined, geo-referenced grid of release points approximately 25-70 m apart, but in no case farther than 100 m apart. The numbers released will be proportional to the local population of *Ae.aegypti* at the trial site. Release rates will be adjusted as the population of *Ae.aegypti* at the trial site declines, to achieve the goals of the investigation (see section 11). Egg and adult mosquito traps will be used to monitor the *Ae.aegypti* population. Egg traps (ovitraps) provide an indirect measure of female *Ae.aegypti abundance* without interference from the released OX513A males. Adult traps directly capture adult *Ae.aegypti.* As *Ae.aegypti* is a mosquito that lives near humans, traps will be located predominantly by domestic dwellings, although other sites (e.g., garages, commercial buildings) may be included. Verbal consent for placing and servicing the traps will be sought from the owner/occupier at the time of placement. If no consent is given then the trap will not be placed in that location.

10.4.2.6 Field Analysis

Samples from the field traps will be returned to a separate laboratory space in the FKMCD facility for their analysis. These samples will include both OX513A and their progeny and local *Ae.aegypti* mosquitoes. All solid wastes from the field laboratory will be treated as GE wastes and frozen (≤-15^{o}C) for over 12 hours prior to disposal by incineration by an external contractor. Liquid wastes are sieved to remove insect parts, which are treated as solid wastes. Samples required for further analysis, such as PCR analysis, will be stored frozen in 70% ethanol prior to shipping to the UK or other suitable laboratory authorized by Oxitec to conduct the work, under the appropriate shipping conditions for the samples (e.g., dry ice if necessary).

The samples returned from the field will be analyzed in a variety of ways:

10.4.2.6.1 Ovitrap analysis

The eggs from the ovitraps will be hatched and the larvae analyzed for the fluorescent marker under a microscope with the appropriate filters for fluorescence. Larvae will be scored for fluorescence and identified as either *Ae.aegypti* or non-*Ae.aegypti*. Larvae will be maintained until positive species identification can be conducted either at late larval stages or as adults using morphological features.

10.4.2.6.2 Adult analysis

The adult traps contain a bag to capture the mosquitoes that fly into them. These bags will be frozen to kill the mosquitoes and *Ae.aegypti* mosquitoes separated from non-*Ae.aegypti* mosquitoes. The *Ae .aegypti* mosquitoes will be analyzed for their sex by trained staff and the numbers of females recorded.

10.4.2.6.3 Testing of functional adult mortality

Eggs from ovitraps, representing the progeny of matings with OX513A in the treatment area will be hatched and tested for the presence of functional #OX513 rDNA construct, by rearing to adulthood. At least a 2-fold increase in mortality of these #OX513 rDNA construct-bearing progeny relative to local non-GE progeny is expected before they reach functional adulthood. Functional adulthood is defined as fully eclosed live adults able to maintain flight. Dead mosquito samples (all lifestages) from traps used in the trial will also be shipped to the UK for analysis to confirm their genotype by PCR methods. The mosquitoes caught in the traps are expected to be either hemizygous for the #OX513 rDNA construct or without the #OX513 rDNA construct i.e., local *Ae.aegypti or* other non- *Ae.aegypti* mosquito species*.* It is possible that some mosquitoes homozygous for the #OX513 rDNA construct will be detected. These would likely be derived from the small number of females (<0.2%) that may be co-released with the male OX513A (as described in 10.4.2.1). Any co-released female will live no longer than a wild *Ae.aegypti* and as there are insufficient sources of tetracycline in the environment, progeny from any matings she makes will die as described in Section 13.3.

11 Investigational Field Trial

11.1 Proposed Field Trial Protocol

The objectives of the proposed investigational field trial are to evaluate the mating ability of OX513A male mosquitoes with local wild type, non-GE *Ae.aegypti* females, to assess the survival to functional adulthood of the resulting progeny inheriting the #OX513 rDNA construct as compared to local non-GE progeny, and to estimate the efficacy of sustained releases of OX513A male mosquitoes for the supression of the local population of *Ae .aegypti* in the described release area in the Florida Keys. This is achieved by the released OX513A *Ae.aegypti* mating with more than one individual of the local females of the same species, passing on the #OX513 rDNA construct to their offspring which is expected to lead to at least a 2-fold increase in mortality of these #OX513 rDNA construct-bearing progeny before they reach functional adulthood in comparison to local non-GE progeny. With sustained releases that are adapted to the numbers of the *Ae.aegypti* in the environment, the suppression of the local population of *Ae.aegypti,* relative to comparator areas is the expected outcome. The protocol developed for the investigational use is summarized in the following paragraphs.

The trial is proposed in three phases:

- Preparation phase; which will involve *Ae.aegypti* rearing optimization in the HRU and environmental monitoring of the *Ae.aegypti* local population in the proposed trial location.
- Rangefinder phase; up to 8 weeks, which will involve the release of adult OX513A male mosquitoes up to three times a week at a constant release rate to determine more precisely the *Ae.aegypti* population in the proposed trial locations. This will also address the two primary objectives or goals of the trial; “does a male OX513A *Ae.aegypti* mosquito mate with

more than one female of the local *Ae.aegypti* population and transfer the #OX513 rDNA construct to their resulting progeny" and "is there at least a 2-fold increase in mortality of these #OX513 rDNA construct-bearing progeny relative to local non-GE progeny before they reach functional adulthood."

- Suppression phase: up to 22 months of sustained release of OX513A adult male mosquitoes up to three times a week, the rate of which will be adapted dynamically during release to achieve suppression of the local population of *Ae.aegypti* in the trial locations. This will allow the secondary objective or goal of the trial to be assessed which is "does sustained release of OX513A result in suppression of the local *Ae.aegypti* population by ≥50%, relative to the comparator area that is not treated with the released OX513A". Monitoring of the release will occur during and post releases using egg and adult trapping methods. The trial may be concluded earlier if the trial objective is met.

12 Environmental Risk Analysis

12.1 Accessible environments

The environments and habitats that *Ae.aegypti* are found in are described below, along with description of the environment found at the investigational trial site.

12.1.1 Aedes aegypti habitat

Ae.aegypti mosquitoes are non-native mosquito species introduced into the United States with human migrations and international trade (Tabachnik, 1991, Gubler *et al.*, 2001, Slosek, 1986). It has limited interactions with ecological systems outside domestic settings in this habitat, although a subspecies of *Ae.aegypti,Ae.aegypti formosa* has been found in tree holes and more sylvan or rural settings in its native Africa (McBride *et al.*, 2014, Brown *et al*., 2011). *Ae.aegypti* occupies two different habitats, aquatic or terrestrial, depending on the life stage of the mosquito. They are regarded as a uniquely domestic or anthropophilic species of mosquito tied closely to human habitations and urban areas; the presence of suitable breeding sites, along with the availability of a human blood meal, strongly influences both the habitat and geographic range of the mosquito.

12.1.1.1 Aquatic habitats

Ae.aegypti eggs are preferentially laid on the surfaces of damp, man-made containers that hold clean, still water or rainwater, such as water storage containers, flowerpots, and waste materials such as tires, cans, and bottles. Breeding sites also can include those that might contain brackish water (defined as less than 30 parts per million (ppm) salinity or 3 g/L) such as boats, or man-made containers at coastal edges, or on beaches (Ramasamy *et al*., 2011). *Ae.aegypti* maintains osmoregulation by increasing the level of free amino acids in the haemolymph and has been reported to not survive in waters with salinity greater than 14 g/L; sea water salinity is generally in the range of 35 g/L (Clark *et al*., 2004). Other potential aquatic

habitats could include standing waste water treatment areas such as septic tanks. A review of the literature in PubMed online conducted in January 2014 indicated only 6 papers describing breeding of *Ae.aegypti* in septic tanks (Somers *et al*., 2011, Burke *et al*., 2010, Mackay *et al*., 2009, Barrera *et al*, 2008, Nwoke *et al*., 1993, Irving-Bell *et al*., 1987). As best described by Burke (2010) and Barrera (2008), septic tanks were more productive for the mosquito when they were uncovered or cracked. A survey of productive containers for mosquitoes was undertaken in Monroe County in 2001 by FKMCD. The survey established that plastic buckets, trash cans, and discarded plastic containers were the most common mosquito breeding sites (Hribar, 2001) and therefore broken and cracked septic tanks are unlikely to be breeding sites in the trial area. Containers that were situated in areas with overhanging vegetation provided more favorable habitats as the breeding site is both shaded from intense sunshine and build-up of heat and provides a ready source of detritus for larval consumption. These waste material containers are usually only sources of breeding sites for mosquitoes during the rainy season in countries with wet and dry seasons, but the eggs are resistant to desiccation and can remain in suitable containers until the following season's rains. This is known as the egg bank.

12.1.1.2 Terrestrial habitats

Adult *Ae.aegypti* occupies terrestrial (land-based) habitats. Male adults require three kinds of resources: a) access to plant sugars for food, b) mates, and c) resting sites. Female adults require the same three resources as well as a bloodmeal and oviposition sites to lay eggs. All of these resources can be obtained in the domestic urban or peri-urban environments, without the need for the mosquito to fly long distances, which is probably why *Ae.aegypti* has become so well adapted to this human environment and rarely flies spontaneously for distances greater than 200 meters, as described in 12.3 of this document.

12.1.2 Monroe County Florida

Monroe County is at the southernmost tip of Florida and is composed of 3,737 square miles of which approximately 73% is water. Tourism is the main industry with over 94.7 million visitors to Florida in 2013, an increase of 3.5 percent over 2012[12]. Monroe County is comprised of portions of the Everglades National Park, Big Cypress National Preserve, and several other important biodiversity refuges (National Key Deer Refuge, Great White Heron National Wildlife Refuge and the National Marine Park, which comprises of sea-based biodiversity resource encompassing the majority of the Keys). Monroe County has a sub-tropical climate with a mean daily temperature of 83.4°F (range max 87.4 – min 79.4°F) and rarely falling below 65°F at night. Precipitation varies throughout the year ranging from 1.51 inches in Feb 2011 to 5.45 inches in Sept of the same year (NOAA[13]) with a relative humidity of around 76%. Climate data is summarized in Figure 8.

[12] http://www.flgov.com/2014/02/14/gov-rick-scott-another-record-year-for-florida-tourism/ [accessed 14 Jan 2015]

[13] http://www.srh.noaa.gov [Accessed 27 Sept 2012]

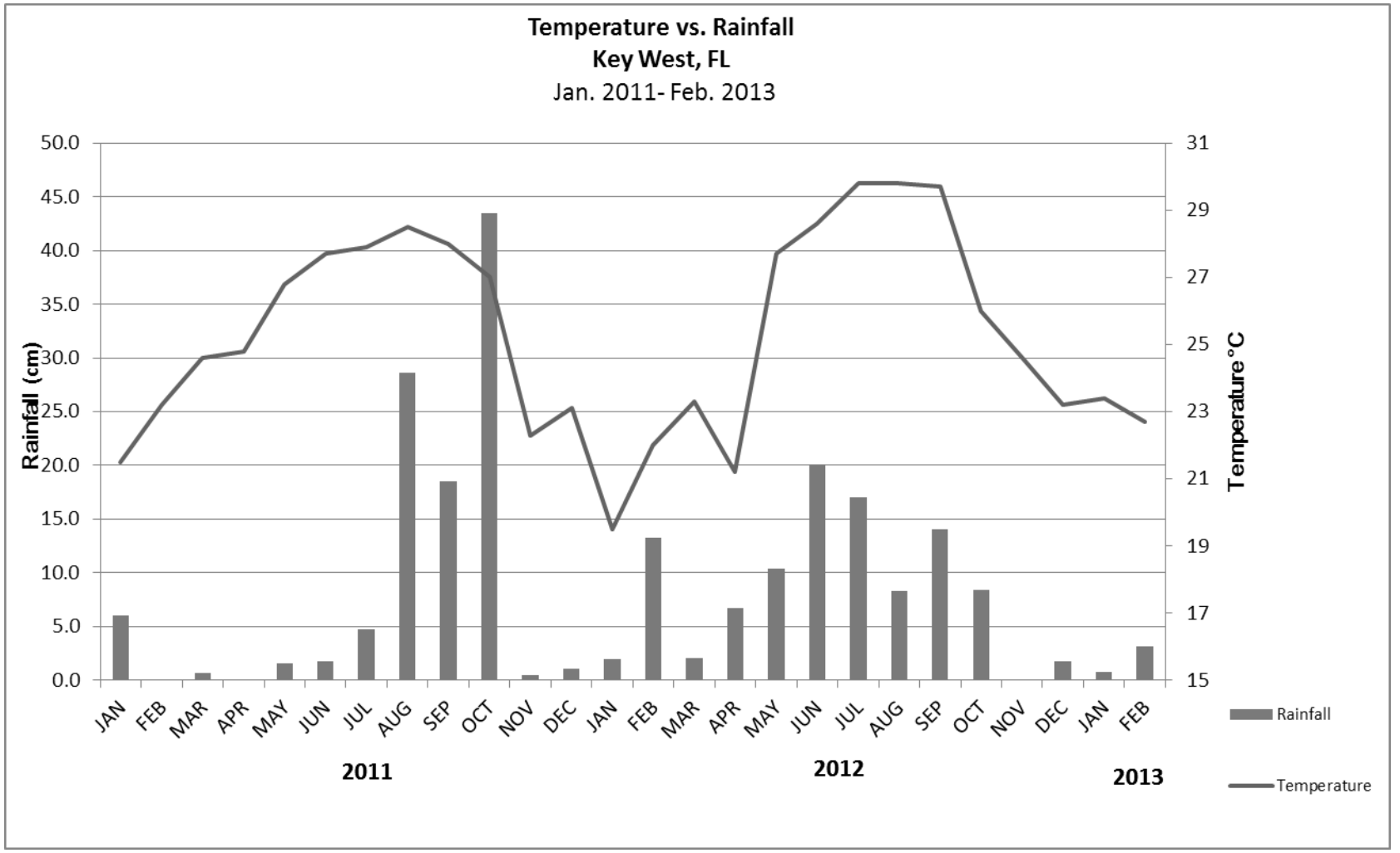


Figure 8 Temperature vs Rainfall, Key West, Florida. Jan 2011-Feb 2013

Key West data is used as it is based on the meteorological station at the airport. Key Haven is close enough to be similar. http://climatecenter.fsu.edu/products-services/data/local-climatological-data

12.1.2.1 Occurrence of natural disasters

Monroe County is one of the most vulnerable counties in the United States to hurricanes, with a historical average of a Category 1 hurricane passing within 75 nautical miles of the Florida Keys every 4.5 years[14]. The historical average for a Category 3 storm passing within 75 nautical miles of the Keys, which requires mandatory resident evacuation, is every nine years. Hurricane season extends from June to November with most of the hurricanes making landfall in the Keys occurring in the month of September[15]. Storm surge as a result of hurricane activity has historically ranged from 6-17 ft in height, with little of Key West predicted as remaining un-flooded at the lower figure of 6 ft of storm surge (Figure 9). Key Haven was flooded following Hurricane Wilma in 2005 as were most of the "Lower Keys"[16]. There are more up-to-date FEMA interactive maps[17] available for storm surge but as most of the Keys are at or slightly above sea level, storm surge flooding is a potential hazard in all locations.

The HRU is located in Marathon, in a Category 4 hurricane-protected building and a hurricane preparedness plan is in place, where adult insects will be killed within 36 hours of a hurricane strike predicted by the U.S. National Weather Service.

A hurricane also has the potential to interrupt the investigational field trial for extended time periods. If this is the case, then either the timeframe of the study may need to be extended to allow sufficient sustained releases of OX513A to suppress the local population of *Ae.aegypti* or the investigational field trial will be abandoned, depending on the severity of the disruption encountered.

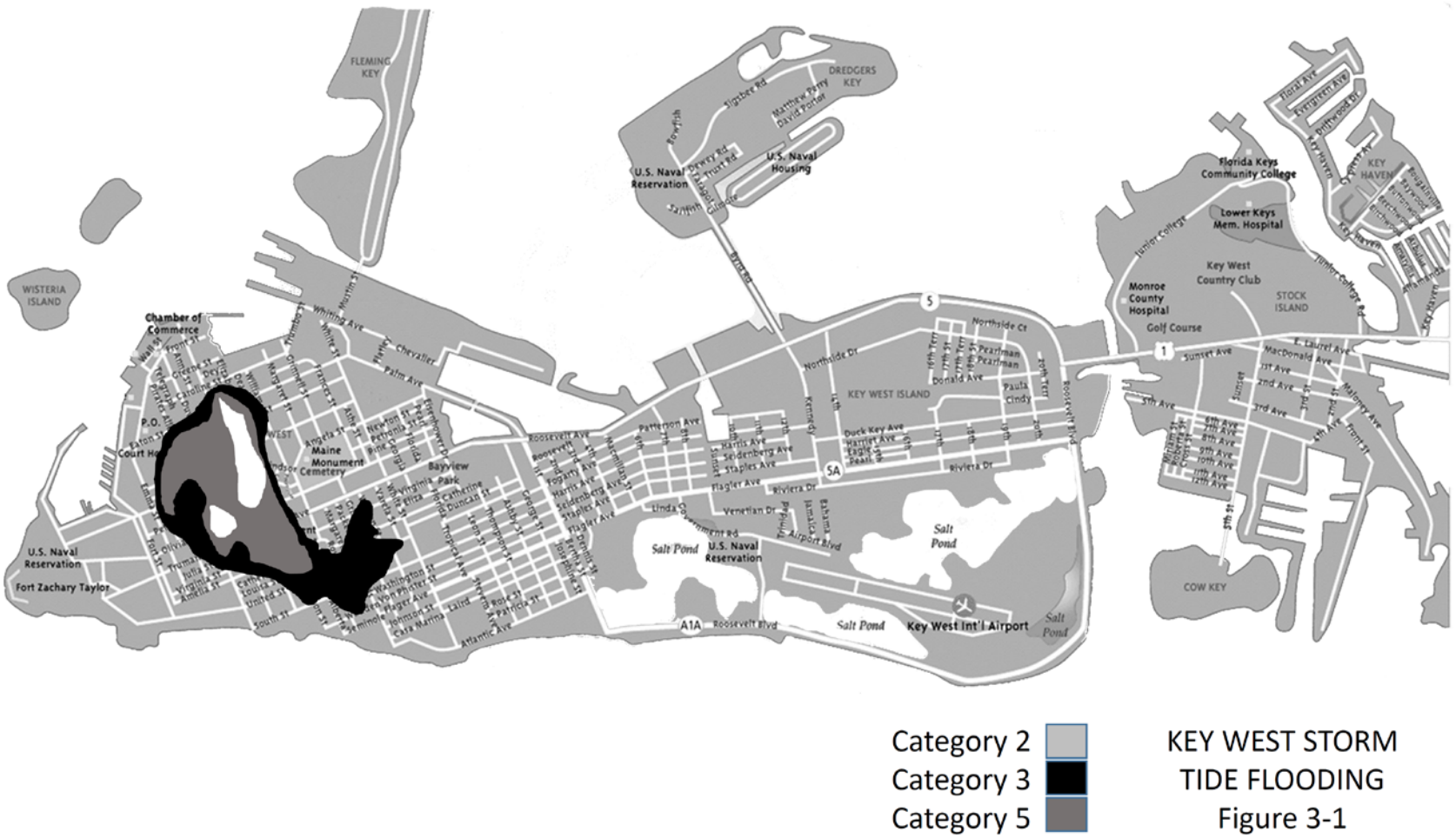
[14] http://www.nhc.noaa.gov/HAW2/english/basics/images/cat1_gulf.gif. [Accessed 27 Sept 2012]

[15] http://www.aoml.noaa.gov/hrd/tcfaq/E20.html [Accessed 27 Sept 2012]

[16] http://www.srh.noaa.gov/key/?n=wilma

[17] http://gis.fema.gov/REST/services/FEMA/Surge/MapServer [Accessed 3 Oct 2013]

Figure 9 Storm Surge Flooding Map for Key West.



Source: The image is re-drawn from Lower South East Florida Hurricane Evacuation Study Technical Assessment Summary for Monroe County Florida Keys 1991. Category 2 storm surge would cover the whole area (mid grey) apart from the black; dark grey and white areas; a Category 3 storm would inundate the mid grey area and include the black area of the map and a Category 5 storm would inundate the whole area with the exception of the small white areas in the black area.

12.1.2.2 Biological and ecological properties

12.1.2.2.1 Threatened and endangered species

A threatened and endangered species habitat analysis has been carried out for Monroe County (attached – *Appendix B*) and the proposed release area, Key Haven, also known as Racoon Key. A total of 43 threatened, endangered, or candidate species were identified in this area, many of which were marine species. There was no habitat overlap with their habitat and the domestic or peri-domestic environment. The Stock Island Tree Snail is the only species found in the physical vicinity of the proposed trial site. An assessment has been conducted according the United States Fish and Wildlife Service (USFWS) criteria[18] to determine likely impacts from the study on this species. Using the criteria checklist from the Stock Island Tree snail Assessment guide, (reproduced below), it was determined that the use of OX513A is not likely to adversely affect (NLAA) as no removal or modification of habitat is proposed in this trial.

Criteria from the Stock Island Tree Snail Assessment Guide (USFWS):

A. *The parcel **IS** in a known location of the Stock Island tree snail, in the species focus area and/or on the RE parcel list..... **go to B***

B. *The applicant proposes no removal or modification of the Stock Island tree snail's native habitat (hammock and beach berm)..... **NLAA***

None of the critical habitats of the identified species overlap with the peri-domestic/domestic habitat of *Ae.aegypti,* meaning that the released mosquitoes won't occupy the same habitat as these threatened and endangered species.

12.1.2.2.2 National Wildlife Refuges (NWR)

The National Key Deer Refuge headquarters is located on Big Pine Key, which is 100-miles south of Miami and 30 miles north of Key West on Highway US-1, and 26 miles from Key Haven. It was established in 1957 to protect and preserve Key deer and other wildlife resources in the Florida Keys. The refuge is located in the lower Florida Keys and currently consists of approximately 9,200 acres of land that includes pine rockland forests, tropical hardwood hammocks, freshwater wetlands, salt marsh wetlands, and mangrove forests. These natural communities are critical habitat for hundreds of endemic and migratory species including 17 federally- listed species such as Key deer, lower Keys marsh rabbit, and the silver rice rat.

The Great White Heron refuge is also administered as part of the Key Deer Refuge, and is only accessible by boat. It was established in 1938 as a haven for great white herons (which are only found in the Florida Keys), migratory birds, and other wildlife. The refuge is located in the lower Florida Keys and consists of

[18] http://www.fws.gov/verobeach/ConservationinKeysPDFs/20130729_updated%20Stock%20Island%20Tree%20Snail%20Assessment%20Guide.pdf

almost 200,000 acres of open water and islands that are north of the primary Keys from Marathon to Key West. The islands account for approximately 7,600 acres and are primarily mangroves with some of the larger islands containing pine rockland and tropical hardwood hammock habitats. This vast wilderness area, known locally as the "backcountry," provides critical nesting, feeding, and resting areas for more than 250 species of birds.

The mosquito fauna of both national Deer Key and Great White Heron refuge have been evaluated; *Ae.aegypti* was found “rarely” which was defined as a total of less than 20 specimens in the total refuge (Leal and Hribar, 2012).

Three species of sea turtles rely on the backcountry for feeding and nesting. Endangered Green sea turtles and threatened Loggerhead sea turtles are the two documented species that successfully nest in the refuge. Hawksbill sea turtles are known to feed in seagrass beds throughout the refuge, but nesting has not been observed. Sea turtles mainly consume marine sponges, crustacea, and sea plants and are not known predators of *Ae.aegypti*. The Key West National Wildlife Refuge is another reserve that is administered as part of the Key Deer Refuge. It is only accessible by boat and comprises of more than 200,000 acres with only 2,000 acres of land. The area is home to more than 250 species of birds and is important for sea turtle nesting. The islands are predominately mangrove with a few beaches and salt ponds.

Another refuge that comes under the administration of the Key Deer Refuge is Crocodile Lake National Wildlife Refuge. It is located near Key Largo, approximately 40 miles south of Miami, and 94 miles from Key Haven. It was established in 1980 to protect critical breeding and nesting habitat for the endangered American crocodile and other wildlife. The refuge is located in North Key Largo and is currently comprised of 6,700 acres including 650 acres of open water. It contains a mosaic of habitat types including tropical hardwood hammock, mangrove forest, and salt marsh. These habitats are critical for hundreds of plants and animals including six federally-listed species. It is closed to general public use due to its small size and the sensitivity of the habitats and wildlife to human disturbance. Access to the refuge is by Special Use Permit only. The six federally endangered and threatened species indigenous to the refuge are highly susceptible to noise disturbance. The habitats they rely on for their survival can be adversely impacted by human traffic. It is highly unlikely that released mosquitoes could travel this far (i.e., tens of miles), as their dispersal by spontaneous flight is less than 200 m, and as there are no human habitations in the refuge, it is unlikely to form an unattractive habitat for *Ae.aegypti, as Ae.aegypti* is predominantly associated with human activity (Brown *et al*., 2011).

12.1.2.2.3 Conclusion

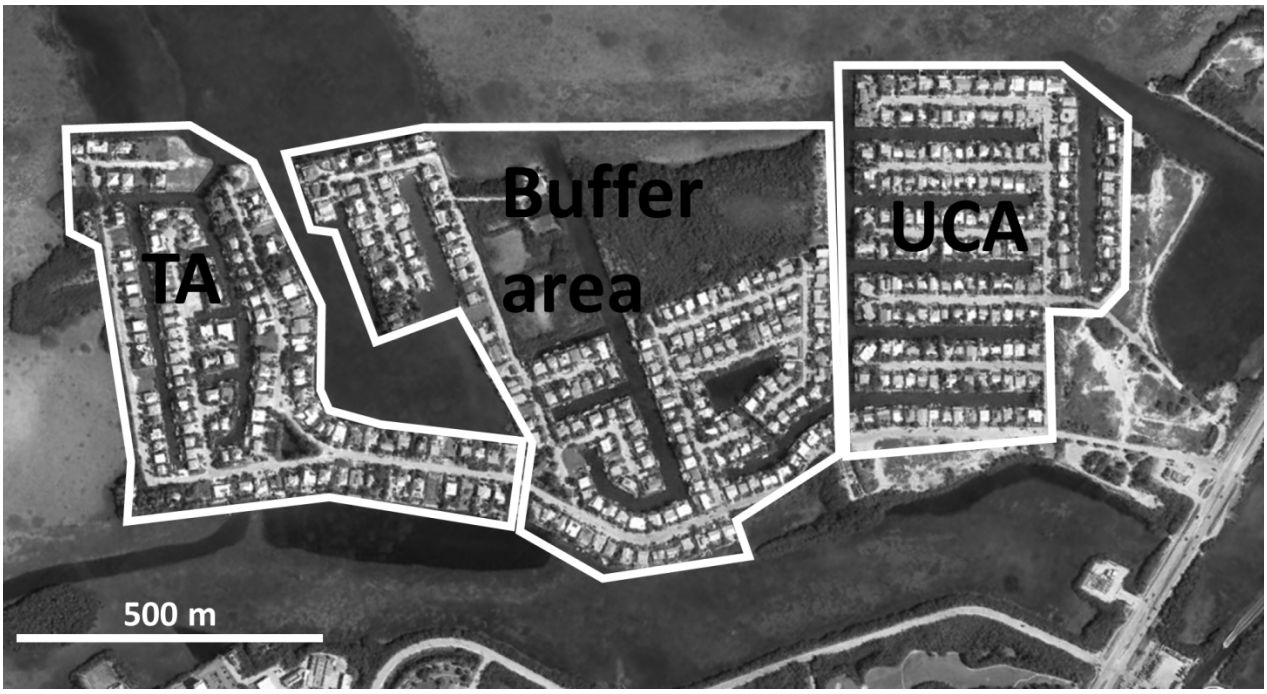
It is therefore concluded that release of OX513A will not affect threatened and endangered species or their habitats in Monroe County as there is no habitat overlap between the Key Haven release site and the habitat of these species.

12.1.2.3 Proposed release site

The proposed release site is located within Monroe County, on Key Haven, which has also been known as Racoon Key (identified areas in Figure 10)[19]. The release site is a Key that is surrounded by sea water with a small land attachment to the main island highway and hence an area that is quite isolated from the potential immigration of other *Ae.aegypti* which could comprise the success of the investigational trial.

The proposed site for evaluation of OX513A will be divided into two areas of similar size separated by a buffer zone (Figure 10). The area to receive releases of OX513A mosquitoes is identified as the Treatment Area (TA). The Untreated Comparator Area (UCA) is also identified in Figure 11, below. The Key Haven site has been monitored for *Ae.aegypti* since 2012, with both ovitraps and adult traps. FKMCD indicate that with all the current control measures (source reduction, larviciding, and adult insecticide) used over the whole of the Florida Keys that control of *Ae.aegypti* is only up to 50% effective (FKMCD 2014, personal communication).

Figure 10 Proposed Trial Area on Key Haven.



Proposed site for investigational release of OX513A mosquitoes. Areas identified are Treated (TA), Buffer, and Untreated Control Areas (UCA), respectively.

[19] There is another island in the Keys known as Racoon Key (24°44'48"N, 81 °29'28"W) which is located northwest of Big Torch Key.

12.1.2.3.1 Environment

The Monroe County Master Plan for Future Development on Stock Island and Key Haven (2006) describes in detail the land use and environmental condition of the site and pertinent information is summarized below, with the full report available at http://www.monroecounty-fl.gov/DocumentCenter/Home/View/1291.

According to the 2000 Census, single family homes comprise 41% of the housing types in Stock Island (SI) and Key Haven (KH) communities, with 64% of those single family homes located in KH. KH is exclusively developed with single family homes. There are different land use zoning categories in the KH and SI communities. The main land use zoning categories are residential, commercial, industrial, and public, although KH does not have any industrial zoning due to the residential nature of the island. There is only one commercial zone on KH, being a single gas station on the north side of US1 at the entrance to Key Haven. SI industrial use is predominantly maritime (e.g., boat repair, launching and maintenance, recreational fishing etc.). The present-day size and development pattern of SI and KH are primarily a result of dredge and fill activities. Much of this filling and development occurred since 1950. Because the Islands' history is so heavily human-influenced, there are few truly "natural" areas or native plant or animal species except the tree snail and occasional crocodile or alligator. The American crocodile is a threatened species living in brackish or saltwater according to USFWS[20]; whereas alligators are a fundamental part of Florida's swamps, rivers, and lakes.

Historically, Stock Island supported the largest population of Stock Island Tree Snails (*Orthalicus reses*), a tree-living snail. Habitat destruction and modification, pesticide use, and over-collection lead the U.S. Fish and Wildlife Service to include the tree snail on the list of threatened in July of 1978 (43 FR 28932). The population continued to decline through construction and increasing urbanization (USFWS South Florida Multi-Species Recovery Plan[21]). Beginning in October of 2000, the Stock Island tree snail had been relocated to public and private property throughout the Florida Keys and remaining populations are currently being monitored and tended to. USFWS[22] designates suitable habitat as hammock and beach berm. The USFWS species assessment guide has been utilized to determine if the proposed project could have an impact on the Stock Island Tree Snail (see Section 12.1.2.2.1).

The Monroe County Planning Department brought in tiered land characterization in 2002 (Goal 105)[23] with a view to determining priority for acquisition of land by the County, either for conservation or for affordable housing. Tier 1 lands are classified as the most environmentally sensitive, Tier 3 land as the least environmentally sensitive, as it is predominantly built upon and is where future building infill is to be

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[20] http://myfwc.com/wildlifehabitats/managed/american-crocodile/
http://myfwc.com/wildlifehabitats/managed/american-crocodile/ [accessed 26 Mar 2015]

[21] http://www.fws.gov/verobeach/MSRPPDFs/StockIslandTreeSnail.pdf [accessed 27 Jan 2015]

[22] http://www.fws.gov/verobeach/ConservationinKeysPDFs/20130729_updated%20Stock%20Island%20Tree%20Snail%20Assessment%20Guide.pdf [Accessed 23 Sept 2013]

[23] http://www.monroecounty-fl.gov/DocumentCenter/Home/View/1291.

directed. Key Haven lands are predominantly classified as Tier 3, with a section in the Middle Key Haven zoned as Native area (NA) and red-flag wetlands[24].

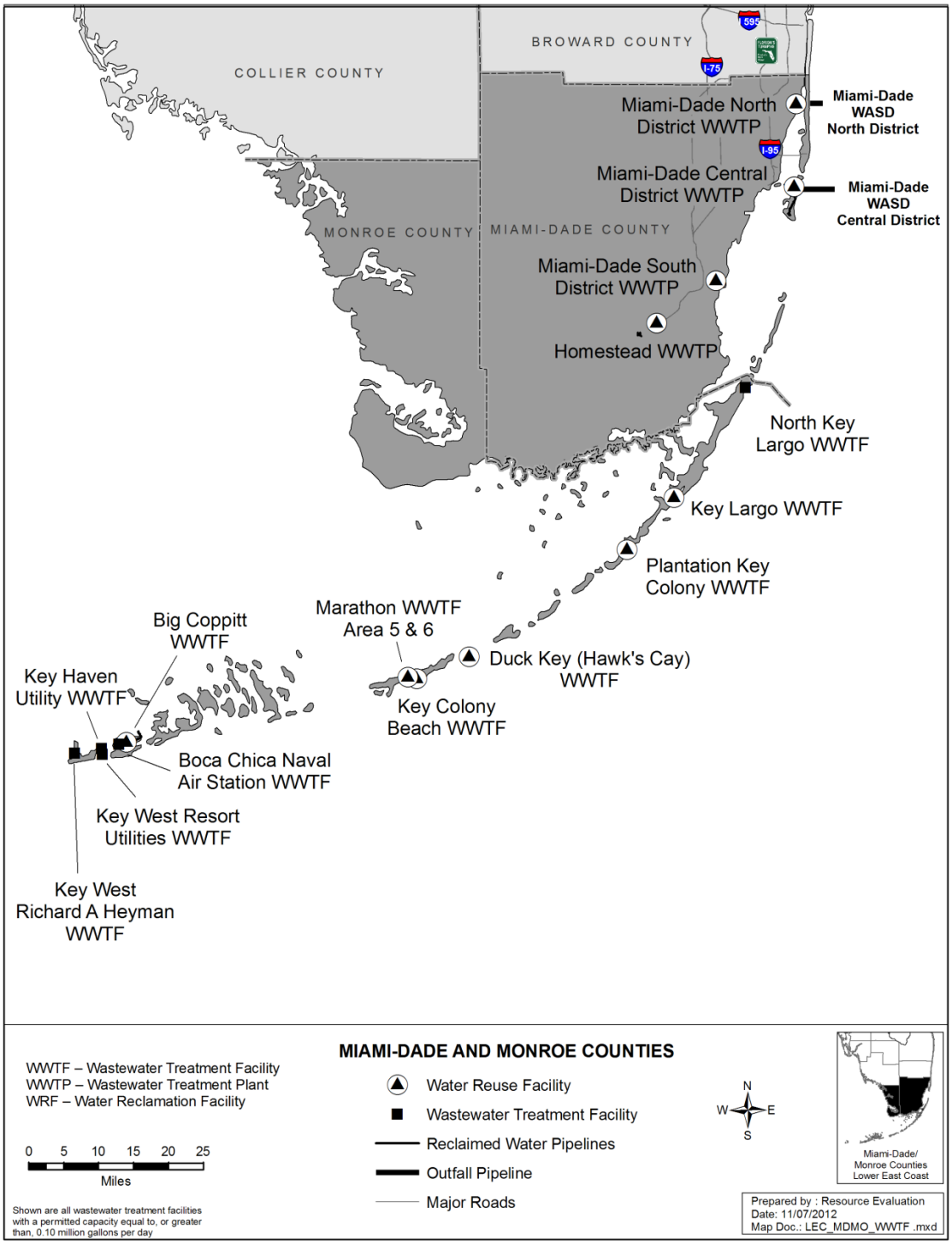
12.1.2.3.2 Water

The Florida Keys Aqueduct Authority (FKAA) is the provider of potable water for all of the Florida Keys. The main source of water for the FKAA is the Biscayne Aquifer with its well field located west of Florida City in Miami-Dade County providing most of the potable water for SE Florida, although the Biscayne Aquifer is designated as non-potable for the Keys due to the high chloride content. FKAA also operates a Reverse Osmosis (RO) plant on Stock Island, and is capable of producing 1.8 million gallons per day of water. The Monroe County Commissioners Resolution 426-2007[25] adopted the South Lower Key Regional Wastewater Treatment plant (WWTP) facilities plan, which was to include services at the Key Haven site. The location of the WWTP in Monroe County are shown in Figure 11, although it is noted in the plan that the Key Haven Utility is expected to be decommissioned in 2016 and its output flows are projected to be diverted to the Key West Resort Utilities WWTP.

[24]" Red-flag wetlands" are defined in the Keys Wetland Evaluation Procedure (KEYWEP) pursuant to Monroe County Code §118.10(4)(F)(1)(I)(AA) as "wetlands that clearly exhibit a high level of functional capacity and lack of disturbance prohibit development under any circumstances",

[25] http://www.minutes-monroe-clerk.com/WebLink8/DocView.aspx?id=131444&page=18&dbid=0 [accessed 6 Jan 2015]

Figure 11 Locations of the Wastewater Treatment plant (WWTP) in Monroe County, including Key Haven WWTF.



Source: 2012 Lower East Coast Water Supply Plan update [http://www.sfwmd.gov/portal/page/portal/xrepository/sfwmd_repository_pdf/lec_app_d_draft%20ext%2012-20-12.pdf - Accessed 12 Jan 2015].

12.1.2.3.3 Facilities

The HRU is proposed to be located in Marathon (Figure 12). The relationship between the HRU in Marathon and the proposed release site is shown in Figure 13.

The distance between Key Haven and Marathon is approximately 50 miles along the main highway linking the Keys (the Overseas Highway-U.S Highway 1). The HRU is located in an industrial zone, with residential housing close to Marathon Airport[26]. Marathon has piped potable water and a centralized sewerage system. The site is in sub-area 2 identified on the Marathon Master Plan[27], and contains a mix of land uses. Behind the Airport is the state owned Blue Heron Park. This pristine tropical hardwood hammock and scrub mangrove area is known habitat for the white crown pigeon and the eastern indigo snake. The park is surrounded by established residential subdivisions and borders the airport property. The marine environment off the coast of Marathon is designated as a National Marine Sanctuary.

Imports of OX513A eggs from the UK are shipped via international air carrier and then once cleared through customs and border protection at a major port are couriered by air to Marathon. This is further described in Section 10.4.1.6.

Figure 12 HRU site at FKMCD Marathon Base.

The FKMCD Marathon site is outlined in yellow, and the HRU is located in the FKMCD buildings.

[26] http://cityofm.tikilive.com/download/download.php?id=795

[27] http://cityofm.tikilive.com/download/download.php?id=2826

Figure 13 Relationships between the Proposed Site of the HRU and the Field Trial Location

TA = treated area, UCA = untreated control area

12.2 Survivability

12.2.1 Influence of abiotic factors on survivability of OX513A Ae.aegypti

The insertion and expression of the repressible lethality trait to *Ae .aegypti* is intended to confer a strong selective disadvantage, i.e., lethality to the strain. The penetrance of the introduced lethality trait in OX513A is approximately 95%, meaning that in the laboratory <5% of the progeny of OX513A males and wild-type females will survive if reared without the dietary antidote, tetracycline (Phuc, 2007). Laboratory conditions represent optimal conditions for the insects: field data indicates that survival is much lower. Mark release recapture studies with OX513A males were conducted in Malaysia (Lacroix et al., 2012) and the Cayman Islands (Winskill et al., 2014) to assess longevity of released males. Decay in recapture rate of males over time allowed estimation of daily survival probability (DSP), from which average life expectancy can be calculated as $-1/\text{Log}_e(\text{DSP})$.

In the Malaysian Study, OX513A average life expectancy was 2.0 (DSP=0.611) and 2.3 (DSP=0.646) days for the non-GE comparator, and therefore did not differ significantly from the non-GE laboratory strain co-released as part of a comparative evaluation. In the Cayman study, four separate mark release recapture

studies were conducted with resulting estimates of average life expectancy ranging between 0.1 (DSP=0.001) to 1.6 (DSP = 0.53) days. No non GE-comparator was released in the Cayman study.

It is possible that survival of the strain could be affected by exogenous tetracyclines in the environment. A review of the potential exogenous tetracycline concentrations that could be encountered in the environment has been conducted from the scientific literature, along with a dose response of the strain to tetracycline under a variety of scenarios (*Appendix C*). The OX513A strain was also examined for changes to the penetrance phenotype in the progeny when females were fed high doses of tetracycline in a blood meal (*Appendix G)*, mimicking the potential concentrations of tetracyclines that could be present in blood, if humans or animals were receiving a therapeutic tetracycline dose. This study is described in section 12.2.1.2.1 and used concentrations approximately 10 times higher than the highest dose found from the literature in human blood. The results showed that there was no increased survival of the OX513A mosquito female offspring if they were to take a blood meal from a human that has recently received a therapeutic dose of tetracycline.

Temperature is also a key factor in the survivability of the *Ae.aegypti;* Oxitec has evaluated the sensitivity of the strain to a range of temperatures, including those outside the known isothermic range of the insect (the isothermic range is reported as between 10°C - 30°C (50°F - 86°F), with optimal survival at 25-27 °C (77°F - 81°F); (Tun-Lin *et al.*, 2000, Hemme *et al*., 2009) to determine if the use of the #OX513 rDNA construct in the insect has any impact on its sensitivity to temperatures and could therefore potentially allow an expansion of its geographic range. The study evaluated larval rearing temperatures of 9, 18, 24, 30, and 37°C (48, 64, 75, 86, and 98.4°F). No survival of OX513A to adulthood outside the *Ae.aegypti* isothermic range at temperatures of 9°C (48°F) and 37°C (98.4°F) was identified (see report in *Appendix D*).

Tolerance to current insecticides is a further potential factor that could impact not only on the survivability of the OX513A strain, but also if the strain was carrying novel insecticidal resistance alleles that could be introgressed into the local population this could also impact on existing control measures for *Ae.aegypti.* Consequently, Oxitec commissioned a study from the Liverpool School of Tropical Medicine to evaluate the susceptibility of the OX513A strain to a range of current chemical control methods, using a standardized insecticide testing regime from the World Health Organization[28] as well as using literature information. The results showed that the OX513A strain was susceptible to discriminating doses of insecticides (temephos, permethrin, deltamethrin, and malathion), and it showed significant resistance to bendiocarb. The level of resistance to bendiocarb was comparable to that seen in the New Orleans (control) strain used (reported in *Appendix E*). A further study was conducted with the OX513A strain in Malaysia (Nazni *et al*., 2009a) which reported that the OX513A was susceptible to the current insecticides in use in vector control programs.

[28] http://whqlibdoc.who.int/hq/1998/WHO_CDS_CPC_MAL_98.12.pdf?ua=1

These studies are summarized in the sections below:

12.2.1.1 Sensitivity to tetracycline

Survival of the OX513A progeny is greatly reduced (to <5%) in the absence of the dietary antidote, tetracycline, due to the expression of the conditionally expressed lethal gene, tTAV. Hence, the response to tetracyclines in the environment can affect survivability of the strain. In order to determine the response of the OX513A strain to tetracyclines, Oxitec conducted a dose response study; the results were examined in light of potential exogenous tetracycline concentrations that might be encountered in the environment (*Appendix C*). Additionally, the strain was examined for longevity without tetracycline in the diet, as the length of time the strain survives in the environment contributes to overall survivability potential (*Appendix F*). Furthermore, the strain was also examined for changes to the penetrance phenotype in the progeny when females were fed high doses of tetracycline in a blood meal (*Appendix G*), mimicking the potential concentrations of tetracyclines that could be present in blood, if humans or animals were receiving a therapeutic dose. These studies and their results are presented in the sections below.

12.2.1.1.1 Dose response study to tetracycline

The response of OX513A strain to different doses of tetracycline has been evaluated in the laboratory, with the objective of the study to identify the lowest concentration of tetracycline that allows for greater survival of OX513A progeny than when reared in the absence of tetracycline. The study evaluated twelve different concentrations of tetracycline in the rearing water ranging from 10pg/mL to 1 µg/mL. Oxitec determined that concentrations of 3ng/mL tetracycline yielded a small but statistically significant increase (p=0.212) in the fraction of functional (flying) adults over those reared without tetracycline, with full rescue of the phenotype occurring above 1 µg/mL (as shown in Table 2). Therefore the no observable effect level (NOEL) was determined to be 1ng/ml.

Table 2 shows the dose response of hemizygous OX513A larvae to different concentrations of tetracycline. Percentages are means of first instar larva (L1) individuals reaching the specified stage based on initial counts of 200 L1s per repeat. Confidence intervals are displayed in parentheses. “Non-viable adults” were defined as dead adults on the water surface, dead adults in the cage, and non-flying adults.

Table 2 Dose Response of Hemizygous OX513A Larvae to Differing Concentrations of Tetracycline.

Tetracycline concentration	Dead pupae	Non-viable adults	Flying adults
1 µg/mL	0.8% (0.0%-1.6%)	6.7% (2.3%-11.1%)	60.9% (54.5%-67.3%)
300 ng/mL	0.4% (0.0%-1.0%)	7.0% (3.0-11.0%)	57.4% (50.4%-64.4%)
100 ng/mL	0.2% (0.0%-0.6%)	15.5% (10.0%-21.0%)	51.1% (44.6%-57.6%)
30 ng/mL	1.8% (0.5%-3.1%)	31.5% (25.9%-37.1%)	42.3% (34.6%-50.0%)
10 ng/mL	13.3% (8.0%-18.5%)	36% (33.3%-38.7%)	30.8% (26.9%-34.6%)
3 ng/mL	36.6% (28.4%-44.8%)	31.25% (29.0%-33.5%)	8.9% (6.6%-11.1%)
1 ng/mL	51.2% (47.4%-54.9%)	18.5% (16.3%-20.7%)	4.3% (3.2%-5.4%)
300 pg/mL	57.7% (52.6%-62.8%)	18.1% (14.7%-21.5%)	3.2% (2.3%-4.1%)
100 pg/mL	57.7% (49.3%-66.1%)	14.9% (10.8%-19.0%)	3.9% (2.4%-5.4%)
30 pg/mL	57.2% (53.0%-61.4%)	15.5% (12.8%-18.2%)	4.8% (4.1%-5.5%)
10 pg/mL	63% (52.9%-73.1%)	12.5% (9.0%-16.0%)	2.5% (1.3%-3.7%)
0	50.2% (45.0%-55.3%)	12.5% (9.2%-15.8%)	3.4% (2.4%-4.3%)
Rows do not add up to 100% as dead larvae fractions are not recorded in these figures.			

A survey of the literature found maximum reported concentrations of tetracylines from field sites around the world as follows: tetracylines 0.096ng mL^{-1} to 1.3ng mL^{-1} (e.g., chlortetracycline 0.04ng mL^{-1} to 0.97 ng mL^{-1}, oxytetracycline 0.7ng mL^{-1} to 1.34ng mL^{-1} and doxycycline 0.07ng mL^{-1} to 0.4ng mL^{-1})(Gulkowska *et al*., 2008, Brown *et al*., 2006; Le-Minh *et al*., 2010; Locatelli *et al*., 2011; McQuillan *et al*., 2002, Watkinson *et al*., 2009).

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 drains from Key Haven and Key West via sewerage, and there are two local water treatment plants (see Figure 11) that could hypothetically hold waters with residues of tetracylines. 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 (Bautiz 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. This is likely an overestimate for *Ae.aegypti* as waste water treatment environments are not typical *Ae.aegypti* larval habitats which include artificial containers such as used car tires, flower vases, water storage vessels and discarded materials in the domestic/peri-domestic environments.

From a review of the accessible environments (Section 12.1 of this document), there are no apparent sources of high concentrations of environmental tetracylines, as there are no commercial farming (land based or marine) enterprises or hospitals in the immediate vicinity of the proposed release site. The nearest hospital/clinic is over 300 m away from the proposed release site separated by an inlet comprising of sea water and vegetation. The inlet with the sea water and the vegetation bordering it provides a geophysical barrier to dispersal of the released mosquitoes through spontaneous flight (Hemme *et al*., 2010; Maciel- de-Freitas *et al*., 2010) especially as there are/ will be sufficient breeding sites (ovitraps) in the release site, so the male mosquitoes don't need to fly far to find the females with which to mate.

The dose-response study presented in Table 2 has demonstrated that tetracycline concentrations at and below 1 ng/mL do not increase the fitness of OX513A larvae, i.e., do not increase the proportion of functional adults. The overall mean percentage of functional OX513A adults reared with no effect from the tetracycline (concentrations 0 to 1 ng/mL) was 3.7% (CI 3.24%-4.18%). The complete study is provided in *Appendix C*. Full rescue of the OX513A individuals (the maximum number surviving to functional adults) was also shown in this data to require tetracycline concentrations that were 746 to 2500 times greater than the maximum value we found in the literature for environmental tetracylines.

12.2.1.1.2 Conclusion

Tetracycline concentrations above the rescue level of 1ng/ml are very unlikely to be found in the typical breeding sites of Ae.aegypti such as man-made containers or uncovered stored water near homes. There are no commercial farms, aquaculture facilities, or hospitals in the immediate vicinity of the release site that have the potential to provide sufficient levels of tetracycline residues. Data from the literature regarding environmental presence of* tetracyline*s and the data reported in Table 2 indicate that OX513A larvae would need to encounter environmental tetracycline concentrations 746 -2500 times greater than the maximum value we found reported in the literature to fully rescue the non-lethal phenotype. Even if the level of tetracycline in the environment was high enough to increase survival, if a female mated with an OX513A male lays her eggs in water with tetracycline and some adults may emerge depending on the tetracycline concentration, then they still carry a copy of the #OX513 rDNA construct meaning that >95% offspring from their mating would die if they didn't encounter sufficient environmental tetracycline again (Harris et al. 2011). As Ae.aegypti prefers to lay eggs in different containers (known as skip oviposition (Rey and O'Connell, 2014)) the probability that they would all contain tetracycline of sufficient quantity to increase survival is very low.

12.2.1.2 Longevity of OX513A reared on/off tetracycline

The longevity of the strain (adult males and females) has been evaluated in the laboratory. The homozygous OX513A strain used for field trials in Brazil was outcrossed to wild-type of the "Latin" background to generate hemizygous eggs. These eggs were hatched and reared in the absence of the antibiotic tetracycline that is required for survival of most OX513A individuals. Emerged, flying adults were collected and housed in single-sex groups. The longevity of these individuals was assessed over a period of more than 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 hemizygous offspring of OX513A males will encounter in the wild. The 1µg/mL dose was selected because it is the minimum dose needed to give rise to the maximum percentage of flying adults (see *Appendix C*), yet well over the amounts of tetracycline animals might encounter in the field as described above. Longevity of homozygous OX513A individuals reared on the standard tetracycline dose of 30 µg/mL was also assessed.

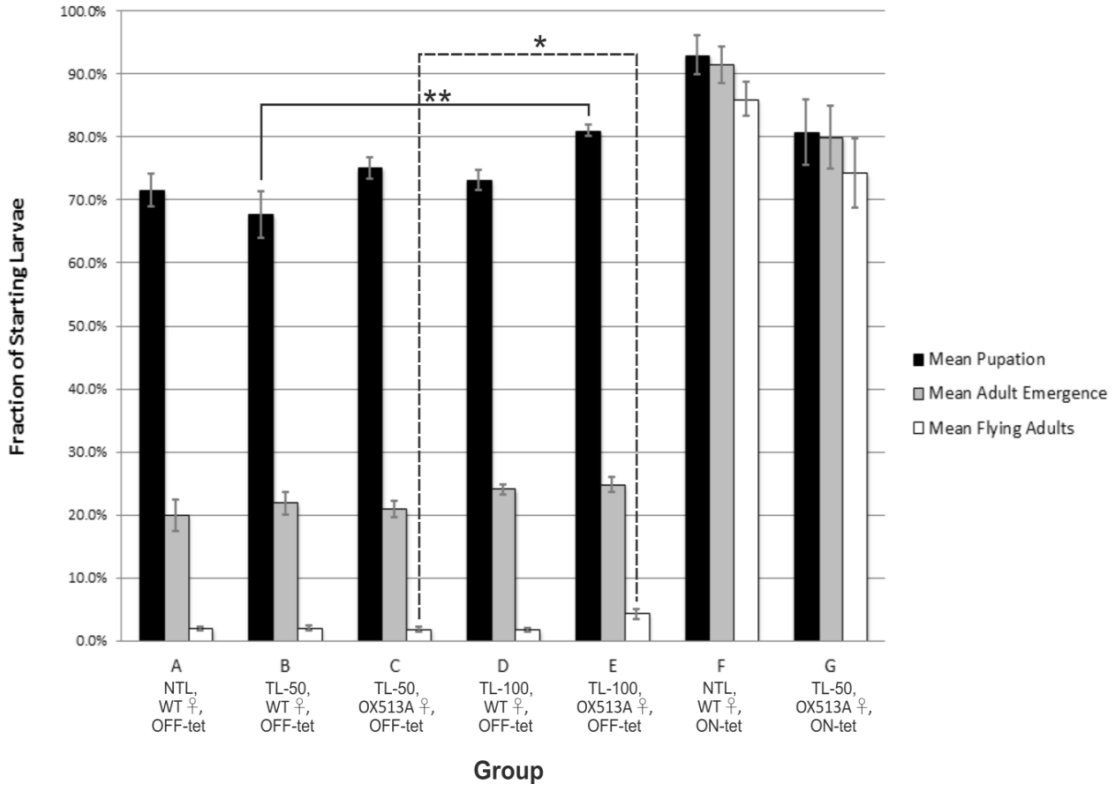
These experiments therefore examine the longevity of the two types of OX513A female most plausibly present in the field – homozygous females inadvertently co-released with homozygous males, and hemizygous progeny of released males that have mated with wild females and survive as a consequence of incomplete penetrance of the lethal trait. The lifespan of OX513A homozygotes and hemizygotes reared on tetracycline was found to be no longer than that of the wild type comparators and the median lifespan of OX513A females was significantly shorter than the wild type comparators (65 days vs.72). As longevity is an important component of vectorial capacity (i.e., ability to transmit disease), shorter lifespan implies reduced vectorial capacity, especially for hemizygous females reared without tetracycline (with a median lifespan of two days relative to a wild-type median lifespan of 68 days). The full report is available at *Appendix F*. Environmental factors are known to reduce daily survival compared to in the laboratory (Joy et al, 2012) and from previous trials with OX513A (Lacroix *et al*, 2012; Winskill *et al*, 2014).This reduction in longevity also implies that the mean fitness of hemizygous OX513A males and females reared without tetracycline is even lower than one would estimate simply by considering survival to adulthood alone.

12.2.1.2.1 The evaluation of the potential for changes in penetrance of the introduced traits on exposure to high doses of tetracycline in blood feeding

As there is a potential for small numbers of female mosquitoes to be released or result from progeny of mating with OX513A males, a study was conducted to test the hypothesis that providing high doses 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) has no effect in the penetrance of the OX513A lethal phenotype observed in their hemizygous offspring. As tetracycline is an antibiotic used as a therapeutic and/or prophylactic agent in human and veterinary medicine, 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 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,). 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). There are no farms in Key Haven although companion animals and humans may be on therapeutic doses of tetracyclines. In the study, Oxitec used concentrations of tetracycline approximately 10 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 (Figure 14).

Figure 14 Summary of results of tetracycline-loaded blood study.



No significant difference for any parameter was observed between the non-tetracycline-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 genetically engineered. OFF-tet: Larvae reared without tetracycline. ON-tet: Larvae reared with tetracycline added to the rearing water.

Oxitec's results (Figure 14) indicate no significant differences in any parameter observed between the non-tetracycline control group and any of the treatment groups, but significant differences were observed in pupation and the numbers of flying adults between two of the treatment groups. The complete study is included in *Appendix G*. These results indicate that the penetrance of the OX513A phenotype in hemizygous 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. Therefore there is no increased survival of the OX513A mosquito in the event that a surviving hemizygous female offspring takes a blood meal from an individual (human or animal) that has recently received a human or veterinary therapeutic dose of tetracycline that could still be at a high concentration in their blood. This study was conducted with concentrations approximately 10 times the highest concentration of tetracycline that is found in human blood and 5 times that found in animal blood.

12.2.1.2.2 Conclusion

Taken together with the longevity data in 12.2.1.2 these results support the assertion that the ability of the strain to survive outside the laboratory is unlikely to be affected by environmental exposure to exogenous tetracycline sources.

12.2.1.3 Susceptibility to chemical insecticides

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, and standard mosquito control will continue to be used, as necessary, during the duration of the proposed field trial (see Section 11). Furthermore, should the OX513A mosquito contain any genes that impart resistance to insecticides, and those genes introgress into wild populations of *Ae.aegypti* via sexual reproduction, deployment of OX513A could result in increased resistance to current chemical controls, which could compromise overall *Ae.aegypti* control in the trial location. Oxitec therefore commissioned a study to evaluate the susceptibility of OX513A mosquitoes to insecticides (See *Appendix E*).

A study commissioned in 2011 by Oxitec (performed by the Liverpool School of Tropical Medicine, LSTM) tested the susceptibility of the OX513A strain to five commonly used insecticides (temephos, permethrin, deltamethrin, bendiocarb, and malathion) and screened the OX513A mosquitoes for the presence of knock-down (kdr) mutations 1016 and 1534, which are associated with resistance to pyrethroids and DDT. A susceptible laboratory strain (*Ae.aegypti* New Orleans) was used as control for the study. Standard WHO procedures and discriminating doses[29] were used, and 100 insects were assayed in each treatment. Temephos (which is a larvicide) 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 are summarized in Table 3.

[29] http://whqlibdoc.who.int/hq/2006/WHO_CDS_NTD_WHOPES_GCDPP_2006.3_eng.pdf [Accessed 8th May 2013]

Table 3 Mosquito Mortality Recorded 24 Hours after Exposure to Insecticide.

		OX513A				NEWORLEANS strain			
Insecticide	**Dose**	**No. tested**	**No. alive**	**No. dead**	**% mortality**	**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 strain was found to be susceptible to discriminating doses of temephos, permethrin, deltamethrin, and malathion, and it showed significant 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 CDC. 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, the only plausible explanations are that either the recommended doses for bendiocarb are inappropriate for this species (as suggested in the original report), or that variation associated with such tests (for example due to inaccurately prepared or old solutions, inconsistent dosing, inaccurate endpoint timing, climatic conditions etc.) had resulted in a corresponding shift in responses of both strains.

Given the above, the key metric of a comparison between the levels of mortality observed in OX513A with those of the accepted susceptible standard remains valid i.e., no significant difference for all compounds. As previously mentioned, the OX513A strain 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 absent in the OX513A strain.

A separate study was conducted in Malaysia by Nazni *et al*., 2009a. This study compared the susceptibility of the strain MyRIDL-513A[30] and the laboratory strain MyWT. Seven insecticides (DDT, Fenitrothion, Malathion, Propoxur, Permethrin, Lambdacyhalothrin, and Cyfluthrin) were tested following standard WHO methods. All of the insects used were 3-5 day old females, and there were 25 adults in each test. 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 *Ae.aegypti* populations).

Taken together these studies provide evidence that OX513A is no more resistant to insecticides than the comparator wild-type strain. As the strain is susceptible to the currently used insecticides (as described in Section 8.1) it may be an advantage in large scale use as there is a theoretical possibility that these insecticide susceptibility alleles in OX513A are introgressed into the insecticide resistant population, making them more susceptible to the currently used insecticides. This could be regarded as an additional potential benefit for the use of OX13A in vector control programs.

12.2.1.4 Temperature

Temperature is a key abiotic factor in the consideration of the survivability of *Ae.aegypti* OX513A, although this can be complicated by the interaction with diet and larval density dependent effects (Couret *et al*., 2014). Worldwide, *Ae.aegypti* is a non-native tropical species with a cosmopolitan habitat extending from 40° N to 40° S latitude. *Ae.aegypti* has an ecological temperature range of 14-30 ^{0}C [~57- 86^{o} F](Brady *et al*., 2014, Brady *et al*., 2013, Hemme *et al*., 2009). The effect of temperature on larval development of *Ae.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 (Christophers *et al*., 1960, Rueda *et al*., 1990). High temperatures alone (>40^{o}C [104^{o}F]) are unlikely to limit the species but low temperatures are limiting with the threshold being around the 15^{o}C [59^{o}F] isotherm. At temperatures lower than 15^{o}C, *Ae.aegypti* become torpid, unable to fly, or move their limbs only slowly (Christophers *et al*., 1960, Rowley and Graham, 1967; Yang *et al*., 2009). Lower temperatures can slow development time to such a degree (where egg-to-adult cycles are longer than 45 days) that the species is prevented from establishing itself in the environment.

Global historical collections and laboratory experiments on this well-studied vector have suggested its distribution is limited by the 10°C [~50^{o}F] winter isotherm[31] (Christophers, 1960), while a more recent and complex stochastic population dynamics model analysis suggests the temperature's limiting value to

[30] 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.

[31] 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.

be more towards the 15°C[~59°F] yearly isotherm (Otero *et al*., 2006). Low temperatures below 10°C [~50°F] are therefore likely to severely limit the geographical range of *Ae.aegypti*, although the protection provided by human habitations may afford some protection from lower temperatures. Scholte *et al*. (2010) indicated that *Ae.aegypti* could not survive winter temperatures in Northern Europe. In a recent study, Thomas *et al*. (2012) found that a tropical strain of *Ae.aegypti* eggs could only survive at a threshold of-2°C [~28°F] for 24 hours before hatching broke down completely. Survival at temperatures below freezing is therefore extremely unlikely from the scientific evidence, and not a temperature that is likely to be encountered in the FL Keys.

12.2.1.4.1 Study on the temperature response of OX513A

The temperature response of the OX513A strain has been evaluated in the laboratory. *Ae.aegypti* larvae, hemizygous for the OX513A construct, were reared at five temperatures ranging between and including 9°C [~48°F] and 37°C [98.6°F]. 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. Oxitec found that OX513A larvae and Latin WT larvae died before pupation when reared at 9°C and 37°C (See *Appendix D* for the full report).

These results demonstrate 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 under laboratory conditions. No evidence was 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 first instar larvae (L1) to functional adult (range 0-2%) (*Appendix D*). 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 be extremely unlikely to expand the habitable geographic range of *Ae.aegypti*.

The geophysical containment of the species is also discussed in Section 14.3.

12.2.1.4.2 Conclusion

Ae.aegypti has a distinctive global distribution which is limited by a number of abiotic factors such as temperature and availability of breeding sites containing fresh water. Survivability of the OX513A strain is impacted by sensitivity to temperature, the antibiotic tetracycline and its analogues, used to control the repressible lethality of the strain, and the susceptibility of the insect to insecticides.

Laboratory studies have indicated that the genetic engineering has not altered the mosquitoes' response to temperatures across a biologically relevant range and consequently no increased distribution of the mosquito is anticipated. Similarly, the sensitivity of the strain to* tetracyclines *has been examined in laboratory conditions. Studies have also been conducted that conclude there is no increased survival of the OX513A mosquito from blood meals spiked with high concentrations of tetracycline, doses that are higher than that would be given to humans or animals therapeutically. Therefore it is unlikely that a surviving

hemizygous female offspring taking a blood meal from an individual (human or animal) that has recently received a human or veterinary therapeutic dose of tetracycline, will imbibe sufficient tetracycline to allow the survival of the mosquito. Therefore, it is highly unlikely that the tTAV protein will be expressed if OX513A mosquitoes encounter the tetracycline levels found in the environment or in human or animal blood (in the unlikely event that a female OX513A mosquito were to bite a human or animal therapeutically treated with tetracycline).

Two studies have shown that the genetic engineering did not affect susceptibility of the OX513A line to currently used insecticides.

In conclusion therefore, the response of OX513A to abiotic factors is likely to be the same as non-genetically engineered Aedes aegypti.

12.2.2 Biotic factors affecting survivability

12.2.2.1 Reproduction

In *Ae.aegypti,* reproduction is sexual with internal exchange of gametes. Mating occurs in aerial swarms, which form around the blood-meal host (Hartberg, 1971). These aggregations are primarily composed of males, with females entering the swarm singly. Pheromones are also involved in swarming behavior (Fawaz *et al*., 2014). Mating occurs in flight, where males and females meet, form a "copula" in mid-air, and mate in a matter of seconds (Hartberg, 1971; Moore, 1979). Key mating behaviors, such as males resonating their antennae to a certain pitch, which the females reproduce by beating their wings at the same specific frequency, are essential to successful coupling between males and females (Cator *et al*., 2011, Cator *et al*., 2009).

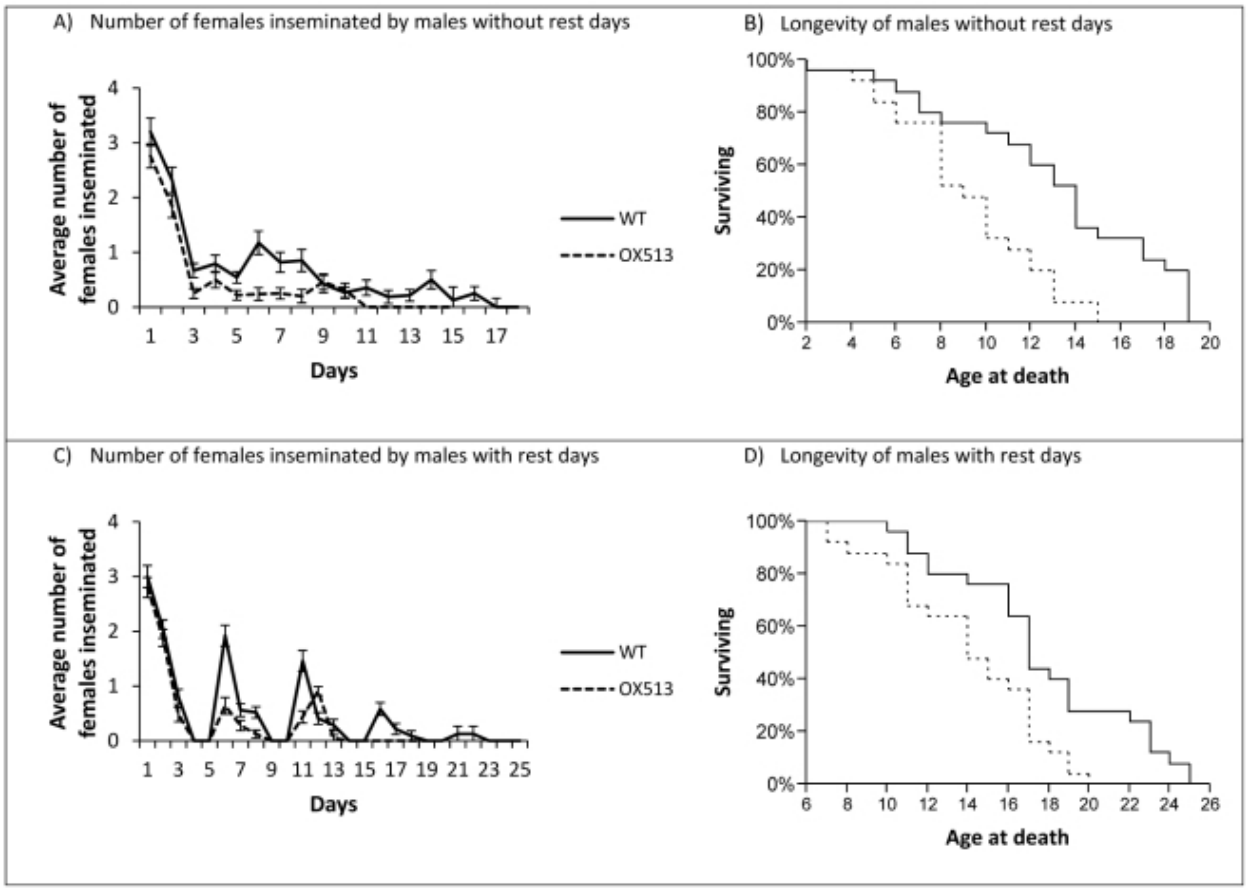
The average adult lifespan is 8-15 days for female mosquitoes and 3-6 days for male mosquitoes (Clements, 2000) although this is highly dependent on temperature, being shorter in tropical regions and longer in more temperate climates, with male mosquitoes not being sexually mature until up to 24 hours post-emergence from the pupal case. The female's behaviors are dependent on her gonotrophic cycle, i.e., response to the host and finding a bloodmeal, digestion of the blood and formation of mature oocyctes, which are then fertilized and oviposited (laid). Although females may go through several gonotrophic cycles in their lifespan as inseminated females store spermatozoa to fertilize a number of egg batches, they are largely regarded to mate only once during their lifetime (Pascini et al., 2012), as seminal fluid proteins are transferred, which render females unreceptive and more refractory to further copulation (Helinski et al., 2012, Avila et al., 2011, Sirot et al., 2008).

The role of male mosquitoes in the reproductive cycle is the insemination of the females. Male reproductive success is dependent on insemination success and reproductive output. During mating, male mosquitoes transfer not just sperm, but also seminal fluid proteins, as described above, that may have profound effects on mated female biology and behavior. Size of male mosquito also influences mating success, with larger males having greater reproductive success than smaller males, mostly likely to sperm depletion (Helinski and Harrington, 2011). Nonetheless, even small males appear to transfer sufficient seminal fluid proteins to prevent further mating of the female (Dickinson and Klowden, 1997).

12.2.2.1.1 Insemination capacity of OX513A males

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, Figure 15).

Figure 15 Insemination Capacity of OX513A Males (from Bargielowski *et al*., 2011a).



Results show distinct differences in the insemination capacity and the cost of mating in males of the genetically engineered OX513A and the WT line. Genetically engineered 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, likely to be a result of mass-rearing, as it is known that mass –rearing can have an adverse impact on fitness parameters relative to wild counterparts (Rao *et al.,* 2014, Rull *et al.*, 2012, Dominiak *et al.,* 2008, Peters *et al.*, 1977).

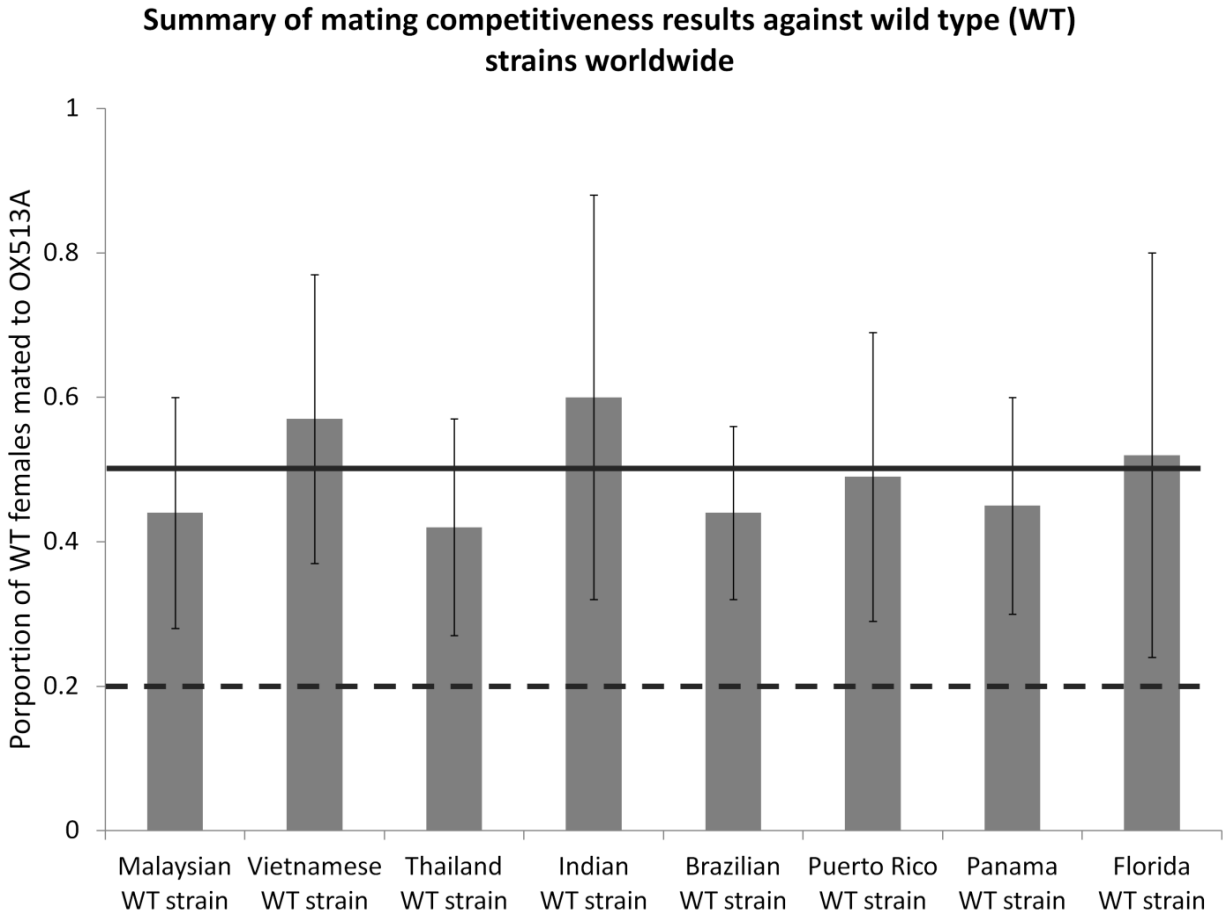
12.2.2.2 Mating competitiveness of the OX513A Ae.aegypti mosquito

In mosquitoes, mating is extremely species-specific. For example, in different species the wing beat frequency can be used for mate detection with the sexes matching the wing beat in harmonics of the flight tone (Cator *et al*., 2009). In *Ae.aegypti,* the male and female wing beat tone converges and they mate in flight. The ability of OX513A male mosquitoes to mate with the wild female mosquitoes at the release site is essential to effect population suppression. Therefore, extensive testing of the OX513A strain mating competiveness in a range of environments has been carried out. This includes studies in laboratory cages and in open field release in the Cayman Islands (Harris *et al*., 2012; Harris *et al*., 2011) and Brazil (*Carvalho et al 2015*).

12.2.2.2.1 Mating competitiveness in the laboratory

Mating competitiveness studies against wild-type strains from around the world have been carried out in a wide variety of laboratory settings. If the OX513A male were equally attractive to the female as a wild-type male, mating competitiveness would be equal to 0.5 (Figure 16). The OX513A strain performed successfully against all the wild-type strains tested regardless of the genetic background as none of the mating competitiveness estimates differ significantly from 0.5. 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 is acceptable for a successful SIT program (FAO/IAEA, 2003).

Figure 16. Summary of 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.

12.2.2.2.2 Mating competitiveness in the field

Mating competitiveness (C) is defined as the relationship between the numerical density of wild-type (*N*) and sterile (*S*) insects and the relative mating success, such that $C = PN/S\ (1 - P)$ where *P* is the proportion of sterile matings, i.e., proportion of fluorescent larvae (Mayer *et al*., 1998; Vreysen, 2005). The 95% confidence intervals were obtained by running a bootstrap statistical analysis (Davison *et al*., 1997, Manly, 2007) on the relative mating success and numerical density of wild-type and sterile insects. All the sustained field releases of OX513A males conducted to date have enabled the estimation of their mating competitiveness. Mating competitiveness is increased when the insects are sexually competitive and of high quality. The process of mass rearing can impact the quality of the insects. The very first releases in the Cayman Islands, which were to demonstrate the proof of principle that Oxitec could produce males, used low rearing densities which gave a mating competitiveness estimate of 0.56 (95% CI:

0.032-1.97, Harris *et al*., 2011). In the following studies the objective was to achieve local *Ae.aegypti* population suppression and with increased mass production to provide sufficient insects for the trial, mating competiveness ranged from 0.0004 to 0.059 (Harris 2012, Carvalho *et al*., 2015). 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 environment 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 local *Ae.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 population had already been suppressed during that period *(Carvalho et al, 2015*).

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 SIT programs, 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*) (Rendón *et al*., 2004; Shelly *et al*., 2007). 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.

Table 4 Summary of Mating Competitiveness Evaluation of the Oxitec OX513A Males in the Wild.

	Location and Date													
	Cayman Islands 2009	Cayman Islands 2010	Itaberaba, Brazil 2011-2012											Mandacaru, Brazil 2012
Mating Competitiveness	**0.560**	**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	1.970	0.210	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

CI – Confidence limit

The data provided in Table 4 are from three different types of typical environments for *Ae.aegypti.* The Cayman Islands data (Harris *et al*., 2011 and 2012) represent a site that was isolated and untreated with conventional insect control measures; the Brazilian Itaberaba site data *(Carvalho et al 2015*) represent a densely populated environment with a high degree of immigration of *Ae.aegypti* from other areas; and the Brazilian Mandacaru environment data 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 *Ae.aegypti,* across different backgrounds and environments.

12.2.2.2.3 Conclusion

The successful mating competitiveness of OX513A with wild Ae.aegypti from different backgrounds and in different housing densities implies that the insertion of tTAV and DsRed2 at the insertion site does not appear to exert any positional effects including alterations in the ability of OX513A to react to specific mating signals from wild Ae.aegypti i.e., the mating competitiveness of OX513A. This means that the highly species specific nature of mosquito reproduction is not compromised by insertion of the #OX513 rDNA construct. OX513A males successfully mating with wild-type Ae.aegypti females results in progeny that carries a repressible lethality trait and consequently would die before reaching adulthood. Based on reproductive behavior of Ae.aegypti, the transmission of the inserted genetic trait by sexual reproduction is limited to the species Ae.aegypti only.

12.3 Dispersion

12.3.1 Dispersal of the OX513A Ae.aegypti mosquito

Spontaneous flight of adult *Ae.aegypti* is limited to around 200 m depending on availability of breeding sites, and hosts from which to take a blood meal (Facchinelli *et al*., 2012,Maciel-de-Freitas *et al*., 2010; Suwonkerd *et al*., 2006), although there are reports of females travelling further, even in urban environments (Halstead, 2008). Roads, water courses, and vegetation represent significant barriers to the movement of *Ae.aegypti* (Hemme *et al*., 2010; Maciel de Freitas, 2009), which is adapted to live in close proximity to human habitations.

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 *Ae.aegypti* in Florida. As an example of passive transport it has recently been reported that *Ae.aegypti* has been detected for the first time in California and that it had likely come from the Southeastern US (Gloria-Soria *et al,* 2014). International Sanitary Regulations (WHO, 2005) require ports and airports to establish programs to control *Ae.aegypti* and other insect disease vectors for at least 400m from point of entry facilities, as a result of this potential for passive transport

Altitude is thought to affect distribution, with an elevation of 6,000-8000 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 *Ae.aegypti* over 2,000 m. The slope of the elevation could also be an influencing factor, with plateaus being more preferable than steep slopes.

Elevation is not a consideration for affecting dispersal of mosquitoes in Monroe County and Key Haven as the majority (>90%) of the land mass is around or just above sea level[32].

Other factors affecting distribution/dissemination of *Ae.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). High temperatures common in desert areas alone, however, are unlikely to limit distribution but the combination of high temperature and low humidity with lack of shade and breeding sites are contributory factors. Landscape or geophysical barriers to movement of *Ae.aegypti* include saltwater, rivers, roads, areas of vegetation without human habitation, and altitude (Hemme *et al*., 2010; Maciel- de-Freitas *et al*., 2010, Maciel de Freitas *et al*, 2009, Navarro, 2010).

Climate (specifically temperature), urbanization, water storage and the availability of breeding sites, are therefore the main factors that influence the distribution, survival and establishment of *Ae.aegypti.*

12.3.2 Data obtained from field release on dispersal of OX513A

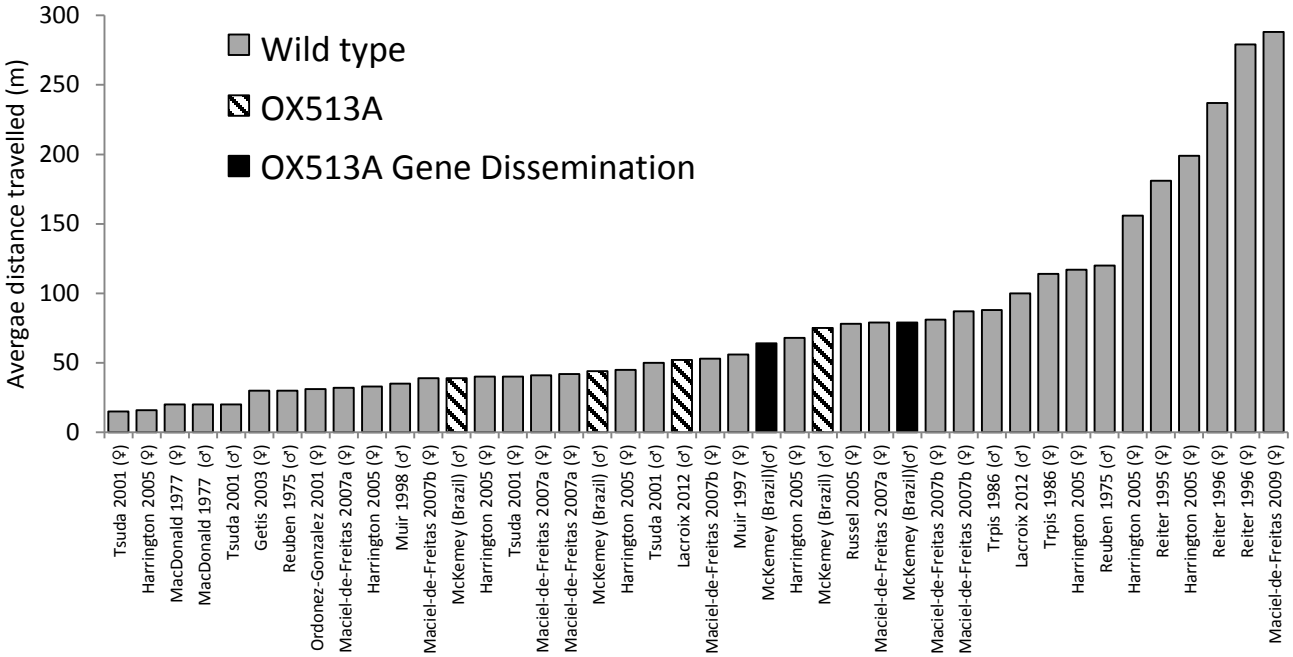
Data on dispersal of the strain has been obtained from previous field trials with OX513A in Malaysia (Lacroix *et al*., 2012). Adult male mosquitoes were released into an uninhabited forested area of Pahang, Malaysia. Their survival and dispersal was assessed by use of a network of traps. Two strains were used, OX513A and a wild-type laboratory strain, to give both absolute and relative data about the performance of the engineered mosquitoes. The two strains had similar maximum dispersal distances (220 m), but mean distance travelled of the OX513A strain was lower (52 vs. 100 m). Life expectancy was similar (2.0 vs. 2.2 days). Recapture rates were high for both strains, possibly because of the uninhabited nature of the site. Neira *et al*., (2014) reported that in Panama marked released WT males had a daily survival probability of 2.3 days, so OX513A falls within this figure for survival.

Longevity of released males is closely associated with their dispersal ability, as dispersal will generally increase with time. 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 (DsRed2) as part of the genetic engineering enabled the evaluation of dissemination of OX513A genes resulting from the release of OX513A males. Oxitec assessed the dissemination of OX513A genes into the environment 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. This differed little for the dispersal of OX513A and males of the comparator strain (recently colonized *Ae.aegypti)* observed at the

[32] http://coastalresilience.org/geographies/florida-keys/future-scenarios-map [Accessed 19 Sept 2013]

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 *Ae.aegypti*, see Figure 17.

Figure 17 Review of Reported Mean Distance Travelled (m) for Wild-Type and OX513A *Ae.aegypti* and Observed Dissemination of OX513A Gene from Male Release.



References for Figure 17: McKemey Brazil (Carvalho *et al,* 2015);Getis *et al*., 2003; Harrington *et al*., 2005; Lacroix *et al*., 2012; MacDonald, 1977; Maciel-De-Freitas *et al*., 2007a; Maciel-De-Freitas *et al*., 2007b; Maciel-de-Freitas and Lourenco-de-Oliveira, 2009; Muir and Kay, 1998; Ordonez-Gonzalez *et al*., 2001; Reiter, 1996; Reiter *et al*., 1995; Reuben *et al*., 1975; Russell *et al*., 2005; Trpis and Häusermann, 1986; Tsuda *et al*., 2001)

12.3.3 Conclusion

The OX513A strain shows a similar dispersion pattern to the unmodified comparator strain in dispersal experiments and falls within the midrange of the reported distances of Ae.aegypti flight from the literature. The daily survival probability is also in the order of 1-3 days which is consistent with the literature for released male Ae.aegypti. Consequently, the insertion of the #OX513 rDNA construct in the OX513A strain has not altered the dispersal or survival range of Ae.aegypti.

13 Evaluation of Potential Impacts

This environmental assessment addresses the potential for significant environmental impacts as the result of the conduct of the field trial. These potential impacts include the following:

- Direct or indirect effects on non-target organisms
- Increase in invasiveness or persistence in the environment
- Potential impact on ecosystem services/ecosystem function
- Potential increase in disease transmission
- Potential for loss of biodiversity
- Potential adverse effects on humans
- Potential for escape from the HRU
- Potential for gene movement and changes in phenotypes of recipient organisms via sexual and non-sexual transfer of genetic material

The impacts are evaluated in terms of their likelihood to occur and the potential consequences if they were to occur. When considering the likelihood of potential impacts, consideration is given to appropriate non- genetically engineered comparators; i.e., the existing mosquito control measures and their consequences on the environment as well as the existing *Ae.aegypti* mosquito and its consequences for human health impacts.

13.1 Likelihood of impacts occurring

The likelihood of escape, establishment, and spread has been evaluated in the sections below.

13.2 Likelihood for escape

The following section examines the potential for escape from the HRU and associated activities and measures that are in place to prevent it.

13.2.1 Containment measures

The main pathway for potential impacts is via inadvertent release outside of the intended rearing or trial sites, namely at the HRU site in Marathon and/or during transport of mosquitoes to the release site in Key Haven.

The OX513A line of *Ae.aegypti* will be hatched and reared to adulthood at the HRU facility (see section 10.4.2). There will be both female and male mosquitoes in the HRU, although the females will be killed at the larvae/pupae separation stage which is conducted in the containment facility and therefore, chances of their escape are extremely low. The mosquitoes will be maintained with a minimum of two levels of physical containment in accordance with ACL2 requirements and those of the US agencies permitting the import (see section 14.1). Every effort is made to avoid inadvertent release by established procedures and

staff training. The most likely threat that could lead to a breach of containment is a hurricane and/or flooding following a storm surge. These are natural events that could potentially cause inadvertent release. In the case of hurricane, there is a hurricane preparedness policy for the HRU that aims to minimize inadvertent release, where insects will be killed within 36 hours of a hurricane forecast. The decision to implement these measures will be taken by the FKMCD program manager and the study director, in accordance with the hurricane management plan.

An assessment of the potential impacts during transport of the insects has been conducted by Oxitec along with potential control measures and is summarized in Table 5 below. Potential impacts are categorized as being “low”, “moderate,” or “likely.”

Table 5 Potential routes for impacts, consequences, and control strategies for the transport of OX513A mosquitoes from the HRU to the release site.

Potential route of impact	Consequence	Control Measures(s)	Potential likelihood for adverse impact to human health or environment
Release of mosquitoes during transport to trial site.	GE mosquito released to environment outside release area.	Secure, shatterproof double containers to be used for mosquito transfer. Insects cannot establish in the environment due to intrinsic biological containment (reliance on presence of tetracycline). Insecticide treatment can be applied if required.	LOW
Vehicular accident during transport to trial site.	GE mosquito released to environment outside release area.	Secure, shatterproof double containers to be used for mosquito transfer. Insects cannot establish in the environment due to intrinsic biological containment (reliance on presence of tetracycline). Insecticide treatment can be applied if required.	LOW
Transport boxes inadvertently lost.	GE mosquito released to environment.	Containers will be in FKMCD or Oxitec staff custody throughout journey, any loss of boxes will be reported immediately, and every effort will be made to recover them. A chain of custody is in place for all transport. Even if not found, insects cannot establish in the environment due to intrinsic biological containment (reliance on presence of tetracycline).	LOW
Boxes dropped whilst loading for transport.	GE mosquito released to environment.	Use secure, shatterproof double containers for mosquito transfer. Insects cannot establish in the environment due to intrinsic biological containment (reliance on presence of tetracycline).	LOW
Boxes stolen.	GE mosquito released to environment.	Boxes will be accompanied by FKMCD or Oxitec staff at all times. Any loss of boxes will be reported immediately and appropriate authorities will be informed of the theft. Insects cannot establish in the environment due to intrinsic biological containment (reliance on presence of tetracycline).	LOW
Mosquitoes passively transported away from trial area (trapped in vehicles etc.).	GE mosquito release to environment outside of release area.	Insects cannot establish in the environment due to intrinsic biological containment (reliance on presence of tetracycline). Insecticides can be used if necessary.	LOW
Release of GE mosquitoes during unpacking.	GE mosquito released to environment.	Staff trained in safe handling procedures, unpacking will only be done within the trial site area, and insects cannot establish in the environment due to intrinsic biological containment (reliance on presence of tetracycline).	LOW

13.3 Likelihood for establishment

For a GE animal to make a significant impact on the environment it must spread and establish in the community in which it is released. The National Academy of Sciences (NAS) therefore defines exposure as the establishment of the GE animal in the community. NRC (2002) identified three variables as important in determining the likelihood of establishment:

1. The effect of the rDNA construct on the fitness of the animal for the ecosystem into which it was released
2. The ability of the animal to escape and disperse into diverse communities
3. The stability and the resiliency of the receiving environment

Overall concern is a product of all three variables, not the sum and, therefore, if the risk of any one of the variables is negligible, the overall concern would be extremely low. An examination of the life-cycle parameters of OX513A in comparison to a wild–type control strain contribute to assessment of the overall fitness of the strain. Fitness of the OX513A should be considered within the context that the intended effect of the expression of the rDNA construct to confer dominant conditional lethality to the strain, i.e., a competitive disadvantage, and the strain will die without access to the tetracycline antidote in its diet.

This section focuses on the fitness of the strain as the ability of OX513A to escape and disperse into diverse communities is covered in Section 13.3. The stability and resiliency of the receiving environment is described in the Section 12.1 on accessible environments.

Fitness is comprised of reproductive potential, mating success, and survival. Of these components, survival has been evaluated in Section 12.2 and will not be addressed here further.

13.3.1 Lifecycle parameters

The lifecycle parameters of the OX513A *Ae.aegypti* have been examined in a study by Lee *et al*., (2009a). Comparative lifecycle parameters of a wild-type laboratory strain of *Ae.aegypti* (WT) and OX513A *Ae.aegypti* (in this study called LA513 although this represents only a name change and not a strain difference) were studied in the laboratory. The following parameters were statistically indistinguishable in both strains: the number of eggs laid, the number of unhatched eggs, the egg-hatching rate, the duration of larval period in all four instars, larval survivorship, pupation, adult eclosion rate, gonotrophic cycle, adult fecundity, adult lifespan and offspring sex ratio. These results indicate that the basic lifecycle parameters and growth rate of the OX513A *Ae.aegypti* were not affected by the genetic engineering and its mating competitiveness was sufficient to enable the successful use of this technology.

Bargielowski *et al*., (2011b) compared the life history characteristics of the OX513A line and a wild-type strain of *Ae.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 *Ae.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 *Ae.aegypti* resulting in smaller adults than the unmodified line. This effect was more pronounced in females than in males.

These life-cycle characterization studies between the product and its conventional counterpart have been used to establish whether unintended changes in the GE mosquito have occurred as a result of the genetic engineering. The results of this comparative safety assessment demonstrated that the only difference of biological relevance identified between the OX513A *Ae.aegypti* strain and the wild-type *Ae.aegypti* mosquito is the expression of the intended proteins (tTAV and DsRed2) and a small fitness penalty.

13.3.2 Mating competitiveness

Mating competitiveness is a key parameter in the assessment of the fitness of the insect. The successful mating competitiveness of OX513A compared with wild-type *Ae.aegypti* implies that the insertion of the rDNA construct has not affected reproductive behavior of OX513A mosquitoes. In addition, the ability of the mosquito to react to the specific mating signals from other *Ae.aegypti* mosquitoes has similarly not been affected. Mating competitiveness has also been assessed prior to field studies in the Cayman Islands (Harris *et al*., 2012; Harris *et al*., 2011) and Brazil (*Carvalho et al, 2015*). Additional mating studies have been conducted in the laboratory using different background strains of *Ae.aegypti* in the laboratory with similar successful results (data provided in Section 12.2.2.2), which suggests that there are unlikely to be differences in mating behaviors of OX513A compared with the local population of *Ae.aegypti* across different backgrounds and environments.

13.3.3 Conclusion on the likelihood for establishment in the environment

Lifecycle parameters, fitness and mating competitiveness have all been assessed in laboratory studies and in the case of mating competiveness in the field as well during previous releases in the Cayman Islands and Brazil. The ability to survive has been addressed in Section 12.2, which concluded that the response of OX513A to abiotic factors is likely to be the same as a non-genetically engineered Ae.aegypti and survival was unlikely to be increased. All the available data indicates that it is extremely unlikely for there to be differences between OX513A and wild-type Ae.aegypti that would change the likelihood for establishment in the environment.

13.4 Likelihood for spread

Spontaneous flight of adult *Ae.aegypti* is limited to around 200 m depending on availability of breeding sites, and hosts from which to take a blood meal (Maciel-de-Freitas *et al*., 2010; Suwonkerd *et al*., 2006), although there are reports of females travelling further even in urban environments (Halstead, 2008). Roads, water courses and vegetation all represent potentially significant barriers to the movement of *Ae.aegypti* (Hemme *et al*., 2010; Maciel de Freitas, 2009), which is adapted to live in close proximity to human habitations.

The species can also be dispersed by human activities such as passive transport on boats, trains, automobiles, and other forms of transport (Gubler, 2006; Lounibos, 2002). Based on the spontaneous flight distance, WHO International Sanitary Regulations require ports and airports to establish programs to control *Ae.aegypti* and other insect disease vectors for at least 400 m from point of entry facilities for this reason.

Other factors affecting the distribution/dissemination of *Ae.aegypti* are temperature, altitude, and presence and type of water storage (Hayden *et al*., 2010; Sharma *et al*., 2008). Altitude is not considered here as the Florida Keys are close to sea level. The following studies and information have been used to determine the likelihood for spread.

13.4.1 Likelihood of rDNA construct spreading to other organisms

13.4.1.1 Likelihood of sexual transfer of genetic material

Ae.aegypti does not form part of a species complex (i.e. a group of insects of similar form that are often indistinguishable at the species level) and matings with closely related mosquito species do not produce viable offspring (Nazni *et al*., 2009b, Harper and Paulson 1994, Leahy, 1967). Nazni *et al*., (2009b) made forced matings in the laboratory between wild-type *Ae.aegypti* and *Ae.albopictus* that yielded eggs in all cases but these eggs were not viable, and when bleached were shown to have no embryos. Lee *et al.,* (2009b) also showed that there was no evidence for successful interspecific mating of OX513A *Ae.aegypti* with wild-type *Ae.albopictus.* More recently a study showed that there is cross species insemination in the field between *Ae.aegypti* and *Ae. albopictus* (Tripet *et al*., 2011) but these interspecific matings encounter many barriers and only low frequencies of this type of mating appear to occur (a single *Ae. albopictus* was found to have *Ae.aegypti* sperm in this study; and three *Ae.aegypti* females were inseminated by *Ae. albopictus*), but no viable progeny resulted. Movement of the genetic elements in OX513A by vertical or sexual transfer to other mosquito species is therefore likely to be only a rare event in nature, and even if movement does occur this is unlikely to produce viable offspring. This is corroborated by the examination of the dispersion of the fluorescent marker gene as described in Section 12.3.

13.4.1.2 Likelihood of non-sexual transfer of genetic material

Non-sexual transfer (NST) of genetic material describes the movement of genes between independent co-existing organisms from different species. It does not include the transfer of genes through sexual reproduction mechanisms i.e., breeding[33]. Non-sexual transfer of genetic material 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). NST from multicellular (eukaryotic) organisms, such as plants or insects, to other organisms is

[33] Non-sexual transfer of genetic material is sometimes referred to as horizontal gene transfer, most correctly when discussing transfer of genetic material between bacteria or other microorganisms.

remarkably rare, occasionally being detected under optimized laboratory conditions, but at frequencies expected to be lower than background rates in natural or field conditions (Crisp *et al*, 2015, Keese, 2008).

Specifically with regard to OX513A mosquito, it has been shown in section 12.2.2.1 that sexual transfer to other species is unlikely to produce viable offspring due to both complex mating barriers and the lack of release of gamete materials. These mating barriers have the effect of restricting the genes to that species, in contrast to many other higher organisms that release genetic material into the surrounding environment, such as plants releasing pollen, fungi releasing spores, or milt in fish.

The potential for the introduced genes to be transferred to other organisms by oral ingestion of the mosquitoes by predators as well as the potential that genes could be transferred if a female mosquito bites a human or animal has also been assessed in the following sections:

13.4.1.2.1 Acquisition of genes through oral ingestion or blood feeding

One potential hazard could be that the blood meal taken by the female mosquito in the laboratory might provide an opportunity for transfer of mosquito genes to the individuals that have been bitten. Mosquitoes have been feeding on humans and other mammals for millennia, estimated to be more than 100 million years. Complete genome sequences are now available for several mammalian species, including humans, and several mosquito species, including *Ae.aegypti*; there is no evidence of gene transfer via blood feeding. Even if this hypothetically were to occur, even at extremely low frequencies, one would see DNA sequences from humans in human-feeding mosquitoes, from birds in bird-feeding mosquitoes and so forth and *vice versa* under the even more implausible hypothesis of DNA transfer from mosquito to host.

More generally, in the case of birds eating mosquitoes (and humans unintentionally swallowing them), animals do not incorporate DNA from their food into their genome. Because nucleic acids, including DNA, are present in the cells of every living organism, including every plant and animal used for food by humans and animals, and do not raise a safety concern as a component of food, nucleic acids are presumed to be generally recognized as safe (GRAS) for food consumption (57 FR 22984, 22990, May 29, 1992). Accordingly, there is no direct food consumption risk associated with exposure to the endogenous *Ae.aegypti* DNA or the #OX513 rDNA 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) or cows (Klotz and Einspanier, 1998) fed with glyphosate tolerant soybean 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 GE crops.

If the organism does acquire a gene through NST, the acquisition might not have any measureable effect on the environment. To have an impact, a significant number of organisms must acquire this new gene to be able to compete with organisms in the environment and establisht (NRC 2002). The likelihood of that depends on the rate of NST, the nature of the gene, the incorporation of the gene into heritable cells, and environmental influences.

Although NST between prokaryotes (e.g., simple organisms such as bacteria) is well-documented, the rate of NST in those populations is extremely rare, occurring at very low frequencies (Thomas and Nielsen, 2005). The occurrence of NST between prokaryotes and eukaryotes is more controversial, very difficult to detect, and likely happens on an evolutionary time scale only (Kurland et al., 2003; Dunning Hotopp et al., 2007, Dunning Hotopp, 2011; Boto, 2014). In a recent study, Crisp et al., 2015 carried out a detailed analysis of 26 species including 10 primates, 12 *Drosophila* species, and four *Caenorhabditis* genomes and simplified analysis of additional 14 species for the evidence of NST between bacteria and metazoans (more complex eukaryotic organisms including animals whose bodies are composed of cells differentiated into tissues). Their results suggest that in humans and primates, for example, NST events appear to be ancient and more likely occurred sometime in one of their common ancestors. These results support the notion that NST events occur at extremely low rates, on an evolutionary timescale rather than within the relatively short timescale of the proposed investigational study, and therefore it is highly unlikely for an NST mediated event related to OX513A mosqutioes to occur.

A potential impact could be from insect gut bacteria acquiring antibiotic resistance genes as they are fed on antibiotics in the laboratory and could spread those genes in the environment. There is no causal pathway for this to occur as gut bacteria are lost during mosquito metamorphosis from larvae to adults (DeMaio *et al.,* 1996; Moll *et al.,* 2001). 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), and pupae and adults are not subsequently treated with tetracycline during the rearing.

Therefore, only hypothetical impacts could occur from dead OX513A material persisting in the environment, but this is highly unlikely as the OX513A dead insects contain no known toxic compounds and consist of ubiquitous proteins, nucleic acids, carbohydrates, and naturally occurring minerals and/or other organic compounds. A wide range of studies have used fluorescent protein markers, including expression in whole animals with neutral outcomes. The following review articles describe some of these studies:

- 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, 155-162

Direct analysis of the effect of fluorescent proteins fed to rats has demonstrated no adverse effects of oral administration. The study was conducted by Richards in 2003.

- Richards *et al*. (2003) Safety Assessment of recombinant green fluorescent protein orally administered to weaned rats. J. Nutr. 133, 1909-1912.

DsRed2 protein has also been subject to an Early Food Safety Evaluation by the FDA CFSAN for use in maize, as described in Section 13.6.2.

Similarly the conditional lethal element, known as the tTA system developed by Gossen and Bujard (1992) and subsequent variants, has been widely used both *in vitro* and *in vivo* for over a decade. Low-level expression of tTA or its variants has been widely used and thought to be innocuous; whereas a high level expression is thought to be deleterious to cells, likely due to transcriptional "squelching" (Gill and Ptashe, 1988; Lin 2007) and/or interference with ubiquitin-dependent proteolysis. It is the interference of high levels of protein accumulation in the cell that is likely to cause cellular death in the absence of tetracycline. When tetracycline is supplied the cellular machinery leading to an over accumulation of the protein is turned off.

Although some potential symptoms of toxicity have been reported in transgenic mice expressing high levels of tTA or its variants (Whitsett and Perl, 2006) other papers have observed no apparent toxicity:

- Zhou *et al*. (2009) Developing tTA transgenic rats for inducible and reversible gene expression. *Int. J. Biol. Sci.* **5**, 171-81.
- Barton *et al*. (2002) Modified GFAP promoter auto-regulates tet-activator expression for increased transactivation and reduced tTA-associated toxicity**.** *Brain Res. Mol. Brain Res.* **101**, 71-81.
- Chen *et al*. (1998) Transgenic animals with inducible targeted gene expression in brain. *Mol Pharmacology* **54**, 495-503.

Further studies on the tTAV and DsRed2 proteins, including feeding studies in animals are described in Section 13.4 and its sub-sections.

13.5 Interactions with other organisms

Ae.aegypti is considered uniquely domestic amongst the mosquito species, being closely associated with humans. It is a non-native species in the US present predominantly in the Gulf Coast States (Lounibos, 2002), and has therefore not co-evolved with other organisms in the ecosystem and does not represent a keystone species on which other organisms rely for food. It is continually suppressed by control methods such as the use of insecticides and breeding site source reduction. These methods already reduce the *Ae.aegypti* population to low levels, with an average reduction by chemical intervention of 27.2% (Ballenger-Brown and Elder, 2009) but are increasingly ineffective due to the buildup of resistance mechanisms to the chemicals in use (Maciel de Freitas *et al*., 2014 Marcombe *et al*., 2011). The use of chemical control methods may also be considered to have a greater environmental impact on other organisms than the result of the suppression of *Ae.aegypti* using OX513A. For example, pyrethroid based sprays are considered a potential toxicity hazard to aquatic organisms (Pierce *et al.*, 2005) and as they are non-discriminatory may harm beneficial insect species as well. Recent research however indicates that this risk may have been overstated (Phillips *et al*., 2014). In a recent risk assessment conducted for the release of *Ae.aegypti* carrying the intracellular bacterium, *Wolbachia*, a group of experts concluded that *Ae.aegypti* was unlikely to have interactions with natural ecosystems, it was unlikely that the other species rely heavily or even moderately on *Ae.aegypti* as a food item or provider of ecosystem services (Murphy *et al*., 2010). Reduced *Ae.aegypti* populations are already achieved as a result of current mosquito control

practices. Consequently interactions with other organisms in the environment are extremely limited and therefore have only been briefly addressed below.

13.5.1 Competition with other mosquito species (conspecifics).

Several species of mosquito can co-occur in the same water-filled containers (aquatic breeding sites), where they are competing for resources such as food. Larval competition, inter- or intraspecific, may have important effects on the growth, survivorship, and reproductive success of these species (Juliano and Lounibos 2005). Therefore, larval conditions may have a significant impact on overall population growth. Those species that can maintain positive population growth under interspecific conditions of greater density or lower resource availability than a competitor are likely to be more successful in their breeding. OX513A conditional lethality trait expression occurs towards the fourth instar and pupal life stages and therefore enables the developing larvae to compete with conspecifics for resources. By competing for breeding sites and resources in this way and not dying earlier, for example at the egg stage, this has the effect, as it would for other conspecific mosquitoes not carrying the rDNA construct, of reducing the overall numbers of mosquitoes in the breeding environment.

Adult male mosquitoes will actively compete with one another to mate with females in the environment. The proposed releases will involve a higher number of OX513A males released to the local *Ae.aegypti* male population at the trial site, which will enable the Oxitec mosquitoes to attain over 50% of the matings. Continued release of Oxitec males is then anticipated to result in population suppression at the release site. The numbers of mosquitoes released will be adapted to during the course of the trial to maintain over 50% of the female matings with OX513A.

13.5.2 Predators of Ae.aegypti

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 and Canyon, 2009). Because *Ae.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 *Ae.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 *Ae.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, which is where the investigational trial is proposed.

Potential routes of exposure involve different ecological guilds[34] of organisms. These guilds are summarized in Table 6.

Table 6 Summary of Guilds Potentially Exposed to the OX513A *Ae.aegypti.*

Terrestrial	Aquatic
Predators	Predators
Parasitoids	Decomposers
Pollinators	
Decomposers	

In the consideration of the possible ecological consequences of mosquito control using OX513A, a key issue is whether *Ae.aegypti* provide any ecological role in the environment. *Ae.aegypti* mosquito is an urban or domestic mosquito closely associated with human habitations. Non-target organisms in these areas are not usually threatened, endangered, or species that the population value and from the analysis of the threatened and endangered species (*Appendix B*) this is confirmed as there is no habitat overlap for these species with the domestic urban environment. From a review of the scientific literature conducted in PubMed, no papers were identified where a predator was found to be dependent on *Ae.aegypti* alone as a food source. Additionally, *Ae.aegypti* is a non-native insect (Slosek, 1986) and is regularly subjected to other control methods such as insecticide treatment and source reduction, it is highly unlikely that any predator is co-dependent upon it. Therefore, it is highly unlikely any predator species is dependent on *Ae.aegypti* for its survival in the food chain and as a consequence there is likely to be negligible impact on non-target organisms.

Nonetheless, in consideration of possible impacts of the release of OX513A, non-target organisms are included in the risk analysis below. Non-target 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 *et al*., 2009, Blum *et al*., 1997; USFWS 2004).

13.5.2.1 Predatory mammals

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 *Ae.aegypti* mosquitoes. *Ae.aegypti* mosquitoes are active in the day (Gubler and Clark, 1995) whereas bats are active at dawn and dusk. Furthermore, a study conducted on bats found that mosquitoes were not always available as diet to bats and therefore make up only a small fraction of their diet. This was due to their small size, poor detectability by low frequency echolocation,

[34] Ecological guilds are a group of species that exploits the same kinds of resources in comparable ways. These can be unrelated species competing for the same resources e.g., insects that pollinate plants compete for the same nectar sources.

and variable field metabolic rates (Gonsalves *et al*., 2013). The American Mosquito Control Association (AMCA) also review the role of bats for mosquito control on their website[35], 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) analyzed 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). This is also confirmed by a study from Feldhamer et al, 2009 where the prey of eight different insectivorous bats was analyzed. Therefore, due to the temporal separation in activity periods and that the mosquito is likely to form only a small part of the bat diet it is unlikely that *Ae.aegypti* OX513A will impact on bats in a significant way.

13.5.2.2 Predatory birds

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 as a consumer of mosquitoes is the Purple Martin (*Progne subis*), the largest species of martin in North America; however both the AMCA and the Purple Martin Conservation Association[36] declare that this is not supported by scientific fact. The facts are that there is temporal 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[37]. Therefore due to the temporal separation in activity periods and that the mosquito is likely to form only a small part of the diet it is unlikely that *Ae.aegypti* OX513A will impact on insectivore birds in a significant way.

13.5.2.3 Predatory amphibians

Amphibian predators such as frogs and reptiles such as salamanders do not interact with *Ae.aegypti* or other adult mosquitoes in sufficient number for mosquito control[38]. Reptiles do have the capacity to consume mosquito larvae, and a study showed that in the laboratory 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 *et al*., 2008). However, there are unlikely to be salamanders in the same breeding sites as *Ae.aegypti, as Ae.aegypti* is more

[35] http://www.mosquito.org/faq

[36] http://www.purplemartin.org/update/MosCont.html

[37] http://www.purplemartin.org/update/MosCont.html

[38] http://www.michigan.gov/emergingdiseases/0,4579,7-186-25805_25824-75797--,00.html

associated with human habitats, and salamanders are associated with seasonal pools and wetlands. Blum *et al*., 1997 found that through the diet analysis of anurans (newts) that mosquitoes made up only 0.16% of the anuran diet's content.

13.5.2.4 Predatory invertebrates

Invertebrate predators form another group that is known to predate on mosquito larvae, in particular the predator mosquito species *Toxorynchites*, which has been recognized as a potential biological control organism for *Aedes* species. Their use in biological control has been problematic due to establishment and concurrence of oviposition sites (Collins and Blackwell, 2000). In Florida, *Toxorynchites rutillus* is present, most commonly found in tree-holes, bromeliads, and other ephemeral containers. It was reported present in the Florida Keys for the first time in 2013, where 9 specimens were found in Key Largo (Tambasco and Hribar, 2013). 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 *Ae.aegypti* or related species, but again they are generalist predators and not reliant on a single species of mosquito for their food source.

13.5.2.5 Studies on mosquito predators

To determine potential impacts on predator species, two studies have been conducted in which the predator species (invertebrate predator *Toxorhynchites and fish (Poecilla species)* were fed OX513A larvae at high levels of dietary incorporation (70-100% of their diet) for extended periods (up to 28 days). These studies showed no adverse effects on either of the non-target predatory species. These studies and the scientific literature reviewed above indicate that introduction of the rDNA construct in *Ae.aegypti* is unlikely to impact predators that might eat OX513A in the environment.

13.5.2.5.1 Studies on Toxorhynchites species

Toxorhynchites species are predatory mosquitoes whose larvae feed on small aquatic organisms. These species have been evaluated for biological control of mosquito larvae (Nyamah *et al*, 2011, Collins and Blackwell, 2000). They are relatively large 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 *Ae.aegypti* 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). Another two experiments were set up as controls. The *Toxorhynchites* species were also fed a diet of wild-type *Ae.aegypti* and OX513A *Ae.aegypti* reared on tetracycline, the dietary antidote to the conditional-lethal gene. Single *Toxorhynchites* larvae were placed into individual cups and 20 *Ae.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. *Toxorhynchites* 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 group that was not supplemented with tetracycline during their aquatic development phase; *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 in the presence of tetracycline (t=−3.3, p<0.002). Why this result occurred is unknown but there were no significant differences in any other measured parameters.

There was no evidence that the development, fecundity, or longevity of the two *Toxorhynchites* species were adversely effected by the OX513A larvae. 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. This study is published by Nordin et al., 2013 in an open access journal[39].

13.5.2.5.2 Study on fish (Poecilia species)

A laboratory toxicity study was conducted by SynTech Research France, under GLP conditions, on guppy fish *Poecilia reticulata (Actinopterygii: Poeciliidae)*; according to OECD No. 204 (1984) modified for oral route of exposure (Ythier, 2012). Guppies (20-26mm at the start of the test) were exposed to a mix of freshly defrosted larvae and pupae from OX513A and a non-GE control over a period of 14 days in laboratory conditions. During the study, the fish were fed with OX513A or the non-GE control mosquito in the fish diet, daily, at the rate of 700g mosquitoes/kg diet, following a rangefinder study. The natural ratio 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 per cent of the initial fish weight. 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 group was 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). Results are shown in Table 7 found immediately below; the study is appended (*Appendix H*).

[39] Nordin, O., Donald, W., Ming, W.H., Ney, T.G., Mohamed, K.A., Halim, N.A., Winskill, P., Hadi, A.A., Muhammad, Z.S., Lacroix, R.*, et al.* (2013). Oral ingestion of transgenic RIDL *Ae.aegypti* larvae has no negative effect on two predator Toxorhynchites species. PLoS One *8*, e58805.

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

Test item	*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-GE mosquitoes/kg diet)	10	22.44	198.3
OX513A (700 g GE mosquitoes/kg diet)	0	23.2	212.9
LR 50 / ER50 [g GE mosquitoes/kg diet]	> 700		
LOER [g GE mosquitoes/kg diet]	> 700		
NOER [g GE mosquitoes/kg diet]	700		

GE = genetically engineered

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 700 g GE mosquitoes/kg diet and the LOER and LR50/ER50 were estimated to be > 700 g GE mosquitoes/kg diet.

Ae.aegypti and parasitoids.

No specific parasitoids are known to be associated with *Ae.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 *Ae.aegypti* in the laboratory, they have not been found infecting natural populations (Wise de Valdez, 2007).

Ae.aegypti as a decomposer.

Ae. aegypti larval development is in an aquatic environment and predominantly man-made breeding sites (such as water containers, plant pots, discarded soda cans), which frequently contain detritus which is metabolized by the microbial communities. Although there is limited research in this area, it is thought that *Ae. 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 *Ae.aegypti* in

breeding sites (Otero et al., 2006). As the microorganisms break down the detritus, there are number of metabolites and volatile compounds that act as attractants to gravid mosquitoes and stimulate egg laying in containers which are enriched with bacteria (Ponnusamy et al., 2008). Although *Ae. aegypti* occupy man-made or artificial containers where plant and animal detritus is broken down, it is unlikely that the mosquito itself is contributing to the direct decomposition of the material. However, in one study Yee et al., 2007 showed that animal detritus could be directly consumed by mosquitoes in breeding sites. 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.

Ae.aegypti as a resource for decomposers.

A few organisms are known decomposers of *Ae.aegypti*; fungi such as *Metarhizium anisopliae*, a well-known entomopathogenic fungus[40] and *Beauveria bassiana* are capable of infecting *Ae.aegypti* eggs (Leles, 2012). Entomopathogenic fungi have been tested as biocontrol agents for the control of *Ae. aegypti* and other mosquitoes (Scholte et al 2007; Kanzok et al, 2006). These fungi are soil dwelling and reported to be in agricultural soils in Florida (Beavers, 1983) but are also commercially available as biological control agents that have been tested in the Florida environment for the integrated pest management of orchard crops (Lacey and Shapiro-Ilan, 2003). No reports have been found of the occurrence of these fungi specifically in the soils of the Florida Keys from an internet search on Google Scholar and Pubmed using the key terms of "soil, Florida Keys, Metarhizium anisopliae, Beauvaria bassiana", but it is possible that they could be present. However soils in the Florida Keys are shallow lying directly on limestone bedrock so are less likely to have high organic matter levels that would encourage soil dwelling fungi.

Ae.aegypti as a pollinator.

Although female *Ae.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. *Ae.aegypti*, for example, is known to react positively or negatively to different floral scents and to prefer green flowers as reviewed by Argue (2012). Details of the relationship between plant species and *Ae.aegypti* specifically has not been observed in this study. *Ae.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 sugar sources from potted plants or plant species that are found around houses as part of their preferred existence around humans (Martinez-Ibarra *et al*., 1997). There is limited information on the pollination of plant species by mosquitoes in

[40] Entomopathogenic fungi are parasitic fungi that can kill or seriously disables insects, usually by infecting them with spores that can bore through the cuticles of insects, killing them.

general, and no reports that *Ae.aegypti* is a pollinator for any plant species. Despite feeding on plant nectar, it is likely that mosquitoes transfer pollen to some extent although there is little scientific information on this. *Ae. communis* and *Ae. canadensis* canadensis are known as pollinators of an orchid in Northern Canada, *Habenaria obtust* (Thien, 1969), a plant species not found in Florida. This lack of pollination activity may be because, as a non-native 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 (Elder and Lamche, 2004; Wheeler *et al.,* 2007 and 2009; Gubler, 2011; Brathwaite Dick *et al*., 2012; Monteiro *et al.*, 2014) 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.

13.6 Analysis of the potential toxicity and allergenicity of the introduced proteins

13.6.1 Bioinformatics studies of the novel proteins expressed in OX513A

Because wild *Ae.aegypti* mosquitoes can trigger allergic reactions via bites in humans (Doucoure *et al*., 2012), and there is the potential to have small numbers of female *Ae.aegypti* carrying the rDNA construct in the environment as a result of the mating or a small amount of females being released, two questions were pursued:

1. *Does the tTAV or DsRed 2 protein have a degree of homology with proteins that are known to be toxic or allergenic?*
2. *If tTAV or DsRed 2 were found to have allergenic potential, would exposure into or through the skin resulting from a mosquito bite represent a greater risk to human health than a bite from an existing mosquito?*

The evaluation of the amino acid sequence similarity of novel proteins with known toxins and allergens is the first step in the safety analysis. FAO/WHO guidelines (Codex, 2003 and 2009) have been developed specifically for this purpose. The Codex Alimentarius Guidelines have been designed for the safety of foods produced with genetically engineered organisms and hence an oral route of exposure.

Subcutaneous (injected under the skin, as in the case of a mosquito bite) routes of exposure in the context of the safety of recombinant proteins, have been widely researched in the context of recombinant vaccines, including that of the tetravalent dengue vaccine (Dayan *et al*., 2013, Osorio *et al*., 2014). Additionally, the World Allergy Organization regards recombinant proteins as promising new approaches to target allergy immunotherapy (Canonica *et al*., 2014). Three studies (Eifan, 2010, Keles, 2011, and Yukselen, 2012) 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, 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, and therefore it is Oxitec's view that the Codex Alimentarius focus on amino acid sequence similarity of the protein with known toxins and allergens should be equally applicable to both oral and injection routes of exposure. This view is further supported by expert opinion (*Appendix I).*

13.6.1.1 *tTAV* toxicity and potential allergenicity assessment

The potential toxicity and allergenicity of the tTAV protein was assessed using a bioinformatics study (conducted independently by Dr. Rick Goodman of the University of Nebraska, USA a leading expert on allergenicity of products from genetically engineered organisms) with the amino acid sequence and publicly available protein sequences of known toxins according the Guidelines of Codex Alimentarius (Codex, 2003 and 2009) *(Appendix J*). The tTAV protein is a synthetic fusion protein and therefore search was broken into component parts relating to the donor organisms from which the synthetic sequences are derived; namely *Escherichia coli* and the VP16 protein from Herpes simplex virus. The study included the following analysis on toxicity and allergenicity in accordance with the Codex Guidelines:

- Scientific literature search strategies in the PubMed database using key search terms "E.coli","VP16", "Herpes", "allergy" and " allergen", "toxin" and " toxicity".
- Amino acid sequence of tTAV search strategies (FASTA3; BLASTP algorithm) using Allergenonline version 13 and NCBI Entrez protein databases.

The predicted amino acid sequence of tTAV is given in Figure 18 below:

Figure 18 Amino Acid Sequence of the tTAV protein

<tTAV
MGSRLDKSKVINSALELLNEVGIEGLTTRKLAQKLGVEQPTLYWHVKNKRALLDALAIEM
LDRHHTHFCPLEGESWQDFLRNNAKSFRCALLSHRDGAKVHLGTRPTEKQYETLENQLAF
LCQQGFSLENALYALSAVGHFTLGCVLEDQEHQVAKEERETPTTDSMPPLLRQAIELFDH
QGAEPAFLFGLELIICGLEKQLKCESGSGPAYSRARTKNNYGSTIEGLLDLPDDDAPEEA
GLAAPRLSFLPAGHTRRLSTAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDS
PGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDEYGG

Potential toxicity was evaluated by comparison of the amino acid sequences of the TetR N-terminal (208 amino acids) and the VP16 C Terminal 129 amino acids against the NCBI database using BLAST and keyword search query limits ("toxin" or "toxic") in 2011 and repeated in Sept 2013 with key word search terms of "toxin" and "toxicity".

DsRed2 is a marker protein which is expressed constitutively in the developmental stages of the OX513A mosquito. DsRed is a naturally occurring fluorescent protein which was originally found in various *Discosoma spp*. DsRed2 was artificially developed from DsRed to enhance the fluorescence and improve the solubility, which in turn increases the sensitivity of detection (Shagin, 2004; Bevis, 2000; Matz, 1999; Lukyanov, 2000; CLONTECHniques, 2001). The DsRed2 is from Clontech Laboratories (Figure 20). In OX513A, there are three additional amino acids (MAR) at the N-terminus, which are from a cloning linker sequence.

Figure 19: DsRed2 Amino Acid Sequence of the DsRed2 protein

MASSENVITE FMRFKVRMEG TVNGHEFEIE GEGEGRPYEG HNTVKLKVTK GGPLPFAWDI LSPQFQYGSK
VYVKHPADIP DYKKLSFPEG FKWERVMNFE DGGVATVTQD SSLQDGCFIY KVKFIGVNFP SDGPVMQKKT
MGWEASTERL YPRDGVLKGE THKALKLKDG GHYLVEFKSI YMAKKPVQLP GYYYVDAKLD ITSHNEDYTI
VEQYERTEGR HHLFL

13.6.2 DsRed2 Potential Toxicity and Allergenicity Assessment

The DsRed2 marker protein has been evaluated in a New Protein Consultation by the FDA CFSAN in the USA for human safety, and they raised no objections to its use in corn plants (Pavely and Fedorova, 2006; FDA, 2010). This involved an assessment of the amino acid sequence using bioinformatics analyses in accordance with the Guidance provided by Codex (2003), the lability of the protein in simulated gastric fluid (SGF) and an examination of the gene source and history of exposure, as well as the toxicity of the protein using bioinformatics analysis. Additional information on the lack of toxicity of DsRed2 is given in Section 13.6.2 of this document, including oral studies in rats (Richards *et al*., 2003). It has been further evaluated in an EA by the United States Department of Agriculture[41] which concluded that the corn transformation event that contained the DsRed2 gene was unlikely to become a plant pest risk. An additional EA on a GE pink bollworm expressing fluorescent genes similar to DsRed2 has also been conducted (USDA, 2001)[42] and concluded in a Finding of No Significant Impact (FONSI) on the environment. Furthermore DsRed2 and members of its GFP family, has been widely used in many organisms for non-invasive *in vivo* and *in vitro* monitoring of disease states and pathways and they appear to be well tolerated. A search in Pubmed using the search terms "DsRed2; animal: human" returned over 60 papers, when conducted on 5 Feb 2015. When "toxic**" was added to the search terms, no papers were returned.

13.6.3 Bioinformatics assessment results

Potential allergenicity assessment examined the sequence alignment of the tTAV and DsRed2 protein sequences with protein sequences in the database by 80 amino acid segments to determine potential IgE binding epitopes and potential for cross-reaction with other allergens where a match of >35% homology with a known allergen would signal further investigation for cross-reactivity.

- All alignments either identified with tetracycline controlled regulatory elements or their components or were linked to author laboratory affiliation rather than identification of allergenic sequences.
- Potential for IgE cross-reactivity with similar proteins; the current internationally accepted paradigm is that the threshold for a level of homology that might be relevant for cross-reactivity is 35% amino acid identity over any stretch of an 80 amino acid sequence (Codex, 2003). This is a very

[41] http://www.aphis.usda.gov/brs/aphisdocs/08_33801p_dpra.pdf [Accessed March 19 2013]

[42] http://www.gpo.gov/fdsys/pkg/FR-2006-04-19/html/E6-5878.htm [Accessed March 14 2013]

conservative guideline, but will probably identify nearly every protein that is sufficiently similar. The complete sequence of the tTAV and DsRed 2 proteins were used to search the allergenic sequences of Version 13 of the Food Allergy Research and Resource Program (FARRP) Allergenonline.org[43] database, the only public, peer reviewed allergen database available for safety evaluation.

A second test used the conservative criteria of >35% identity over any 80 amino acid section. No matches were identified demonstrating lack of probable cross-reactivity to any known allergens. A further analysis was conducted using the precautionary search for any match of any eight (8) amino acid segments to any known allergen in the Allergenonline database, which was also negative.

The study was initially conducted in 2011 and repeated in 2013 as new information is being added to the database regularly. Both studies reached similar conclusions. The updated study from 2013 is therefore included in *Appendix J*.

The study concluded that results of the bioinformatics analysis of tTAV and DsRed2 protein amino acid sequences indicated that there was no more risk of allergy or toxicity that was greater than a typical dietary protein. There were no matches with more than 50% identity over the full sequence length and there were no matches of >35% identity to 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 comparisons are highly conservative and did not identify sequence similarities that would suggest the proteins are allergens or are sufficiently similar to an allergen to cause cross-reactivity. Neither were any matches to known or putative protein toxins identified. These results together indicated that additional testing was not required to evidence possible cross-reactivity as no hazard was identified.

The study in *Appendix J* concluded that although the Codex Guidelines are primarily intended to evaluate 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. The results indicated that there was no convincing evidence was found to suggest tTAV or DsRed2 proteins expressed in OX513A mosquitoes represent risks of allergy or toxicity to humans or other mammals, if the well-defined Codex oral allergy assessment approach is used (*Appendix J*). For the reasons stated above (Sections 13.6.1.1 and 13.6.2), we believe that this analysis is appropriate for both oral and non-oral routes of exposure. We therefore find that tTAV and DsRed2 are non-toxic or allergenic to human or animal health or the environment. Although Oxitec has concluded that there is likely to be no toxic or allergenic reaction from a mosquito bite carrying the tTAv or DsRed2 proteins, because risk is a function of both exposure and hazard, Oxitec provided an additional study on whether the introduced proteins can be detected in OX513A female mosquito saliva.

[43] http://www.allergenonline.org [accessed 22 Jan 2013].

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

Saliva from *Aedes* species mosquitoes contains secreted proteins which play a role in sugar and blood feeding[44]. 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) (Racioppi *et al,* 1987; Valenzuela *et al*, 2002). There is an amino acid signal sequence typically associated with proteins that are secreted into saliva. In addition, Capurro et al., 2000 confirm 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. This signal sequence is cleaved during the process of protein secretion into saliva in mosquitoes (e.g., James *et al*., 1991, Stark and James, 1998). tTAV contains no such signal sequence for secretion nor does it have any sequences with homology to such signal sequences; therefore, tTAV is not anticipated to be found in the saliva of OX513A. 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. Evidence from the bioinformatics analysis in section 13.6.1 shows there is no potential hazard identified.

The Laboratory of Malaria and Vector Research, National Institutes of Health (NIH) conducted a preliminary study to determine whether the synthetic protein tTAV was capable of being expressed in the OX513A female mosquito salivary glands through indirect reporter gene based assays that show qualitative results. The NIH is a world leading body for biological research and the study director a recognized authority on mosquito salivary proteins (Ribeiro et al, 2007, Calvo et al, 2007). The results of this preliminary study which are not included here, prompted further quantitative assessment of the potential risk of a bite from a female OX513A mosquito.

13.6.4.1Study on detection of tTAV and DsRed2 in the saliva of female OX513A

To build on the preliminary assessment conducted at the NIH, and to quantitatively assess the expression of the protein in the saliva, a further saliva study was conducted at Oxitec Ltd, using some of the same reagents as the NIH study. Homozygous adult female *Ae.aegypti* expressing the #OX513 rDNA construct were reared to adulthood in the presence of doxycycline. Saliva was collected from these insects as well as from comparator non-GE *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) approach. Sample integrity was confirmed using an antibody detecting a secreted salivary protein in mosquitoes, Aegyptin, as in the previous study conducted at NIH. Aegyptin detection was also used as a basis to

[44] http://what-when-how.com/insects/salivary-glands-insects/

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 LOD for recombinant tTAV (rtTAV) was determined to be 0.8 ng and the LOD for recombinant DsRed2 (rDsRed2) was determined to be between 2.5 and 5.0 ng.

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 the volumes on saliva collected during this study (270 µl of pooled saliva collected from approximately 300 *Aedes aegypti* adult females homozygous for the #OX513 rDNA construct).The study report is provided in *Appendix K*

13.6.5 Conclusion on the toxicity and allergenicity potential of the introduced proteins

Data and information has been presented that indicates the proteins expressed by the inserted rDNA construct in OX513A *Ae.aegypti* strain are not intrinsically toxic and are non-toxic to other organisms. However, it is the specific and intended effect of the insertion of the rDNA constructs that progeny of matings with released male OX513A *Ae.aegypti* will die due to over-expression of the tTAV protein and the disruption of the cellular transcriptional activity, in the absence of suitable concentrations of tetracycline or its analogues. The results of the feeding studies with three mosquito predator species (two predatory invertebrates from *Toxorhynchites* species and the guppy fish) provide further evidence of a lack of direct toxicity effects of the rDNA construct in the mosquitoes, when fed at rates in excess of usual dietary consumption.

The introduced proteins, tTAV and DsRed2 are not expected to be expressed in the saliva of the few female adult mosquitoes that result from matings with OX513A males, as neither protein has a sequence for secretion nor do they have any sequences with homology to such signal sequences. In order to present a potential risk to human health, tTAV and /or DsRed2 proteins 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. Evidence from the bioinformatics analysis in section 13.6.1 shows there is no potential hazard identified. Therefore to determine if there was likely to be exposure to either of these proteins from saliva, studies were conducted on the saliva from homozygous female OX513A adults.

A preliminary study was conducted to determine whether the synthetic protein tTAV was capable of being expressed in the OX513A female mosquito salivary glands through indirect reporter gene based assays that show qualitative results. To build on the preliminary assessment conducted at the NIH, and to

quantitatively assess the expression of the protein in the saliva, a further saliva study was conducted at Oxitec Ltd, using some of the same reagents as the NIH study.

This study showed that neither tTAV nor the DsRed2 proteins produced by the rDNA construct were detectable in the saliva of homozygous OX513A female mosquitoes by western blot analysis at and above the limit of detection determined in the study (0.8 ng for tTAV and 2.5-5.0 ng for DsRed2 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. Although there is no evidence (from a literature search in Pubmed and Google Scholar conducted April 2015) how much saliva is injected in a single bite from a mosquito, this study equates to saliva collected from approximately 5.5 female mosquitoes. An individual female is likely to bite a human host several times in her lifetime (Harrington *et al*, 2014, Canyon *et al* 1999) and therefore the amount of saliva tested may represent a greater or lesser number of bites than those from 5.5 females. As there is no detectable tTAV or DsRed2 protein in the saliva, exposure to the introduced proteins is negligible and the bite of a female OX513A is expected to be the same as a bite of a non-GE *Ae.aegypti* female.

Taken together this evidence indicates there is no direct exposure of humans to the introduced proteins, and therefore the bite of a female OX513A is predicted to be the same as the bite of a non-GE mosquito and consequently any potential risk is determined to be negligible.

14 Measures used to minimize potential impacts

14.1 Physical containment

Physical containment measures are implemented at HRU to prevent unintentional or inadvertent escape from contained facilities in accordance with measures proposed by the Arthropod Containment Guidelines level 2 (ACL2[45], Benedict *et al*., 2003) These include both primary and secondary level containments and are summarized below and in Figure 20.

14.1.1 ACL2: Standard Practices

The following information is from the ASTMH Committee on Medical Entomology ACL2 Guidelines for safe working practices for the use of infected, uninfected and genetically engineered arthropod species in contained use. Oxitec relies upon these Guidelines in running its insectaries and external entities, such as the CDC, use them when conducting insectary inspections for import permits under CFR 71.54.[46]

- *Location of Arthropods*. Furniture and incubators containing arthropods [e.g., mosquitoes] are located in such a way that accidental contact and release by laboratory personnel, custodians, and service persons is unlikely. This is achieved by locating any arthropods in dedicated rooms, closets, and incubators out of the traffic flow or similar measures.
- *Supply Storage*. The area is designed and maintained to enhance detection of escaped arthropods. Equipment and supplies not required for operation of the insectary should not be located in the insectary. All supplies for insect maintenance that must be kept within the insectary are located in a designated area and closed storage is used where possible. Doors and drawers are opened only for access. Insect diet is kept in sealed containers.
- *General Arthropod Elimination.* Accidental sources of arthropods from within the insectary are eliminated. This is accomplished by cleaning work surfaces after a spill of materials, including water that might contain viable eggs. Pools of water are mopped up immediately.
- *Primary Container Cleaning and Disinfestation.* In addition to cleaning cages and containers to prevent arthropod escape, practices are in place such that arthropods do not escape by inadvertent disposal in primary containers. Cages and other containers are appropriately cleaned

[45] These Guidelines were produced by the American Committee on Medical Entomology and published in 2002. These Guidelines describe safe working practices for the use of infected, uninfected and genetically engineered arthropod species in contained use. They are followed broadly both inside and outside the USA by arthropod researchers and CDC inspects premises holding vectors in accordance with them. They are available at http://www.astmh.org/AM/Template.cfm?Section=ACME&Template=/CM/ContentDisplay.cfm&ContentID=1450.

[46] http://www.cdc.gov/od/eaipp/inspection/docs/Import_Permit_Checklist_ACL-2.pdf [Accessed 31 Mar 2015]

to prevent arthropod survival and escape (e.g., heated to over the lethal temperature or killed by freezing). Autoclaving or incineration of primary containers is recommended for containers.

- *Primary Container Construction*. Cages used to hold arthropods are non-breakable and screened with mesh of a size to prevent escape. Containers are preferably autoclavable or disposable. Openings designed to prevent escape during removal and introduction of arthropods are used.
- *Disposal of Arthropods*. Living arthropods are not to be disposed of. All wastes from the insectary (including arthropod carcasses, and rearing medium) are transported from the insectary in leak-proof, sealed containers for appropriate disposal in compliance with applicable institutional or local requirements. All life stages of arthropods are killed before disposal. Material is killed with hot water or freezing before flushing down drains that are fitted with sieves. All waste from the insectary is frozen at below -15^{o}C prior to disposal via incineration.
- *Primary Container Identification and labelling.* Arthropods are identified adequately. Labels giving species, strain/origin, date of collection, responsible investigator, and so on are firmly attached to the container). Vessels containing stages with limited mobility (e.g., eggs, pupae) are securely stored.
- *Prevention of Accidental Dispersal on Persons or via Sewer*. Before leaving the insectary and after handling arthropods, personnel wash their hands, taking care not to disperse viable life stages into the drainage system. If materials are disposed of via the sewer, all material is destroyed by heat or freezing followed by incineration. Air curtains are used as appropriate.
- *Pest Exclusion Program*. A program to prevent the entrance of wild arthropods (e.g., houseflies, cockroaches, spiders) and rodents effectively precludes predation, contamination.
- *Escaped Arthropod Monitoring*. Investigators assess whether escapes are occurring by instituting an effective arthropod trapping program to monitor the escape prevention program. Oviposition traps, ground-level flea traps, oil-filled channels surrounding tick colonies, light traps for mosquitoes and so on are recommended. The Guidelines also recommend exterior monitoring particularly in the case when exotic arthropods are used. Records of exterior captures are maintained.
- *Source and Harborage Reduction*. Harborage and breeding areas are eliminated. Furniture and racks in the insectary are minimized and can be easily moved to permit cleaning and location of escaped arthropods. Equipment in which water is stored or might accumulate (e.g., humidifiers) is screened to prevent arthropod access, or contains chemicals to prevent arthropod survival.
- *Notification and Signage*. Persons entering the area are aware of the presence of arthropod vectors. The hazard warning sign identifies the arthropod species, lists the name and telephone number of the responsible person(s), and indicates any special requirements for entering the insectary (e.g., the need for immunizations or respirators).
- *Procedure Desig*n. All procedures are carefully designed and performed to prevent arthropod escape.
- *Safety Manual.* A safety manual is prepared, approved by the IBC or senior management, and adopted. The manual contains emergency procedures, standard operating procedures, waste

disposal and other information necessary to inform personnel of the methods for safe maintenance and operation of the insectary.

- *Training.* Laboratory personnel are advised of special hazards and are required to follow instructions on practices and procedures contained in the safety manual. Adherence to established safety procedures and policies is made a condition of employment and is part of the annual performance review of every employee. Personnel receive annual updates and additional training as necessary for procedural or policy changes. Records of all training are maintained.
- *Access Restrictions*. Routine access is limited to trained persons and accompanied guests.
- Service persons are made aware of the hazards present and the consequences of arthropod release and contact with agents that may be present. Transfer of arthropods between manipulation and holding areas is in non-breakable secure containers.
- *Escaped Arthropod Handling*. Loose arthropods must be killed and disposed of, or recaptured and returned to the container from which they escaped.
- *Accidental Release Reporting*. An accidental release procedure is in place. This includes contacts and immediate mitigating actions. Accidents that result in release of GE arthropods from primary containment vessels must be reported immediately to the insectary director who is responsible for ensuring that appropriate and documented action is taken to mitigate the release and written records are maintained.
- *Movement of Equipment*. All equipment must be appropriately decontaminated and disinfested before transfer between rooms within the insectary, and before removal from the insectary.

14.1.1.1 Safety Equipment (Primary Barriers)

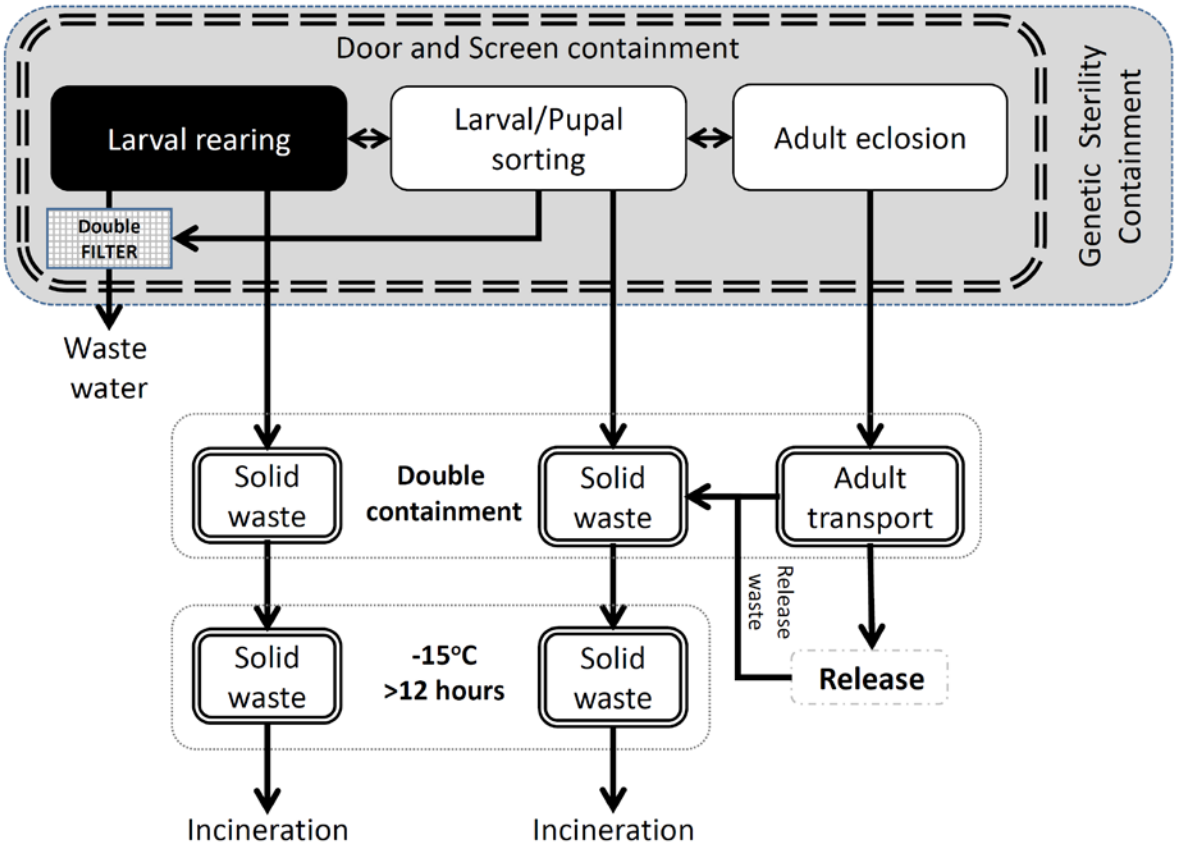
- *Eye and Face Protection*. Appropriate face/eye and respiratory protection are worn by all personnel entering the insectary.
- *Gloves.* Gloves are worn when handling blood, and associated equipment and when contact with potentially infectious material is unavoidable.
- *Torso Apparel*. White laboratory coats, gowns, and/or other protective equipment are worn at all times in the insectary.
- *Personal Clothing*. Clothing should minimize the area of exposed skin (e.g., skirts, shorts, open-toed shoes, sandals, tee shirts are inadvisable since this can increase the risk of attracting and being bitten by a loose arthropod).

14.1.1.2 Facilities (Secondary Barriers)

- *Location of Insectary.* The insectary is separated from areas that are open to unrestricted personnel traffic within the building by at least two self-closing doors that prevent passage of the arthropods.
- *Insectary Doors.* Entrance to the insectary is via a double-door vestibule that prevents flying and crawling arthropod escape. The two contiguous doors must not be opened simultaneously.
- *Additional barriers.* Potential points of egress, such as air ventilation units are screened with insect proof mesh.

- *Insectary Window.* The insectary windows are sealed shut where present, and are of hurricane rated glass.
- *Interior Surfaces.* The insectary is designed, constructed, and maintained to facilitate cleaning and housekeeping. The interior walls are light-colored so that a loose arthropod can be easily located, recaptured, or killed. Gloss finishes, ideally resistant to chemical disinfectants and fumigants, are recommended. Floors are light colored, smooth and uncovered. Ceilings are as low as possible to simplify detection and capture of flying insects.
- *Floor Drains*. Floor drains are modified to prevent accidental release of arthropods by use of metal screens small enough for the trapping of all arthropod stages (e.g., mosquito larvae).
- *Plumbing and Electrical Fixtures*. Internal facility appurtenances (e.g., light fixtures, pipes, ducting) are minimal since these provide hiding places for loose arthropods. Penetrations of walls, floors, and ceilings are minimal and sealed/caulked. Light fixtures are sealed, and accessed from above. HVAC Ventilation is appropriate for arthropod maintenance, but does not compromise containment of the arthropod. Appropriate filter/barriers are installed to prevent escape of arthropods; air curtains are located in vestibules to the laboratory.
- *Sink*. The facility has a hand-washing sink with hot water and with suitable plumbing to prevent arthropod escape.
- *Illumination*. Illumination is appropriate for arthropod maintenance but does not compromise arthropod containment, impede vision, or adversely influence the safety of procedures within the insectary. Lighted (or dark) openings that attract escaped arthropods are avoided.

Figure 20. Summary Schematic of Containment Measures for Egg Production Facility in the US



14.2 Biological containment

Any escapees from the HRU will be homozygous for the OX513A insertion and as the rDNA insertion is >95% penetrant in the laboratory, it is anticipated that >95% will die in the environment as there is no access to the required concentration of tetracycline to allow survival. Laboratory conditions represent optimal conditions; the survival in the environment is expected to be lower due to the harsher environmental conditions encountered. Some evidence from this has been obtained from experiments conducted in Malaysia and the Cayman Islands. Mark release recapture studies with OX513A males were conducted in Malaysia (Lacroix et al., 2012) and the Cayman Islands (Winskill et al., 2014) to assess longevity of released males. Decay in recapture rate of males over time allowed estimation of daily survival probability (DSP), from which average life expectancy can be calculated as $-1/\text{Log}_e(\text{DSP})$.

In the Malaysian study, OX513A average life expectancy was 2.0 (DSP=0.611) days and 2.3 (DSP=0.646) days for the non GE comparator, and therefore OX513A average life expectancy did not differ significantly from the non-GE laboratory strain co-released as part of a comparative evaluation. In the Cayman study, four separate mark release recapture studies were conducted with resulting estimates of average life expectancy that were shorter than observed in Malaysia, ranging between 0.1 (DSP=0.001) to

1.6 (DSP = 0.53) days for the OX513A mosquito. No comparator non-GE strain was co-released in this study.

14.2.1 Potential for the failure of the biological containment

It is theoretically possible that non-specific mutations or alterations in the genome of the OX513A mosquito alters the expression of the lethality trait, which could result in the failure of the lethality trait to act in the absence of tetracycline and in offspring between OX513A males and wild type female crosses surviving. The other possibility is that environmental concentrations of tetracycline are sufficient to rescue the phenotype from the lethality trait. This has been addressed in Section 14.3.4. The insertion of the rDNA construct in OX513A has remained stable over many generations even under mass rearing conditions. The releases will be predominantly male mosquitoes; these are sorted from the females with an accuracy averaging over 99.9% (Carvalho *et al*., 2014; Harris *et al*., 2012). Any survival of the male mosquitoes is not anticipated to increase the biting or disease transmission at the release site as male mosquitoes do not bite.

The efficacy of lethal trait expression is assessed by comparing the mortality of the OX513A (scored by fluorescence and confirmed by PCR) and wild type progeny, as described in the proposed field trial protocol (see Section 11.1). If these results indicate that there is no statistically significant difference in mortality, then the trait will be regarded as not having the desired efficacy. Lack of efficacy has not been seen in any previous releases in the Cayman Islands, Panama, or Brazil. If in the unlikely event that the trait is not effective during the investigational period, it will be detected as described above, the trial will be stopped, and additional mosquito control measures can continued to be applied such as the use of larvicides or adulticides.

14.3 Geographical/geophysical containment

Ae.aegypti can survive in the environment in Florida, where it is regarded as an invasive species by some (Juliano and Lounibos 2005; and CDC[47]) but for the purposes of this EA will be referred to as a non-native species. It is the intention of the proposed investigational use for the OX513A males to mate with females of the wild population of *Ae.aegypti* at the proposed release site. The geographical/geophysical containment measures include

- Temperature;
- Water storage and rainfall;
- Salinity of the water surrounding the release site; and
- Insufficient tetracycline in the environment and breeding sites that has the potential to reverse the lethality trait in the environment.

Each of these elements and their effect on containment are discussed further below:

[47] http://www.cdc.gov/dengue/entomologyecology/ [Accessed 30 Mar 2015]

14.3.1 Temperature

The effect of temperature on larval development of *Ae.aegypti* has been well studied. Studies showed that larvae have an ecological temperature range of 10-30^{o}C (~50^{o}F -86^{o}F) (Tun-Lin, 2000). Larval development is a function of temperature, which also affects adult size, dry weight and ovariole number, all of which fall as the temperature rises (Clements, 2000). High temperatures alone (>40^{o}C)[104^{o}F] are unlikely to limit the species but low temperatures are limiting with the threshold being the 10-15^{o}C (~50-59 ^{o}F) isotherm. At temperatures lower than 15^{0}C (59^{o}F), *Ae.aegypti* becomes torpid, unable to fly, or moves its limbs only slowly. 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. *Ae.aegypti* does not appear to enter a true diapause, although the eggs are able to survive in dry conditions for several months.

Temperature sensitivity of the OX513A strain has been investigated and is reported in Section 12.2.1.4.

14.3.2 Water storage and rainfall

Ae.aegypti eggs have the potential to remain as viable eggs for several months if the environmental conditions are suitable. Access to water will induce egg hatching. The storage of water in uncovered vessels for personal use such as washing and drinking can serve as attractive oviposition sites for female mosquitoes if the water sources are not covered, or the cover is routinely removed.

In the Florida Keys, there is piped water to houses and therefore the only containers that could provide breeding sites are those that are filled with rainwater, or deliberately filled with tap water and left out. FKMCD makes regular surveys of containers in the area and advises residents to tip out all containers that they might have on their land. Additionally, the larvicide Bti is used in any container that is found to be productive for larvae. There have been some reports of *Ae.aegypti* larvae being found in the surface clear water layer of septic tanks (Hribar, 2011, Burke, 2010) , but this is unusual and usually occurs where the lid is cracked or broken, providing access to the female as an oviposition site. Key West and surrounding areas in Monroe County have eliminated 99.9% of septic tanks[48] and uses mains sewerage as the major means of waste disposal.

14.3.3 Salinity of the ocean surrounding the release site

The release site is surrounded by saline ocean waters and inlets. *Ae.aegypti* are reported not to survive in sea water at salinity levels between 14 g/L and 35 g/L, although they have been found to survive to a limited extent in brackish waters (Ramasamy *et al*., 2011) with lower saline levels (3 g/L) as reviewed in Section 12.1.1.1. Some of these environments with brackish waters are likely to include standing water in boats which are expected to be found in the trial area, although these are also the same breeding sites

[48] Monroe County Engineering Division: Keys Wastewater Plan Nov 2007 http://www.monroecounty-fl.gov/DocumentCenter/Home/View/478 [accessed 6 Jan 2015]

that are examined for *Aedes* control using conventional means such as insecticides. FKMCD recommends that standing water is removed from boats[49].

14.3.4 Insufficient tetracycline in the environment

Tetracylines in the environment can come from human and animal therapeutic uses as well as prophylactic use in intensive animal rearing as a growth promoter, although this practice is in decline and several countries have banned its use, such as the European Union (EU, 2005). Tetracycline was first approved for human use in the United States in 1957 and was one of several oral tetracyclines used at that time (oxytetracycline, chlortetracycline,) many of which are no longer available or are used in veterinary medicine only. More modern forms of tetracycline include doxycycline and minocycline which are much more commonly used and have similar indications. Currently, tetracycline is most frequently used for upper respiratory and skin and soft tissue infection. Tetracyclines for veterinary therapeutic use are used widely for the prevention of infection. They have also been used in the USA for growth promotion purposes in food animals, although FDA has issued a policy discouraging use for such purposes (see Guidance for Industry 152[50]). According to an Animal Health Institute survey in 2007 (AHI, 2008) over 10 million pounds of tetracyclines are used in the livestock industry in the US. Tetracycline has known environmental toxicity to fish with a lethal concentration of 186.9-258.9 mg/l (US.Pharmocopeia, 2014). The sensitivity of the OX513A strain has been evaluated in Section 12.2.1.1 and will not be repeated here, but in summary, concentrations of 1 µg/mL are required to fully rescue the phenotype from the lethality trait.

From a review of the accessible environments (Section 12.1 of this document), there are no apparent sources of high concentrations of environmental tetracyclines; as there are no commercial farming (land based or marine) enterprises or hospitals in the immediate vicinity of the proposed release site. The nearest hospital/clinic is on Stock Island and is over 300 m away from the proposed release site separated by sea water and mangrove vegetation which is likely to pose a significant barrier to dispersal of the released mosquitoes through spontaneous flight. *Ae.aegypti* breeds in ephemeral water, in containers, tires, gutters etc. and these are extremely unlikely to be contaminated with sufficient quantities of tetracyclines to cause failure of the lethality trait. Potential failures of the trait have been examined in Section 14.2.1.

[49] http://keysmosquito.org/programs-domestic-field-ent-services-offshore-trucks-aerial/
http://keysmosquito.org/programs-domestic-field-ent-services-offshore-trucks-aerial/

[50] GFI 152: CVM GFI #152 Evaluating the Safety of Antimicrobial New Animal Drugs with Regard to Their Microbiological Effects on Bacteria of Human Health Concern; GFI CVM GFI#209 The Judicious Use of Medically Important Antimicrobial Drugs in Food-Producing Animals (PDF - 251KB).

15 Consequences of potential escape, establishment and spread

15.1 Consequences for the environment

There are many factors that contribute to the consequences of potential escape, establishment, and spread of OX513A *Ae.aegypti*. These factors include both environmental variables and interactions with the genetic makeup of OX513A. As previously discussed in Section 13.3 *Likelihood for Establishment*, if the likelihood of any one of the variables is negligible, the overall concern would be low. The OX513A mosquitoes contain a strong selective disadvantage; lethality, in the absence of sufficient quantities of tetracylines (which are unlikely to be present in the environment at the release site, or in the wider environment of Monroe County) and, as such, natural selection is expected to act on these attributes. *Ae.aegypti* is already a non-native species in the Florida Keys and is currently insufficiently controlled by both adulticide and larvicides to prevent dengue transmissions. There is no keystone species that is obligate on *Ae.aegypti* and even generalist insectivores consume very small quantities of all mosquitoes (Blum,1997; Lounibos, 2002).

One element of the proposed investigational use is that the trial will be continuously monitored by different (egg and adult) trapping and molecular methods, which will also allow monitoring of the performance of the traits and the detection of other mosquito species that may come into the area under the environmental conditions of the investigational use.

15.2 Consequences for human health

Based on the information provided in this EA, the release of *Ae .aegypti* OX513A is not expected to cause any significant adverse impacts on human health beyond those caused by other mosquitoes (e.g., local reactions at the site of bites). Direct eradication of *Ae.aegypti* is not expected to have any significant adverse impact on human health. An indirect effect that may occur is that the ecological niche *Ae.aegypti* inhabits will be vacated and other mosquito species could move in to the vacated niche. This is not an intrinsic consequence of the use of the rDNA construct in OX513A strain, as the same would be expected to happen with other mosquito control measures as all current control methods for mosquitoes aim to significantly reduce or eliminate the mosquito from an area. Even if another mosquito species such as *Ae. albopictus* were to move into the vacated ecological niche, *Ae. albopictus* is not as good a vector for dengue as *Ae.aegypti* (Lambrechts *et al*., 2010). A review by Gratz (2004) of the vector status of *Ae. albopictus* determined that although there was frequent isolation of dengue viruses from wild-caught mosquitoes, there was no evidence that *Ae. albopictus* is an important urban vector of dengue, except in a limited number of countries where *Ae.aegypti* is absent, i.e., parts of China, the Seychelles, historically in Japan and most recently in Hawaii.

16 Risk Assessment

The environmental risk assessment evaluates the likelihood that ecological impacts may occur as a result of exposure to one or more stressors. Therefore, there must be both an effect (may be referred to as a hazard) and exposure to that potential hazard to have a likelihood of an adverse impact on the environment, where RISK is a function of HAZARD X EXPOSURE.

In its report on Animal Biotechnology Science Based Concerns (NRC 2002) the National Academies of Science defined ecological "harm" as "gene pool, species or community perturbation resulting in negative impacts to community stability". Negative impacts might be direct or indirect such as changes in other factors used or needed by the ecological community. Prioritization of environmental concerns posed by GE animals was considered, determining the likelihood that a GE animal will become established in the receiving community and reported below:

- Fitness -The effect the rDNA construct has on the "fitness" of the animal within the ecosystem into which it is released
- Increased adaptability -The ability of the GE animal to escape and disperse into diverse communities
- The stability and resilience of the receiving community.

For a GE animal to prove a hazard it must spread and establish in the community in which it is released, NAS therefore defines exposure as the establishment of the GE animal in the community. The risk assessment has therefore used this definition of exposure potential.

The risk assessment was conducted using the following steps:

- Identification of potential harms regardless of their likelihood
- Identification of the hazards that could produce potential harms
- Likelihood of exposure (using the definition above)
- Likelihood of harm being realized if exposure occurs
- Determination of risk by the multiplication of the resulting outcomes on harm and exposure.

The identification of potential hazards, likelihood of exposure and potential consequences (likelihood of harm being realized) have been evaluated in the preceding sections of the document and are considered together in the risk assessment.

A four point scale was determined for each of the parameters of likelihood, harm being realized (consequence in the table), and estimation of risk as described in Table 8 below:

Table 8. Estimation of risk matrix

LIKELIHOOD ASSESSMENT	RISK ESTIMATE			
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			

Note: the risk assessment matrix and definitions are taken from the Australian OGTR Risk Analysis Framework[51].

The following definitions were used for the assessment criteria in the risk assessment matrix:

Likelihood Assessment

- Highly likely – is expected to occur in most circumstances
- Likely –could occur in many circumstances
- Unlikely – could occur in some circumstances
- Highly unlikely – may only occur in very rare circumstances

Consequence Assessment

- Marginal – there is minimal or no negative impact
- Minor – there is some negative impact
- Intermediate – the negative impact is substantial
- Major – the negative impact is severe

Risk Estimate

- High – risk is unacceptable unless actions for mitigation are highly feasible and effective
- Moderate – risk is of marked concern that will necessitate actions for mitigation that need to be demonstrated as effective
- Low – risk is minimal, but may invoke actions for mitigations beyond normal practices
- Negligible – risk is insubstantial and there is no present need to invoke actions for mitigation

A risk assessment has been conducted regarding the investigational use of OX513A *Ae.aegypti* for a field trial in Key Haven. Risk assessment is a formal and transparent process which looks at potential hazards and exposure to those hazards. In this case a list of potential hazards (intrinsic properties of the modified insect) was identified and characterized.

A summary of the potential harms envisaged from the investigational use of the OX513A *Ae.aegypti* mosquito are summarised in Table 9, these have been classified as direct or indirect, immediate or

[51] http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/content/riskassessments-1 [Accessed 24 Oct 2013]

delayed and have been grouped according to their likely area of impact: human health, animal health, or environmental health.

A direct harm refers to the primary effects that the use of the OX513A mosquito could have on the environment, including human health; there is no causal chain of events that could lead to the harm. An indirect harm refers to a causal chain of events being established whereby the harm is reached though mechanisms not directly related to insect itself, such as interaction with other organisms, transfer of genetic material or changes in use or management at the release site.

Classifying the harm as immediate or direct will facilitate the monitoring activities. An immediate effect refers to a potential harm that would be expected to be seen throughout the timescale of the release whereas a delayed effect may not be observed in the release period but might become apparent as a direct or indirect effect at a later stage. A number of the potential harms that could theoretically occur indirectly therefore have a scientific causal chain of events leading to the identified harm.

The following risk hypotheses were constructed to assess risks for each of the factors considered to be at potential risk from the release of OX513A *Ae.aegypti*.

- **Animal health;** There are no potential adverse effects on animal health associated with the release of the OX513A *Ae.aegypti* when compared to the current control systems.
- **Human health;** There are no potential adverse effects associated with human health following the release of the OX513A *Ae.aegypti* when compared to the current control systems.
- **Environment;** There are no potential adverse environmental effects associated with the release of the OX513A *Ae.aegypti* when compared to the current control systems.

Identified risks can be further broken down into indirect and direct risks for each of the above factors to enable consideration of the immediate and long term consequences that a release might have on human and animal health or the environment. Specific potential harms associated with the release were identified and the likelihood and consequence of these potential harms were evaluated and are shown in Table 9, where necessary actions that could mitigate these harms were considered. The comments in the table are by necessity of their format only a summary of the information available in the rest of the risk assessment

The risk assessment is summarized in Table 9 and brings together all the information previously presented in the EA regarding potential harms, hazards, likelihoods and consequences along with the data endpoints that have been considered in the analysis.

Table 9 Risk assessment

	Risk Scenario					
Risk category (Direct/ Indirect)	Potential Harm/Benefit	Potential causal pathway to harm	Comments	Consequence	Uncertainty	(Risk estimate)[52]
Environmental health (Direct)	Release of tetracycline in the environment kills bacteria involved in environmental processes	Tetracycline from larval rearing is released into environment in the waste water	The levels of tetracycline in the waste water will be small (grams/week) this is expected to be rapidly broken down in the environment as tetracycline is sensitive to light. A number of studies have shown that tetracycline is rapidly degraded by ultra-violet radiation (Bautiz and Nogueira 2007), in the presence of iron or other metal catalysts (Reyes, Fernadez *et al*., 2006), with total deactivation obtained in 70 min. The use of tetracycline and its fate in the environment was reviewed by Sarmah, Meyer *et al*., 2006, and again found that tetracycline rapidly degrades (with the bulk of degradation taking place on day 1) and a short half-life in the environment (15-30 days in water and up to 9 days in animal manure).	Marginal	Very low	Likely x Marginal (Low)

[52] Risk estimate is a function of consequence and likelihood assessments and based on estimation of risk matrix summarized in Table 8.

	Risk Scenario					
Risk category (Direct/ Indirect)	Potential Harm/Benefit	Potential causal pathway to harm	Comments	Consequence	Uncertainty	(Risk estimate)[52]
Human health (Indirect)	Continued population prevalence of *Ae.aegypti*	Suppression of local *Ae.aegypti* population fails	Immigration of mosquitoes into the trial site could compromise the effectiveness of the suppression objective by OX513A.The treatment site is isolated by at least 200 m from untreated sites by a buffer zone.The proposed trial has entomological objectives does not attempt to look at epidemiologic endpoints.	Marginal	Low	Unlikely x Marginal (Negligible)
Environmental health (Indirect)	Loss of identification of the OX513A larvae	Degradation of the fluorescent marker in the environment	The fluorescent marker DsRed2 has been used in a variety of insects which survive in a range of conditions (temperature and humidity). Studies of the longevity of the DsRed2 marker in Pink Bollworm show that as a marker for identifying test insects it has a robust performance and would be acceptable for use in a SIT program (Simmons, McKemey *et al*., 2011). The expression of the protein continues throughout the mosquito larval development making DsRed2 a clear marker in larval and pupal stages. Molecular biological techniques also provide a further means of identification should the marker not be visible.	Marginal	Low	Highly unlikely x Marginal (Negligible)
Environmental health (Direct)	Death of other mosquito species	The released males have altered reproductive behaviour which enables the	Biological data from experiments conducted and literature shows that cross species mating results in non-viable progeny. Existing data shows there is reproductive isolation between species due to the structure of the genitalia (e.g., between *Aedes* and *Culex)*	Minor	Very low	Highly unlikely x Minor (Negligible)

	Risk Scenario					
Risk category (Direct/ Indirect)	Potential Harm/Benefit	Potential causal pathway to harm	Comments	Consequence	Uncertainty	(Risk estimate)[52]
		mosquitoes to breed with other species.				
Environmental Health (Direct)	Increase of disease transmission	Non-specific mutations or alterations in the genome of the mosquito alter the expression of the lethality trait. Failure of the lethality trait to act in the absence of tetracycline results in offspring between OX513A males and wild type female crosses surviving to adulthood.	The insertion has remained stable over many generations even under mass rearing conditions. The releases will be male mosquitoes; these can be sorted from the females with over 99.9% accuracy based on previous studies. Any survival of the male mosquitoes is not anticipated to increase the biting or disease transmission at the release site as male mosquitoes do not bite. PCR of the fluorescent larvae would indicate whether heterozygous larvae are surviving to adulthood and are capable of mating. If the lethality trait fails, the mosquito can be controlled using alternative techniques such as fogging and use of larvicides. No such instability in the lethality trait has been observed to date.	Minor	Very low	Highly unlikely x Minor (Negligible)
Environmental health (Direct)	Effective mosquito control is compromised	A change in biology of the OX513A male mosquito results in the released mosquitoes not mating with the local females, this leads to failure of the	Mating competitiveness testing of the OX513A male mosquitoes with local *Ae.aegypti* females from various locations has been carried out and in all cases mating has been compatible. Mating success is also a primary objective of the trial. The presence of fluorescent larvae from monitoring will	Minor	Very low	Highly Unlikely x Minor (Negligible)

	Risk Scenario					
Risk category (Direct/ Indirect)	Potential Harm/Benefit	Potential causal pathway to harm	Comments	Consequence	Uncertainty	(Risk estimate)[52]
		releases to result in population suppression	further indicate the mating success of the OX513A individuals. Mosquito control continues to be managed by use of existing insecticides and larvicides.			
Environmental Health *(Indirect)*	Local flora adversely affected	Lethality trait is passed to pollinating insect species. Pollination does not occur compromising the flora.	*Ae.aegypti* is not a pollinator. Biological data from experiments conducted and literature shows that mating in *Ae.aegypti* is extremely species specific and even forced mating with the closest relative *Ae. albopictus* in the laboratory results in non-viable progeny. Existing data and scientific literature shows there is reproductive isolation between species due to the structure of the genitalia (e.g. between *Aedes* and *Culex*).	Marginal	Very Low	Highly unlikely x Marginal (Negligible)
Environmental health (Direct)	*Aedes aegypti* is suppressed in the trial site	Population of *Ae. aegypti* at the release site is suppressed through mating with OX513A	*Ae.aegypti* is widespread throughout all global tropical areas. Even if population eradication could be achieved in one area, it is unlikely that this will impact on the global distribution of the mosquito. The mosquito is currently being controlled by source reduction, biological and chemical methods. Many countries have eradicated *Ae.aegypti* in the past without ecological consequences (Monteiro et al, 2014, Gubler, 2011, Petrie and Wheeler, 2007; Elder and Lamche, 2004). The trial will be stopped once the objective have been achieved and therefore is too short in duration to eradicate *Ae.aegypti.* The	Marginal	Very Low	Highly unlikely x Marginal (Negligible)

	Risk Scenario					
Risk category (Direct/ Indirect)	Potential Harm/Benefit	Potential causal pathway to harm	Comments	Consequence	Uncertainty	(Risk estimate)[52]
			Ae.aegypti population will return following the cessation of releases of OX513*A.* During the trial, population suppression of *Ae. aegypti* will result in lower concomitant nuisance to people residing in the release area.			
Environmental health (Direct)	Increase in geographic host range of the mosquito	The mosquitoes are able to survive at altered temperature range and can invade new habitats. Inadvertent artificial selection in the laboratory strains results in the mosquitoes having a higher/lower tolerance to temperature and the potential to increase their geographic range.	There is no difference in the inherent characteristics of both the OX513A *Ae.aegypti* and the wild type mosquito in lab studies. There is no evidence that the genetic insertion will increase the geographic range of the mosquito.	Marginal	Low	Highly unlikely x Marginal (Negligible)

	Risk Scenario					
Risk category (Direct/ Indirect)	Potential Harm/Benefit	Potential causal pathway to harm	Comments	Consequence	Uncertainty	(Risk estimate)[52]
Human Health (Direct)	Toxic or allergic reaction in humans	Humans ingest the inserted genetic traits and any expressed proteins in the adult mosquitoes by accident, or larvae through accidental consumption of the eggs or early larval stages in drinking water. Humans ingest a tetracycline dose from accidental ingestion of a mosquito. A bite of a female OX513A results in an allergic reaction in humans.	The protein DsRed2 is a commonly used marker which has been evaluated in a food safety study (Pavely and Fedorova 2006) submitted to and approved by the US FDA (FDA, 2010). Using internationally recognised techniques, the inserted proteins are shown to have. A study conducted by Oxitec showed that there was no detectable tTAV or DsRed2 in the saliva of female OX513A at or above the limit of detection (0.8 ng tTAV and 2.5-5.0 ng for DsRed2 in 5ul of saliva) Therefore, there is neither intrinsic toxic or allergenic potential nor exposure to the introduced proteins in OX513A. The direct ingestion of a mosquito would not provide tetracycline to humans as the mosquito adults are not supplied with tetracycline prior to release. The bite of a female OX513A would be the same as a bite from a non-GE mosquito.	Minor	Very Low	Highly Unlikely x Minor (Negligible)

	Risk Scenario					
Risk category (Direct/ Indirect)	Potential Harm/Benefit	Potential causal pathway to harm	Comments	Consequence	Uncertainty	(Risk estimate)[52]
Human health (Direct)	Continued *Ae.aegypti* prevalence with associated consequences.	There is the potential that <0.2% of the releases are female. Inadvertent or accidental release of female mosquitoes or survival of female progeny may result in biting. Biting an infected person may lead to local disease transmission and/or an increase in dengue at the release site.	If a person were bitten by an OX513A female mosquito it would be exactly the same as a bite from a wild one, in fact rather less dangerous in several respects: released mosquitoes are disease-free as they are maintained in conditions and with procedures that prevent contamination with virus and because the dengue virus takes a long time to develop in a mosquito to the point where it can be transmitted, shorter-lived females such as the OX513A females are less likely to pass on diseases. Male mosquitoes do not bite humans.	Minor	Low	Highly Unlikely X Minor (Negligible)
Environmental /Human health (Direct)	Local *Ae.aegypti* could develop new insecticide resistance as a result of breeding with OX513A.	Increased resistance to insecticides is spread throughout the local mosquito population as the OX513A mosquitoes have different insecticide resistances than the local population	Due to long term use of pyrethroids and carbamates for insect control local *Ae.aegypti* populations have existing insecticide resistances. Insecticide resistance studies have shown that the OX513A mosquitoes are susceptible to currently used insecticides. Additionally as they are susceptible they could introgress these alleles into the local *Ae.aegypti* population and increase the susceptibility of the local population to existing insecticides. This would be a potential benefit.	Minor	Low	Highly unlikely x Minor (Negligible)

Animal health (Direct)	Adverse effect on non- target organisms	Insectivorous non target organisms feed on OX513A larvae resulting in potential adverse effects	*Ae.aegypti* are not a keystone species in the ecosystems as they are non-native in all parts of the world except Africa. Consequently nothing is dependent on them as a food source and they are consumed by generalist predators and prey as a small part of their diet. The mosquitoes are already controlled by chemical, biological or source reduction methods and therefore impacts on non-targets are not likely to be greater than those of the existing control mechanisms. There is no habitat overlap of OX513A mosquitoes with threatened or endangered species as *Ae.aegypti* is an urban or domestic mosquito closely associated with human habitats. The DsRed2 protein has been evaluated for food safety by the US FDA (Pavely and Fedorova 2006, FDA, 2010). The expressed proteins have been shown to have no homology to known toxins following bioinformatics evaluations carried out according to international guidelines. In addition, feeding OX513A larvae to two species of carnivorous *Toxorhynchites* mosquito larvae showed no difference in survival of the *Toxorhynchites* mosquito when fed either the wild type larvae or OX513A (Nordin, Donald *et al*., 2013). Feeding studies using the guppy, *Poecilia reticulate* (*Actinopterygii: Poeciliidae*) have also shown that there is no impact on the health of the fish from a diet containing a high incorporation level of OX513A.	Minor	Very low	Highly unlikely x Minor (Negligible)

	Risk Scenario					
Risk category (Direct/ Indirect)	Potential Harm/Benefit	Potential causal pathway to harm	Comments	Consequence	Uncertainty	(Risk estimate)[52]
Animal health (Direct)	Insectivorous animals are adversely impacted by removal (or suppression) of a species from the food chain	Following a successful population suppression of *Ae.aegypti* other food sources are required by insectivorous animals.	The *Ae.aegypti* mosquito lives in and around human habitation living in artificial breeding containers such as flower pots and water storage containers. The mosquito is an non-native species and is not known as the sole food source for any one organism although larval stages could be eaten by amphibians or other species living in the domestic environment (spiders, reptiles, etc). In some instances the larvae could be consumed by fish in the environment. Adult mosquitoes are poor fliers and females are generally found in or around houses, adult mosquitoes are most likely to be eaten by spiders or amphibians although it is possible that some adults could be opportunistically eaten by bats or birds. Additionally, the mosquitoes are currently being controlled by chemical, biological, or source reduction methods and therefore impacts on non-targets are not likely to be greater than those of the existing control mechanisms in the event the trial is a success and reduces local *Aedes aegypti* population significantly.	Minor	Low	Highly unlikely x Minor (Negligible)

	Risk Scenario					
Risk category (Direct/ Indirect)	Potential Harm/Benefit	Potential causal pathway to harm	Comments	Consequence	Uncertainty	(Risk estimate)[52]
Animal Health (Direct)	Adverse effects on ecosystem services.	Release of male mosquitoes leads to an increase in dead mosquitoes. Decomposer organisms suffer from a large increase in dead mosquitoes during the release.	Decomposer organisms are often opportunistic, feeding on detritus when it is found. Biodiversity in soil ecosystems is generally high with a range of organisms assisting in the breakdown of organic matter. Complex interactions involving many species exist above and below ground, many of these species are microscopic and would be extremely difficult to monitor effectively. A number of decomposers could be involved in the breakdown of *Ae.aegypti*, including but not limited to organisms from classes of, Oligochaeta, Diplopoda, Isopoda, Nematodes, Collembola, Acari and Earthworms as well as species of Protozoa, Fungi and Bacteria. Fluctuations in the populations of decomposer organisms will not be monitored throughout the release as the size of the trial is too small for effective monitoring. No adverse effects have been identified in open releases conducted in Malaysia, Cayman Islands, Panama and Brazil.	Minor	Low	Highly unlikely x Minor (Negligible)
Animal health (Direct)	Potential to spread animal infectious diseases.	Alteration of blood meal host preference might arise leading to the female mosquitoes starting to preferentially bite animals over humans	Releases will be >99.9% male mosquitoes which do not bite animals or humans, they do not possess the mouthparts to do so or the stomach morphology to ingest blood. *Ae.aegypti* females would have to bite an animal harbouring an animal disease and transmit to another animal. *Ae.aegypti* is	Minor	Very low	Highly unlikely x Marginal (Negligible)

	Risk Scenario					
Risk category (Direct/ Indirect)	Potential Harm/Benefit	Potential causal pathway to harm	Comments	Consequence	Uncertainty	(Risk estimate)[52]
			mainly associated with humans and preferentially bites them over animals and as the aim of the release is to reduce the numbers of *Ae.aegypti* overall the likelihood is extremely low. No changes in the behaviours of female *Ae.aegypti* have been observed in the laboratory or in other trials conducted elsewhere.			

	Risk Scenario					
Risk category (Direct/ Indirect)	Potential Harm	Potential causal pathway to harm	Comments	Consequence	Uncertainty	(Risk estimate)
Human health (Indirect)	Disease transmission despite *Ae.aegypti* population reduction.	*Ae.albopictus,* the secondary vector of dengue is able to proliferate in the absence of *Ae.aegypti*.	Monitoring of the trial will allow the monitoring for potential presence of *Ae.albopictus.* The duration of the trial is insufficient to allow invasion of *Ae.albopictus* into any vacant ecological niche, which may take a much longer period. *Ae .aegypti* populations are likely to recover to pre-trial numbers after the cessation of releases. Current incidence of Dengue in the Florida Keys is very low.	Minor	Medium	Unlikely x Minor (Low)
Human health (Direct)	Increased pain/itchiness from mosquito bites.	The size of the mosquito is increased and the bites from the females are more painful. Inadvertent artificial selection in the laboratory strains results in larger insects.	Bionomic studies show that OX513A males/females are not different from wild type individuals. Lab studies show there is no physical difference in size between the OX513A mosquito and the wild type. Males don't bite or transmit diseases.	Minor	Very Low	Highly unlikely x Marginal (Negligible)

	Risk Scenario					
Risk category (Direct/ Indirect)	Potential Harm	Potential causal pathway to harm	Comments	Consequence	Uncertainty	(Risk estimate)
Human health (Indirect)	Female OX513A mosquitoes are released during the control program	Increased probability of being bitten by female mosquito.	Pupae can be sex-sorted with greater than 99.9% accuracy and there are robust quality control measures in place regarding pupal sex sorting. In the event that a small number of females are released, they do not carry any virus and there is no evidence that the females are capable of survival times greater than the existing mosquito, which could enhance their ability to acquire and transmit viral infections. A bite from a female would be similar to a bite from a wild mosquito. The absence of tetracycline in sufficient quantities in the environment would significantly reduce the lifespan of female mosquitoes. Should breeding and egg laying result, based on the results of previous studies >95% of resulting pupae are expected to die.	Minor	Low	Unlikely x Minor (Low)

16.1 Uncertainties in the risk assessment

Uncertainty in the risk assessment can come from a variety of sources, such as variability in parameters and the limitations of their understanding. Uncertainty can be reduced by obtaining or generating more data on particular aspects, but the variability of the parameter cannot be reduced by more data as it is a natural phenomenon. The risk assessment presented here is qualitative, relying on published information and scientific study. In qualitative risk assessments, judgment by professionals in the field is used to estimate the degree of uncertainty. For the risk questions posed (below) the uncertainty has been evaluated:

- Can OX513A *Ae.aegypti* escape the confined conditions in which it is reared?

There is a high degree of confidence in the containment measures at the HRU in the Florida Keys is expected. Rearing is conducted in accordance with ACL2 containment levels and the facility has been inspected for compliance by the appropriate federal authorities (e.g., FDA, CDC). Staff working at the HRU will be Oxitec staff with a high degree of experience in handling OX513A and other GE insects in contained conditions. Staff from FKMCD working in the HRU will be trained in the procedures for the rearing of OX513A.

Some uncertainty exists for the occurrence of adverse weather conditions being encountered during the course of the trial and preventing rearing or release. For rearing, this is minimized by the HRU being located in a Category 4 hurricane rated building[53] and a Hurricane Preparedness Policy (00054_01 OX513A Hurricane Preparedness Policy) being in place, where adult and larval insect life stages will be killed within 36 hours of a hurricane warning being issued by NOAA or State Authorities. Even if some OX513A were to escape the containment, they will not live longer than their short lifespan and the introduced lethality trait and the dependence on the presence of tetracycline for survival will prevent establishment in the environment.

- What is the likelihood that OX513A *Ae.aegypti* will survive and disperse once released into the environment?

There is a high degree of confidence that OX513A released males will have limited dispersal, based on results from previous trials of OX513A in other countries and information from the published literature. The uncertainty surrounding environmental survival is greater than that for dispersal (medium degree of confidence) as there are many environmental variables that could influence survival (as described in

[53] A Category 4 hurricane rated building is capable of withstanding a Category 4 strength hurricane on the Saffir-Simpson Hurricane Wind Scale (this is defined as *winds of 130-156mph; Catastrophic damage will occur: Well-built framed homes can sustain severe damage with loss of most of the roof structure and/or some exterior walls. Most trees will be snapped or uprooted and power poles downed. Fallen trees and power poles will isolate residential areas. Power outages will last weeks to possibly months. Most of the area will be uninhabitable for weeks or months.*)

Section 12.2) Data from previous releases conducted with OX513A indicate that survival of released OX513A male mosquitoes is likely to be lower than that of the wild type *Ae.aegypti* mosquito.

- What is the likelihood that OX513A *Ae.aegypti* can reproduce and establish in the environment into which they are released?

There is a high degree of confidence that released OX513A males will mate with local females of the same species as data and information from the laboratory, semi-field[54] and field studies have shown that in all cases OX513A has mated successfully with females of the same species. The potential likelihood to establish in the environment has a medium confidence of uncertainty, because it would require detailed information on each environmental variable that could affect establishment, such as temperature, humidity, larval competition, predation, breeding site, container, vegetation etc. Even if such information were available, the interactions of the environmental factors and the organism itself would still provide a degree of uncertainty in the analysis. Sufficient information from previous field releases of OX513A, where the lifespan of the released insects was approximately 1-3 days (Lacroix *et al*., 2012) 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 is unlikely to establish in the environment.

[54] Semi-field describes a study that has been done in containment, but under natural environmental conditions; i.e., a field house or field cage.

17 Conclusions

Information relevant to the pertinent risk questions described throughout this document and summarized in Section 16, have been presented in this draft EA with the following conclusions:

- Can OX513A *Ae.aegypti* escape the confined conditions in which it is reared?

  The likelihood of escape from confined conditions is negligible.

- What is the likelihood that OX513A *Ae.aegypti* will survive and disperse once released into the environment?

  It is extremely unlikely that OX513A will survive longer than their short lifespan or disperse beyond the proposed trial site, and therefore, the likelihood that OX513A *Ae.aegypti* will survive and disperse is negligible.

- What is the likelihood that OX513A *Ae.aegypti c*an reproduce and establish in the environment into which they are released?

  There is a high likelihood that OX513A *Ae.aegypti* can reproduce, as reproduction with local females is the intended effect of the release. However, there is a low likelihood that they will be able to establish in the environment following reproduction. Reproduction in mosquitoes is extremely species-specific, with complex mating behaviors effectively limiting the transfer of the #OX513 construct to *Ae.aegypti* species, which is the intended effect. The offspring (or progeny) from such matings are extremely unlikely to survive and establish in the environment due to the expression of the self-limiting trait and therefore adverse effects on non-target organisms or other environmental processes such as ecosystem services are likely to be negligible. The impact on the environment and non-target organisms is likely to be less than the use of broad spectrum insecticides for mosquito control.

18 Preparation of EA

This draft environmental assessment was prepared by Oxitec Ltd, with advice and direction from the Center for Veterinary Medicine at the Food and Drug Administration, as well as experts from the Center for Disease Control and the Environmental Protection Agency, who serve as part of the inter-agency team for reviewing Oxitec's submissions under the Coordinated Framework for the Regulation of Biotechnology (1986).

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Appendix A

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Centers for Disease Control
and Prevention (CDC)
Atlanta GA 30333

February 9, 2015

Lawrence Hribar
Florida Keys Mosquito Control District
503 107th Gulf Street
Marathon, FL 33050

RE: Facility Inspection Report Response
Organization: Florida Keys Mosquito Control District

Thank you for your response concerning our report of the inspection of your entity conducted on December 3-4, 2015. All departures noted on the inspection report have been addressed adequately and the Centers for Disease Control and Prevention (CDC), Import Permit Program (IPP) does not require any further response from you at this time.

As the permittee it is your responsibility to ensure that the implemented biosafety measures are commensurate with the hazard posed by the infectious biological agents, infectious substances, and/or vectors to be imported, and the level of risk given its intended use.

If you have any questions concerning this correspondence, please contact the Centers for Disease Control and Prevention (CDC), Import Permit Program (IPP) at 404.718.2077.

Thank you,

Robbin S. Weyant, PhD, RBP (ABSA)
Captain, USPHS (Ret.)
Director, Division of Select Agents and Toxins
Office of Public Health Preparedness and Response

Appendix B

Downloaded
28-JAN-2015

FISH AND WILDLIFE SERVICES:THREATENED AND ENDANGERED SPECIES IN MONROE COUNTY, FL

Group	Name	Population	Status	Lead Office	Recovery Plan Name	Recovery Plan Stage
Birds	Everglade snail kite	FL pop.	Endangered	South Florida Ecological	South Florida Multi-Species	Final
Birds	Cape Sable seaside sparrow	Entire	Endangered	South Florida Ecological	South Florida Multi-Species	Final
Birds	Bachman's warbler (=wood)	Entire	Endangered	South Carolina Ecological		
Birds	Wood stork (Mycteria	AL, FL, GA, MS, NC, SC	Threatened	North Florida Ecological	Revised Recovery Plan for the	Final Revision 1
Birds	Piping Plover (Charadrius	except Great Lakes watershed	Threatened	Office Of The Regional Director	Great Lakes & Northern Great	Final
Birds	Piping Plover (Charadrius	except Great Lakes watershed	Threatened	Office Of The Regional Director	Piping Plover Atlantic Coast	Final Revision 1
Birds	Roseate tern (Sterna dougallii	Western Hemisphere except NE	Threatened	Caribbean Ecological Services	Recovery Plan Caribbean	Final
Birds	Red knot (Calidris canutus rufa)		Threatened	New Jersey Ecological Services		
Fishes	Atlantic sturgeon (Gulf	Entire	Threatened	Panama City Ecological	Gulf Sturgeon	Final
Flowering Plants	Blodgett's silverbush		Candidate	South Florida Ecological		
Flowering Plants	Big Pine partridge pea		Candidate	South Florida Ecological		
Flowering Plants	Wedge spurge (Chamaesyce		Candidate	South Florida Ecological		
Flowering Plants	Sand flax (Linum arenicola)		Candidate	South Florida Ecological		
Flowering Plants	Garber's spurge (Chamaesyce		Threatened	South Florida Ecological	South Florida Multi-Species	Final
Flowering Plants	Florida pineland crabgrass		Candidate	South Florida Ecological		
Flowering Plants	Key tree cactus (Pilosocereus		Endangered	South Florida Ecological	South Florida Multi-Species	Final
Flowering Plants	Cape Sable Thoroughwort		Endangered	South Florida Ecological		
Flowering Plants	Florida prairie-clover (Dalea		Candidate	South Florida Ecological		
Flowering Plants	Florida semaphore Cactus		Endangered	South Florida Ecological		
Flowering Plants	Everglades bully (Sideroxylon		Candidate	South Florida Ecological		
Insects	Schaus swallowtail butterfly	Entire	Endangered	South Florida Ecological	South Florida Multi-Species	Final
Insects	Miami Blue Butterfly (Cyclargus		Endangered	South Florida Ecological		
Insects	Bartram's hairstreak Butterfly		Endangered	South Florida Ecological		
Insects	Florida leafwing Butterfly		Endangered	South Florida Ecological		
Mammals	Key deer (Odocoileus	Entire	Endangered	South Florida Ecological	South Florida Multi-Species	Final
Mammals	West Indian Manatee	Entire	Endangered	North Florida Ecological	Florida Manatee Recovery Plan,	Final Revision 3
Mammals	West Indian Manatee	Entire	Endangered	North Florida Ecological	Recovery Plan Puerto Rican	Final
Mammals	Florida panther (Puma (=Felis)		Endangered	South Florida Ecological	Third Revision of the Florida	Final Revision 3
Mammals	Rice rat (Oryzomys palustris	lower FL Keys	Endangered	South Florida Ecological	South Florida Multi-Species	Final
Mammals	Key Largo cotton mouse	Entire	Endangered	South Florida Ecological	South Florida Multi-Species	Final
Mammals	Key Largo woodrat (Neotoma	Entire	Endangered	South Florida Ecological	South Florida Multi-Species	Final
Mammals	Lower Keys marsh rabbit	FL	Endangered	South Florida Ecological	South Florida Multi-Species	Final
Mammals	Puma (=mountain lion) (Puma	FL	Similarity of Appearance	Office Of The Regional Director		
Reptiles	American alligator (Alligator	Entire	Similarity of Appearance	Office Of The Regional Director		
Reptiles	Hawksbill sea turtle	Entire	Endangered	North Florida Ecological	Recovery Plan for the Hawksbill	Final Revision 1
Reptiles	Hawksbill sea turtle	Entire	Endangered	North Florida Ecological	Recovery Plan for U.S. Pacific	Final Revision 1
Reptiles	Leatherback sea turtle	Entire	Endangered	North Florida Ecological	Recovery Plan for U.S. Pacific	Final Revision 1
Reptiles	Leatherback sea turtle	Entire	Endangered	North Florida Ecological	Recovery Plan for Leatherback	Final Revision 1
Reptiles	Green sea turtle (Chelonia	FL, Mexico nesting pops.	Endangered	North Florida Ecological	Recovery Plan for U.S. Pacific	Final Revision 1
Reptiles	Green sea turtle (Chelonia	FL, Mexico nesting pops.	Endangered	North Florida Ecological	Recovery Plan for U.S.	Final Revision 1
Reptiles	Loggerhead sea turtle (Caretta	Northwest Atlantic Ocean DPS	Threatened	North Florida Ecological	Recovery Plan for the Northwest	Final Revision 2
Reptiles	Eastern indigo snake	Entire	Threatened	Mississippi Ecological Services	Eastern Indigo Snake	Final
Reptiles	American crocodile (Crocodylus	FL pop.	Threatened	South Florida Ecological	South Florida Multi-Species	Final
Reptiles	Gopher tortoise (Gopherus	eastern	Candidate			
Snails	Stock Island tree snail	Entire	Threatened	South Florida Ecological	South Florida Multi-Species	Final

Appendix C

OXITEC

INTERNAL RESEARCH REPORT

1. **Reference Number**: PH-2013-4-V3a

2. **Issuing Date**: 25 February 2016

3. **Title**: Investigating the tetracycline dose response 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): Z Curtis	**Study Supervisor (Signature):** PP
Study Coordinator (Name And Position): Zoe Curtis, Research Scientist	**Study Supervisor (Name And Position):** Luke Alphey, Ph.D., Chief Scientific Officer
Date Signed: 25 Feb 2016	**Date Signed:** 25th FEB 2016

7. Associated Personnel:

Name	Tasks
Zoe Curtis	Study coordination, experimental design, data collection and analysis, report writing
Kelly Matzen, Ph.D.	Experimental design, data collection and analysis, report writing
Peter Winskill	Data analysis
Derric Nimmo Ph.D.	Experimental design
Luke Alphey, Ph.D.	Experimental design, report writing and approval
Camilla Beech	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 determine the lowest concentration of tetracycline that allows greater survival of *Aedes aegypti* heterozygous for the OX513A construct than when reared in the absence of tetracycline.

10. Summary:

Aedes aegypti larvae, heterozygous for the OX513A construct were reared on increasing concentrations of tetracycline from 10 pg/mL to 1 µg/mL. We found that concentrations of 3 ng/mL tetracycline in the rearing water gave a small but statistically significant increase in the fraction of functional adults, with full rescue occurring above 1 µg/mL. Tetracycline concentrations above this rescue level are very unlikely to be found in the typical breeding sites of *Aedes aegypti* (Le-Minh, Khan et al. 2010), therefore the potential for the safety and efficacy of a control programme using OX513A to be compromised is negligible.

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 (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 mating between homozygous RIDL males and wild-type females (and of RIDL females with wild-type males) die during immature stages. This is 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.

Here we analyse the relationship between the tetracycline concentration in the larval habitat and survival of OX513A heterozygotes; in particular the lowest concentration of

tetracycline required in the larval rearing water to rescue OX513A heterozygotes by tTAV repression, resulting in a higher percentage of individuals eclosing as functional adults than if tetracycline were absent. By comparison of the dose-response curve determined in this study with published data on levels of tetracyclines in the environment, we do not anticipate *Ae. aegypti* encountering tetracycline concentrations in the environment that are great enough to rescue the lethality of the OX513A allele. This implies that the fitness of OX513A larvae in the field will be equivalent to that of such larvae reared in the complete absence of tetracycline.

12. **Methods**

Strains

The following strains were used for this experiment:

OX513A (bi-sex lethal RIDL strain): In the absence of tetracycline (tet), 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 high enough tetracycline concentrations, expression of tTAV is repressed, allowing normal development to adulthood (Phuc et al., 2007). OX513A is in the Latin genetic background described below.

Latin wild-type (LWT): A non-transgenic strain collected from Chiapas, Mexico and was transferred to Oxitec from Mexico's Institute of Public Health in 2006.

Insect Rearing

All strains were reared under standard insectary conditions: 27°C [+/- 1°C], 70% [+/- 10%] relative humidity, 12h: 12h light: dark cycle. Larvae were reared at 1 larva/mL in 16oz pots (Roberston Packaging, UK SICC65) and fed by a standard regime of finely ground Tetramin© fish flakes (Tetra GmbH, Germany). Live pupae were counted and placed into cages (15x15x15cm, Bugdorm-Megaview, Taiwan). Dead larvae and dead pupae were counted and discarded. Adults were provided with 10% sucrose solution *ad libitum*. Adult cages were assessed[1] for emergence three days after the last pupa was added.

Tetracycline Concentrations

Twelve chlortetracycline hydrochloride (tet)(Sigma-Aldrich, Gillingham, UK) concentrations were tested; 0, 10 pg/mL, 30 pg/mL, 100 pg/mL, 300 pg/mL, 1ng/mL, 3 ng/mL, 10 ng/mL, 30 ng/mL, 100 ng/mL, 300 ng/mL, and 1 µg/mL. Experimental tet solutions were made from stock solutions; 10 µg/mL, 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL. Stock solutions were prepared for each experimental set up. Concentrations were tested in two experiments; 0 µg/mL to 10ng/mL, and 0 µg/mL with 1ng/mL to 1 µg/mL. An overlap in the concentrations was included between the two experiments to ensure consistency between the results. LWT was reared as controls at 0 and 1 µg/mL tetracycline.

[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).

Statistical Analysis

Data were analysed using the RStudio software package version 0.97.237 (RStudio, USA) running version 2.15.0 of the R statistical software. Dose response modelling was performed using the DRC package. EC_{50} calculated using the Weibull model. Significance testing between tet concentrations was performed using a using a t-test.

13. RESULTS

OX513A heterozygous larvae were reared at 12 different tet concentrations, ranging from 10 pg/mL to 1 µg/ml, in addition to 0 µg/ml, with five repeats of 200 larvae at each concentration. Latin WT were reared at 0 and 1 µg/mL to assess background effects, independent of the OX513 construct. OX513A results are summarized in Table 1.

Tetracycline concentration	Dead pupae	Non-viable adults	Flying adults
1 µg/mL	0.8% (0.0%-1.6%)	6.7% (2.3%-11.1%)	60.9% (54.5%-67.3%)
300 ng/mL	0.4% (0.0%-1.0%)	7.0% (3.0-11.0%)	57.4% (50.4%-64.4%)
100 ng/mL	0.2% (0.0%-0.6%)	15.5% (10.0%-21.0%)	51.1% (44.6%-57.6%)
30 ng/mL	1.8% (0.5%-3.1%)	31.5% (25.9%-37.1%)	42.3% (34.6% 50.0%)
10 ng/mL	13.3% (8.0%-18.5%)	36.0% (33.3%-38.7%)	30.8% (26.9%-34.6%)
3 ng/mL	36.6% (28.4%-44.8%)	31.25% (29.0%-33.5%)	8.9% (6.6%-11.1%)
1 ng/mL	51.2% (47.4%-54.9%)	18.5% (16.3%-20.7%)	4.3% (3.2%-5.4%)
300 pg/mL	57.7% (52.6%-62.8%)	18.1% (14.7%-21.5%)	3.2% (2.3%-4.1%)
100 pg/mL	57.7% (49.3%-66.1%)	14.9% (10.8%-19.0%)	3.9% (2.4%-5.4%)
30 pg/mL	57.2% (53.0%-61.4%)	15.5% (12.8%-18.2%)	4.8% (4.1%-5.5%)
10 pg/mL	63.0% (52.9%-73.1%)	12.5% (9.0%-16.0%)	2.5% (1.3%-3.7%)
0	50.2% (45.0%-55.3%)	12.5% (9.2%-15.8%)	3.4% (2.4%-4.3%)

Table 1: OX513A heterozygous larvae reared at different tetracycline concentrations. Percentages are means of L1 individuals reaching the specified stage based on initial counts of 200 L1's per repeat. Confidence intervals are displayed in parentheses. Non-viable adults include; dead adults on the water surface, dead adults in the cage and non-flying adults.

OX513A larvae reared at tet concentrations at or below 1ng/mL did not give rise to a significantly greater percentage of flying adults than larvae reared in the absence of tet (0 µg /mL) (p=0.212 [2]). Tet concentrations in excess of 1ng/mL however, allowed a greater

[2] In the Curtis et *al.* (2015) this p value is reported as p=0.19. This difference is due to the number of decimal places of the raw data when analysed (i.e. 0.00 or 0.000).

percentage of flying adults. Figure 1 shows the percentage of flying adults increasing with greater tet concentrations, with the Weibull model showing the plateau beginning to appear at 1µg/mL. This indicates that concentrations at or slightly above 1 µg/ml will give rise to the maximum percentage of flying adults. The EC_{50} (half maximal effective concentration; the concentration of tet which induces a response halfway between the baseline and maximum) for flying adults is 13 ng/mL (CI 9.6-16.6 ng/mL).

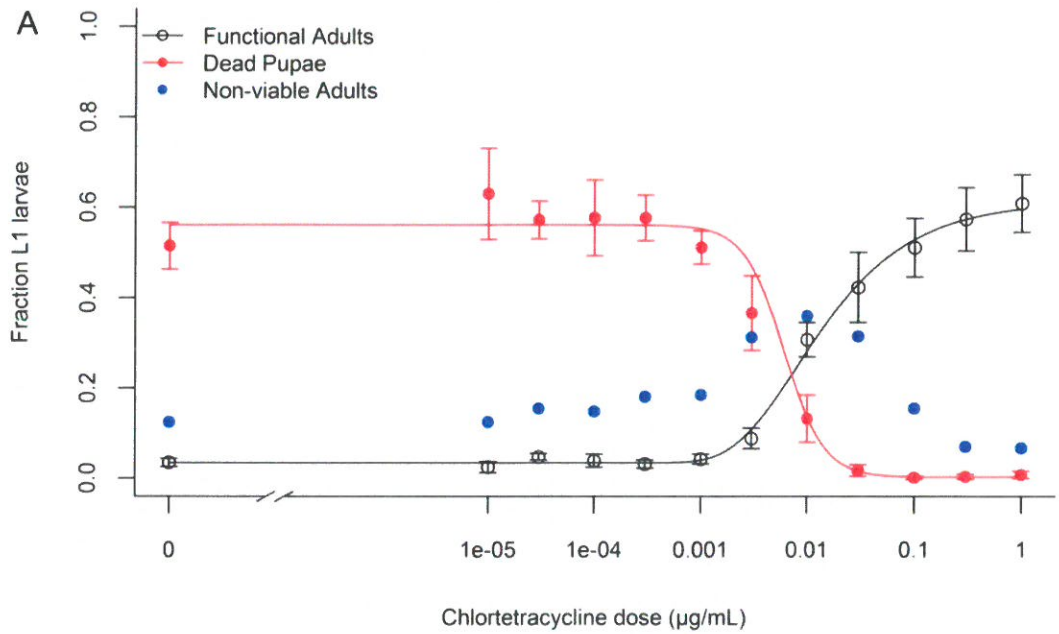

Figure 1: OX513A heterozygous larvae reared at different tetracycline concentrations. Proportion of first instar larvae represents the fraction of individuals reaching the indicated stage based on 200 starting individuals in each repeat. Points represent means from Table 1, and lines represent the model used to calculate the EC_{50} dose. Non-viable adults are not modelled. Error bars represent 95% confidence intervals.

Figure 1 demonstrates that increasing tet concentration increases the fitness of OX513A individuals, as expected. Up to 1 ng/mL, no increase in the proportion of flying adults was found relative to 0 ng/mL. At concentrations above 1ng/mL, where the flying adult percentage begins to deviate from 0 µg/mL tet, the dead pupae fraction begins to decrease but with a corresponding increase in non-viable adults[3]. Continuing towards greater tet concentrations, the non-viable adult percentage peaks at 30ng/mL tet before dropping, shifting towards a greater percentage of flying adults. This demonstrates that tet concentrations above 1ng/mL have incremental rescue effects on OX513A resulting in an average increase in fitness represented by an increased proportion of flying functional adults.

[3] Non-viable adults are a composite of dead adults on the water of the eclosion container, dead adults on the floor of the cage and non-flying adults; all these classes 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 mate, hosts and oviposition sites.

14. **Discussion and Conclusions:**

Determining the lowest concentration of tet which allows greater than the nominal (0 ng/mL tet) percentage of functional adults allows an assessment of the likelihood that larvae will develop in habitats containing tet at or above this concentration, and also the consequence in terms of increased average fitness if they do, e.g. in terms of the numbers of potential functional females expected to emerge, as well as the efficiency and speed of suppression of the *Ae. aegypti* population. Reported maximum concentrations of tet, sampled from field sites around the world, were recorded as 110 pg/mL to 970 pg/mL (e.g. Le-Minh, Khan *et al.* 2010 Locatelli *et al* 2011, Brown *et al* 2006, McQuillan *et al* 2002). The experiments reported here have shown that tet concentrations at and below 1 ng/mL do not increase the fitness of OX513A larvae, i.e. do not increase the proportion of functional adults. The overall mean functional adult number of OX513A reared with no effect from the tet (concentrations 0 to 1ng/mL) was 3.7% (CI 3.24%-4.18%).

Taken together with data from the literature regarding environmental presence of tetracycline's, the data reported here show that OX513A larvae will not encounter tet concentrations in the environment high enough to allow >5% functional adults. We conclude that it is highly unlikely that the safety and efficacy of a control programme would be compromised by reported levels of environmental contamination with tetracycline.

15. **Literature:**

Brown, K.D., Kulis, J., Thomson, B., Chapman, T.H., Mawhinney, D.B. (2006) Occurrence of antibiotics in hospital, residential and dairy effluent, municipal wastewater, and Rio Grande in New Mexico. *Science of the Total Environment* 366: 772-783

McQuillan, D., Hopkins, S., Chapman, T., Sherrell, K., Mills, D. (2002) Drug residues in ambient water: initial surveillance in New Mexico, USA 7th Annual New Mexico Enviromental Health Conference, Alburquerque, New Mexico.

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.

Le-Minh, N., S. J. Khan, et al. (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).

Appendix D

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): Z Curtis	**Study Supervisor (Signature):** PP
Study Coordinator (Name And Position): Zoe Curtis, Research Scientist	**Study Supervisor (Name And Position):** Luke Alphey, Ph.D., Chief Scientific Officer
Date Signed: 25 Feb 2016	**Date Signed:** 25TH FEB 2016

7. Associated Personnel:

Name	Tasks
Zoe Curtis	Study coordination, experimental design, data collection and analysis, report writing
Sam Spence	Data collection
Derric Nimmo, Ph.D.	Experimental design
Kelly Matzen, Ph.D.	Experimental design, data analysis, report writing
Luke Alphey, Ph.D.	Experimental design, approval
Camilla Beech	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.

Richardson, K., Hoffmann, A.A., Johnson, P., Ritchie, S. and Kearney, M.R. (2011) Thermal Sensitivity of *Aedes aegypti* from Australia: empirical data and prediction of effects on distribution. Journal of Medical Entomology 48 (4), 914-923

Appendix E

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 F

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): Kelly Matzen, Ph.D. Senior Scientist	**Study Supervisor (Name And Position):** Luke Alphey, Ph.D. Chief Scientific Officer
Date Signed: 25 Feb 2016	**Date Signed:** 25 FEB 2016

7. **Associated Personnel:**

Name	Tasks
Kelly Matzen, Ph.D.	Study coordination, experimental design, data collection and analysis, report writing
Marco Neira, Ph.D.	Experimental design, approval
Luke Alphey, Ph. D.	Experimental design, report editing, study supervisor and approval
Heather Haines	Data collection
Camilla Beech	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.

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[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.

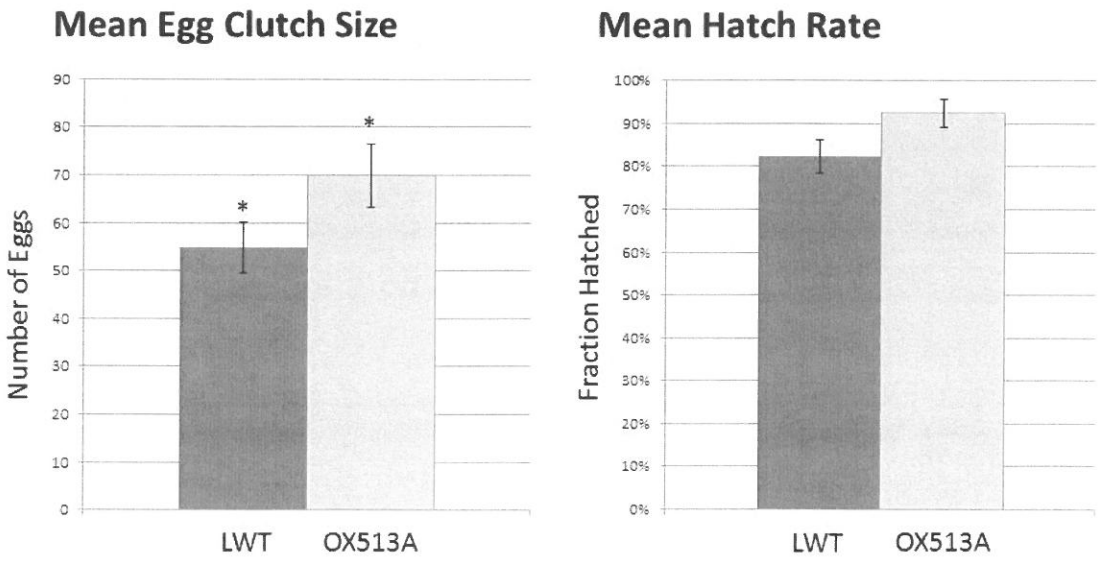

Figure 1: Fecundity study results. * p=0.001. The 95% confidence interval is shown for egg clutch size, and the standard error of the mean for hatch rate.

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).

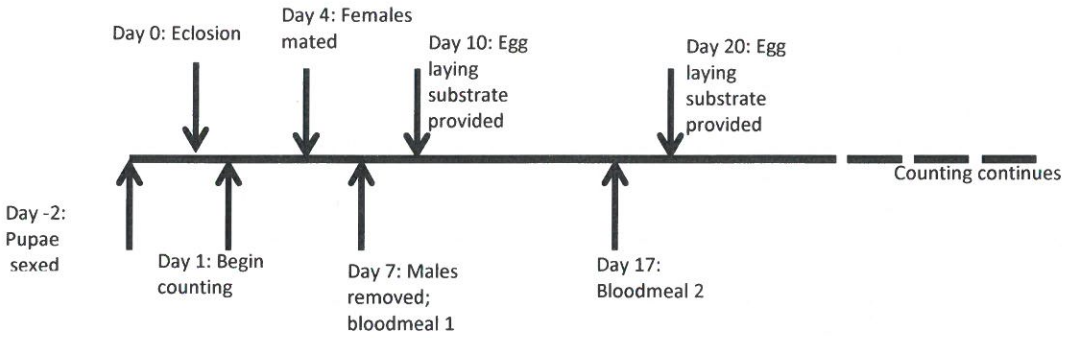

Figure 2: SFFigure 2: Schematic of female longevity experiments. Males were allowed to eclose and then counted each day, starting on Day 1.

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.

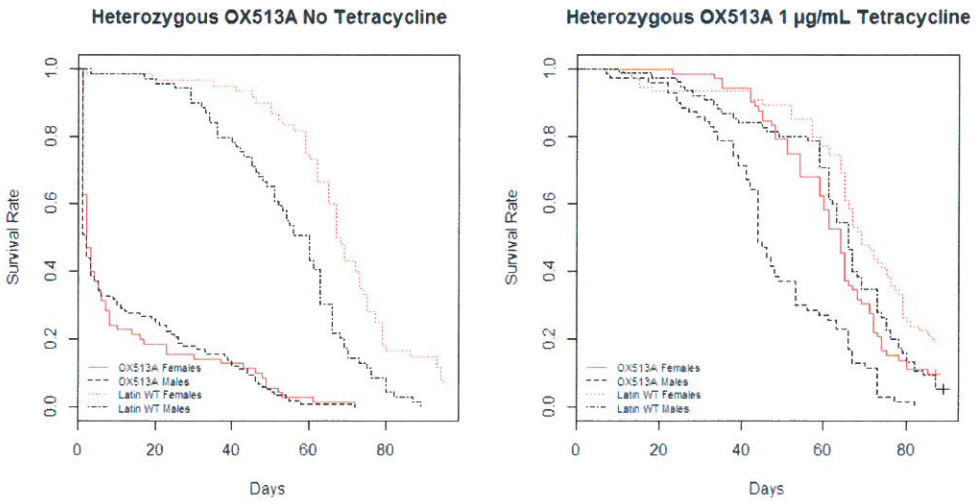

Figure 3: Survival curves for heterozygous OX513A reared without tetracycline or with 1 µg/mL in the larval rearing water.

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.

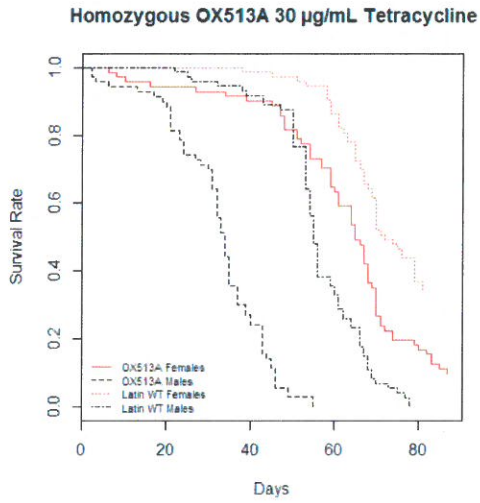

Figure 4: Survival of homozygous OX513A individuals that were reared with 30 µg/mL tetracycline compared to LWT reared under the same conditions.

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:**

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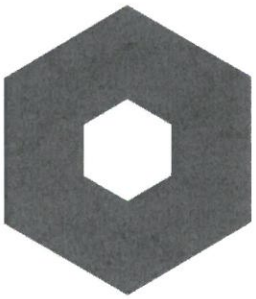
Appendix G



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): PP	**Study Supervisor (Signature):** PP
Study Coordinator (Name And Position): Marco Neira, Ph.D. Senior Scientist	**Study Supervisor (Name And Position):** Luke Alphey, Ph.D. Chief Scientific Officer
Date Signed: 21 APRIL 2015	**Date Signed:** 21 April 2015

7. Associated Personnel:

Name	Tasks
Marco Neira, Ph.D.	Study coordination, experimental design, data analysis, report writing
Luke Alphey, Ph.D.	Experimental design, report writing and approval
Pamela Baker	Insectary work, data collection, report writing
Kelly Matzen, Ph.D.	Insectary work, data collection, statistical analysis
Tim Harvey-Samuel	Statistical analysis
Heather Haines	Insectary work
Camilla Beech	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.

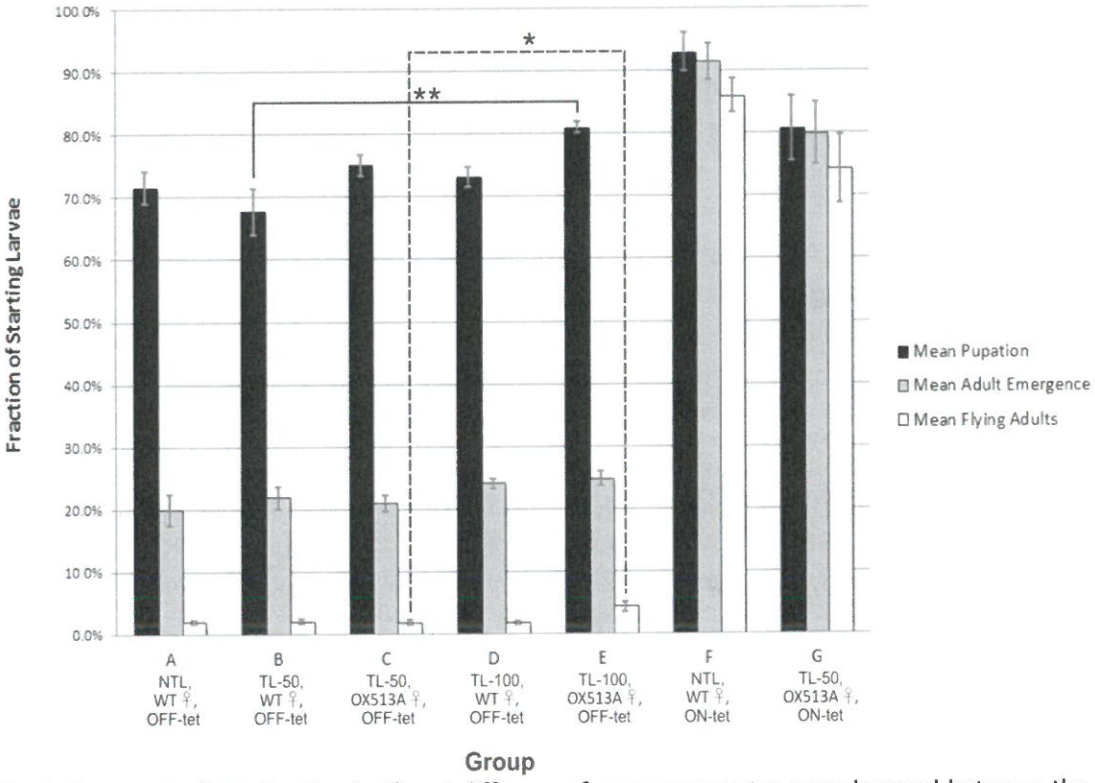


Fig. 1. **Summary of results**. No significant difference for any parameter was observed between the non tet-loaded control group (A) and any of the treatment groups (B-E). Significant differences were only observed in pupation between groups B and E ($p<0.01$), and in the number of flying adults between 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:

Agwuh, K.N., and MacGowan, A. (2006). Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines. The Journal of antimicrobial chemotherapy 58, 256-265.

Bimazubute, M., Cambier, C., Baert, K., Vanbelle, S., Chiap, P., and Gustin, P. (2011). Penetration of oxytetracycline into the nasal secretions and relationship between nasal secretions and plasma oxytetracycline concentrations after oral and intramuscular administration in healthy pigs. Journal of veterinary pharmacology and therapeutics 34, 176-183.

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.

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.

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.

Appendix H

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

SynTech Research France S.A.S.
613 Route du Bois de Loyse
F-71570 La Chapelle de Guinchay
France

TEST SITE

SynTech Research France S.A.S.
1095 Chemin du Bachas
F-30000 Nîmes
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

TABLE OF CONTENTS

LIST OF TABLES

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: Poeciliidae)		
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 03 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

Certifient 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 - DGALN, Service de l'industrie, bureau de la chimie - 12, rue Vellor - 75732 Paris cedex 12
Téléphone : 01 53 44 96 10 Télécopie : 01 53 44 91 72

MINISTÈRE DE L'ÉCOLOGIE DU DÉVELOPPEMENT DURABLES ET DE L'INDUSTRIE

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 I

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).

James JM, Crespo JF (2007) Allergic reactions to foods by inhalation. Curr. Allergy Asthma Rep. 7, 167-174.

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 J

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 QGAEPAFLFGLELIICGLEKQLKCESGSGPAVSRARTKNNYGSTIEGLLDLPDDDAPEEA 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.

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7.0 Appendix 1, AllergenOnline Database, version 13, February, 2013 (see attached PDF).

Appendix K

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
Camilla Beech	Oxitec Ltd, UK	Study Sponsor, report editing and approval
Kelly Matzen	Oxitec Ltd, UK	Responsible for insect rearing and saliva extraction
Sian Spinner	Oxitec Ltd, UK	Insect rearing and saliva extraction
Gwilym Phillips	Oxitec Ltd, UK	Insect rearing and saliva extraction
Pam Gray	Oxitec Ltd, UK	Insect rearing and saliva extraction
Tarig Dafaalla	Oxitec Ltd, UK	Study co-ordination, experimental design, data collection
Stephen Joyce	Oxitec Ltd, UK	Data collection, report editing
Lorraine Tomlin	Oxitec Ltd, UK	QMS support, report writing
Simon Warner	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.

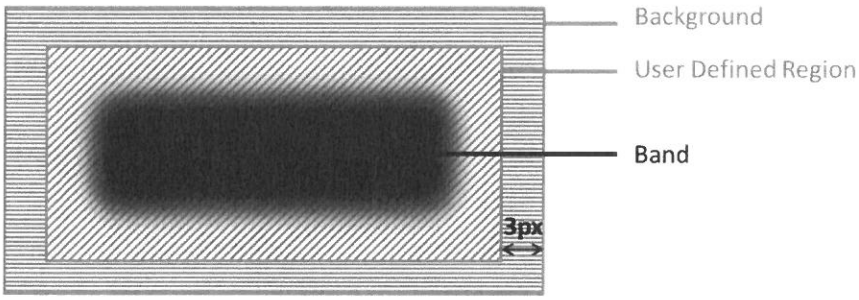

Figure 1a: Schematic representation of a 'UDR' (diagonal stripes), the 'Background' which is a perimeter 3 pixels wide surrounding the 'UDR' (horizontal stripes) and a 'Band' within the 'UDR'. 3px abbreviation denotes the three pixel perimeter.

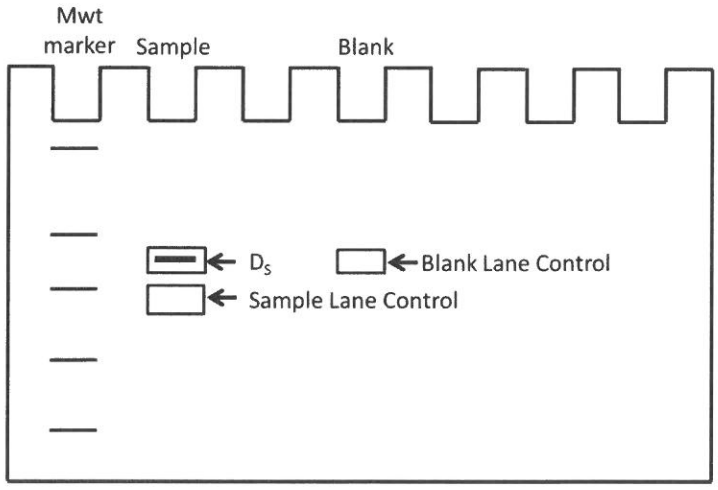

Figure 1b: Diagram of a gel showing how a user defined the UDR's which will give the Total Densities for the Band (D_S), a Sample Lane Control (D_{SLC}) and the Blank Lane Control (D_{BLC}).

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

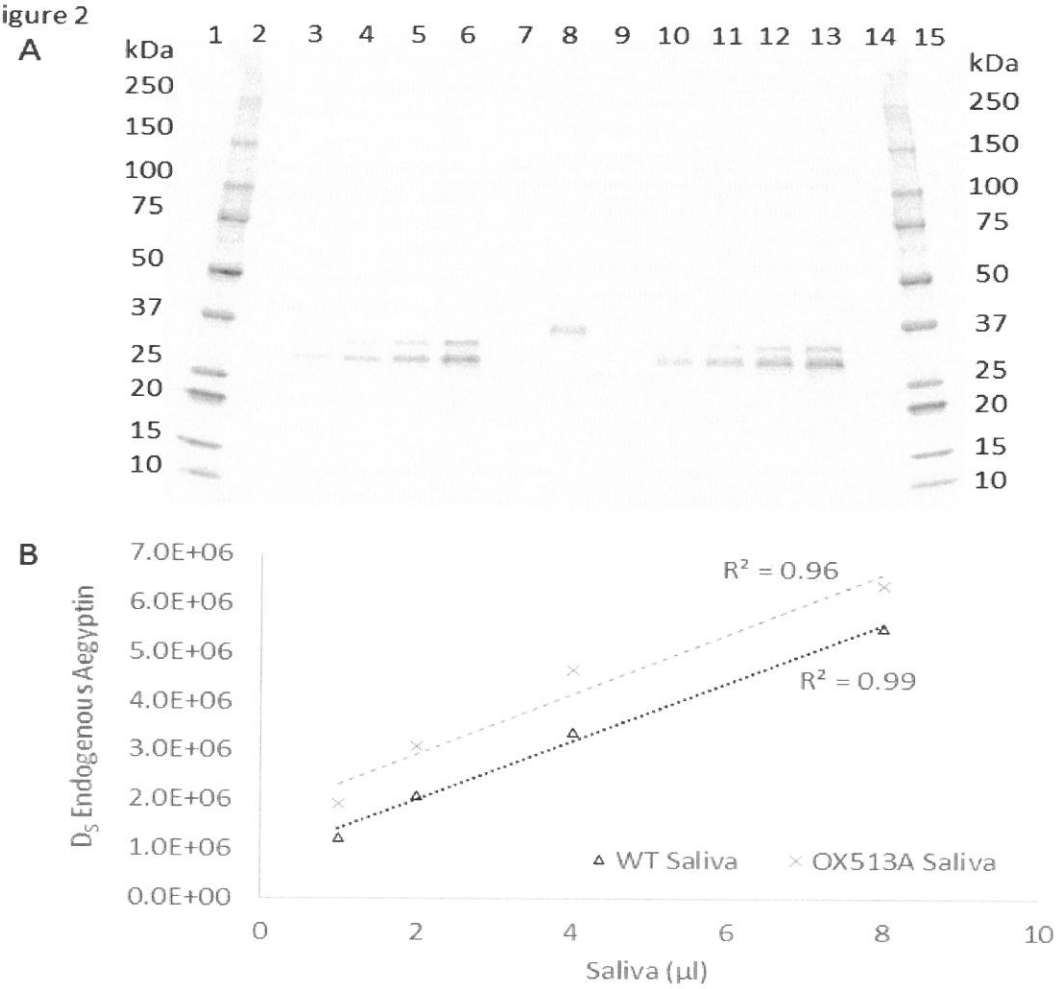

Figure 2: Western blot analysis demonstrating equivalence of endogenous Aegyptin signals in OX513A and WT Saliva

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

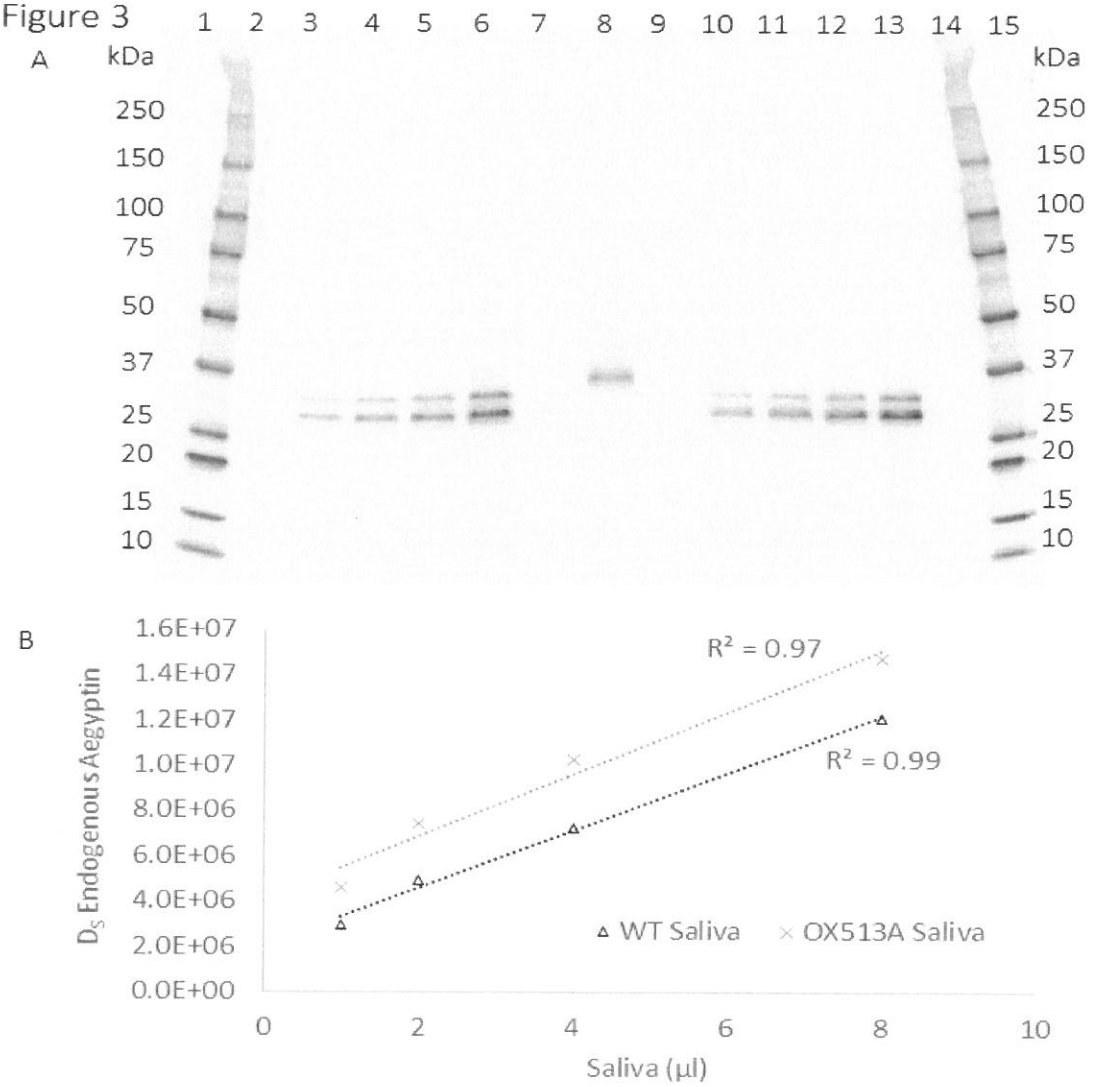

Figure 3: Western blot analysis demonstrating equivalence of endogenous Aegyptin signals in OX513A and WT Saliva (Replicate)

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

TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

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

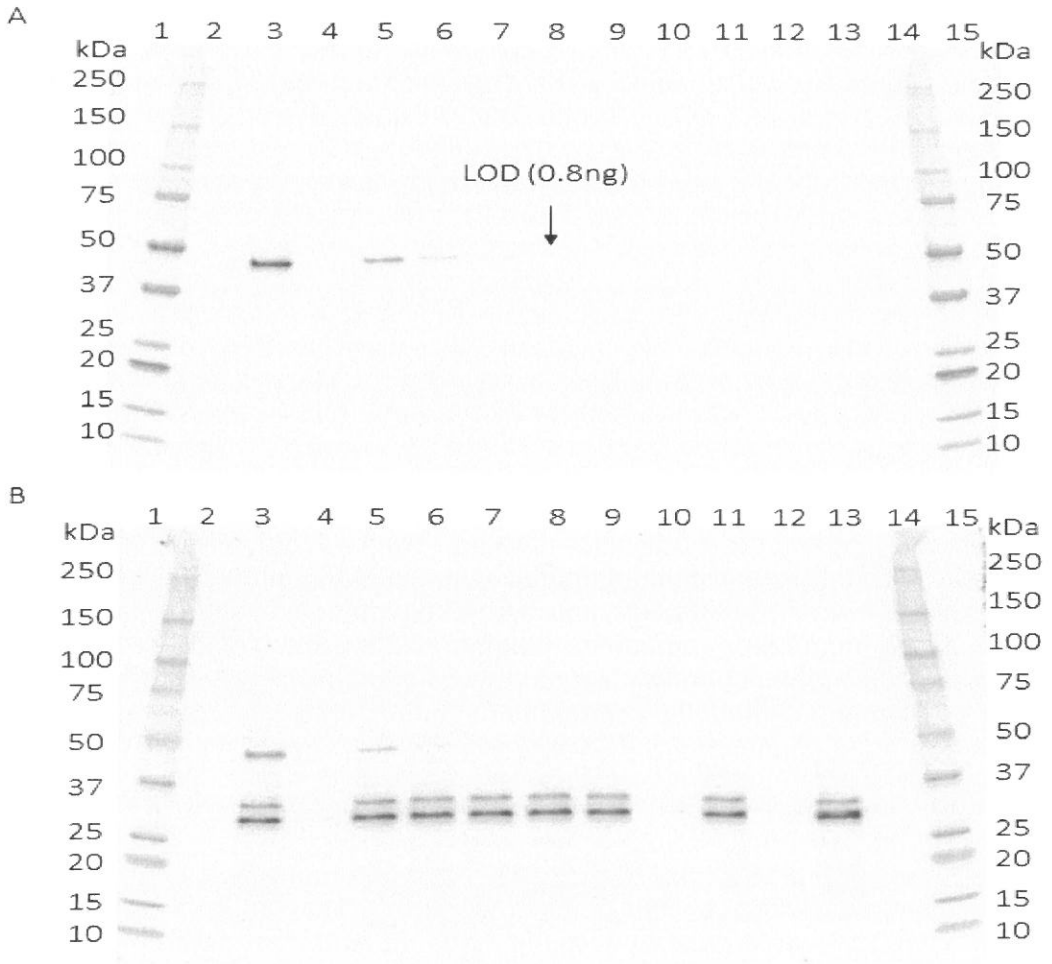

Figure 4: tTAV protein cannot be detected in OX513A saliva using western blot analysis

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

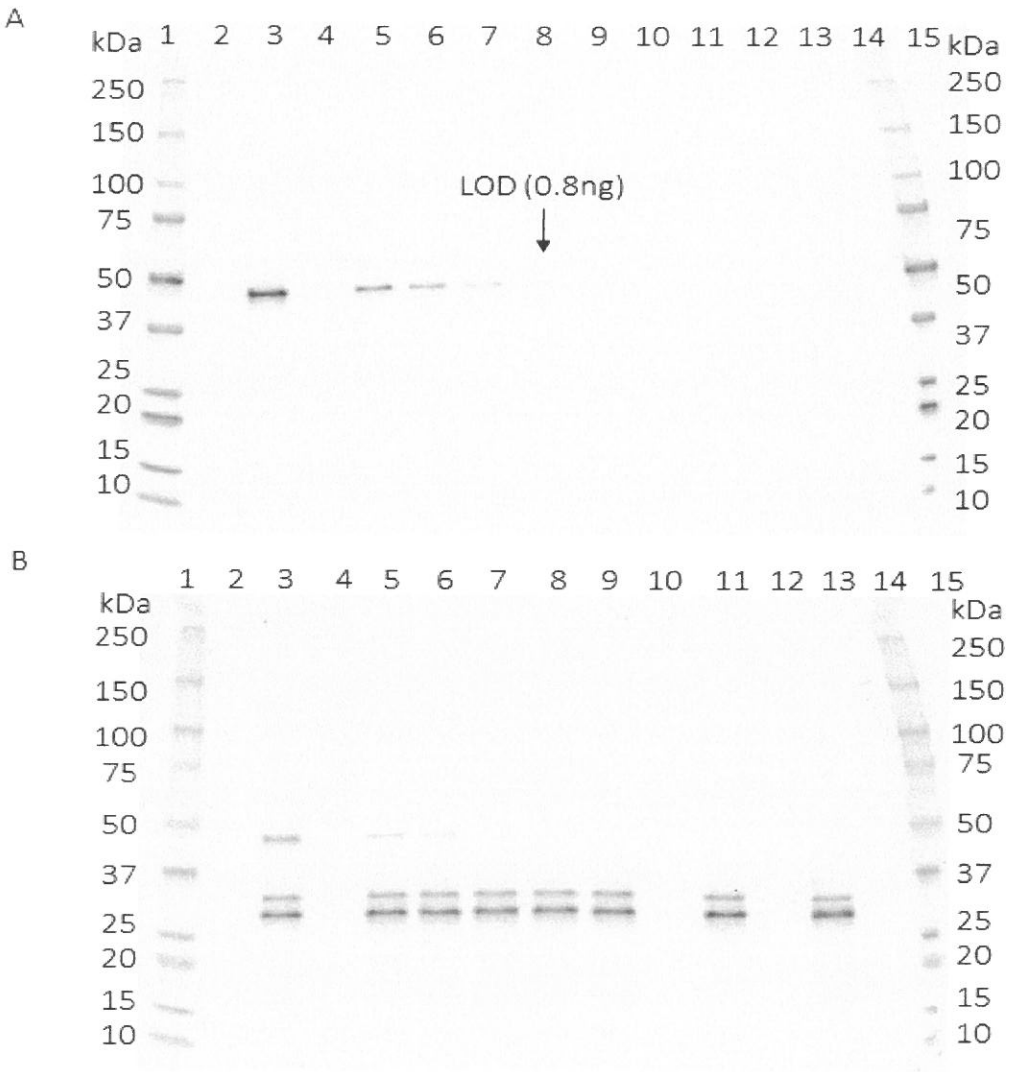

Figure 5: tTAV protein cannot be detected in OX513A saliva using western blot analysis (Replicate)

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

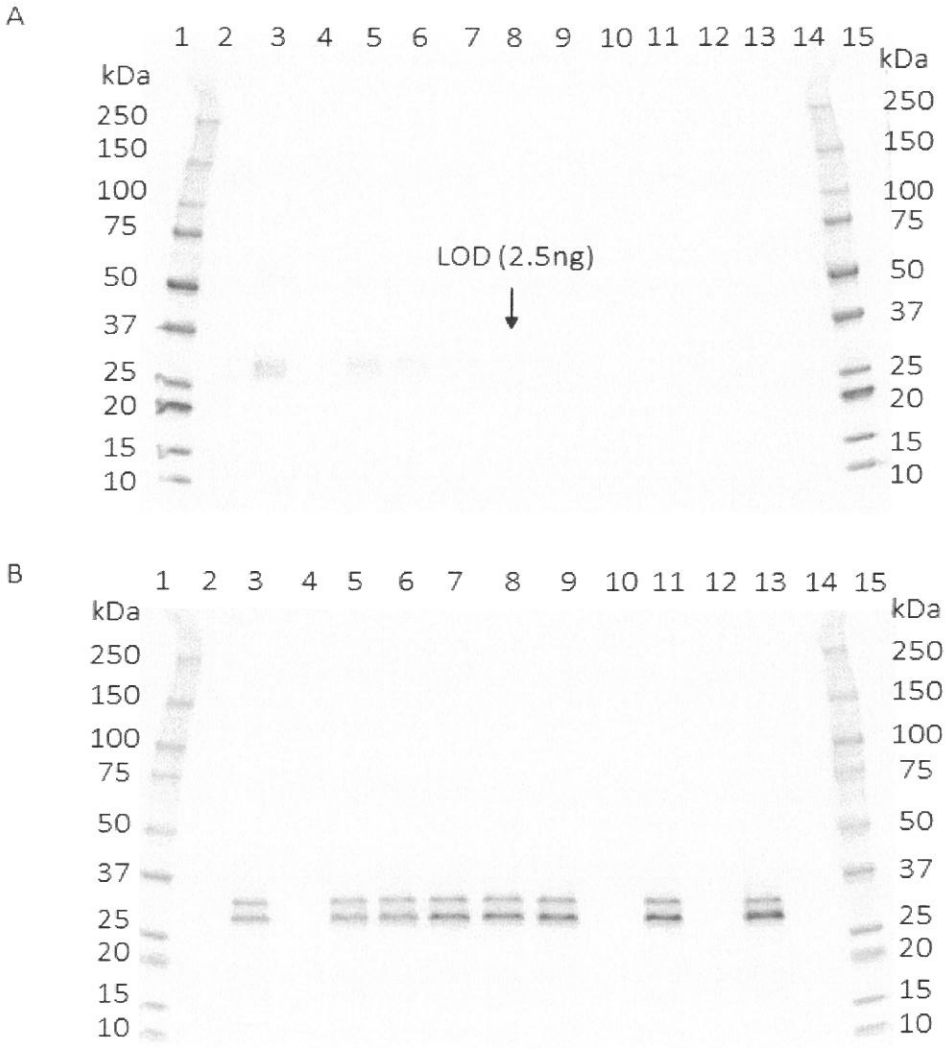

Figure 6: DsRed2 protein cannot be detected in OX513A saliva using western blot analysis

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

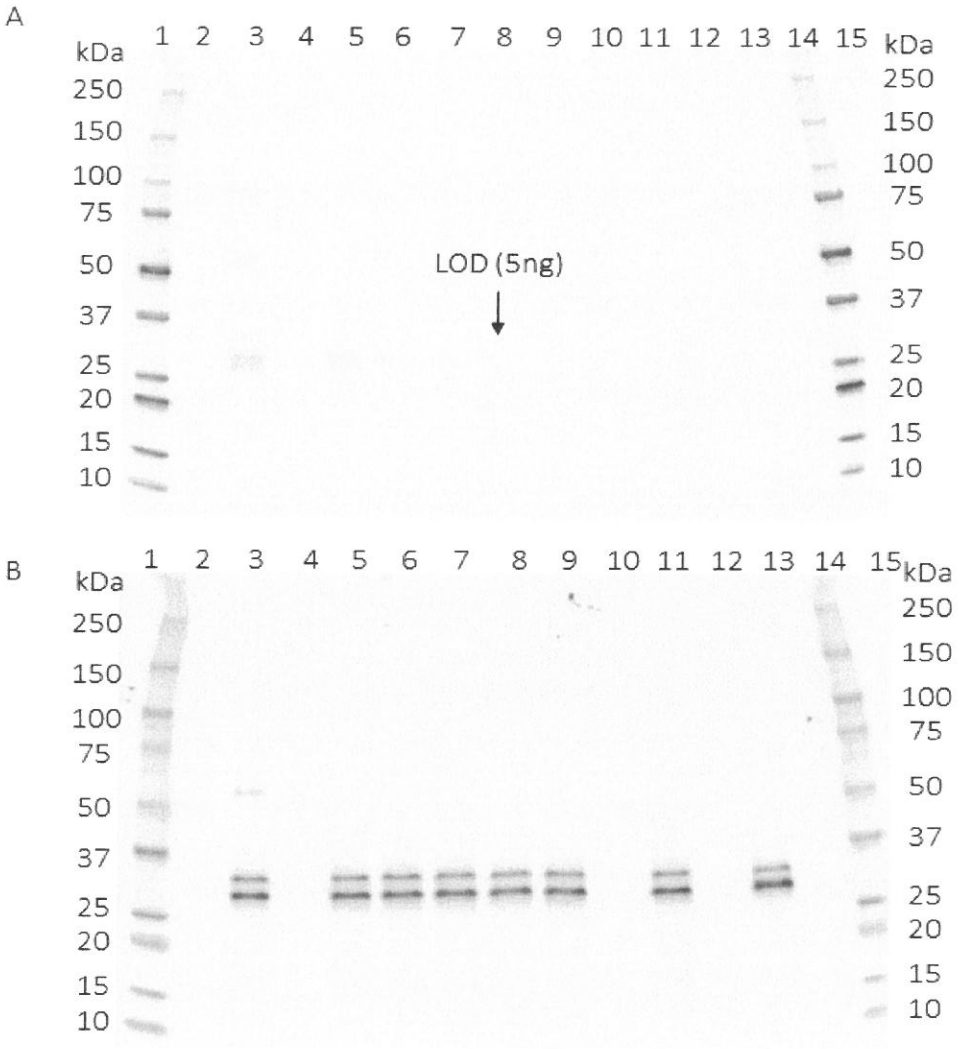

Figure 7: DsRed2 protein cannot be detected in OX513A saliva using western blot analysis (Replicate)

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

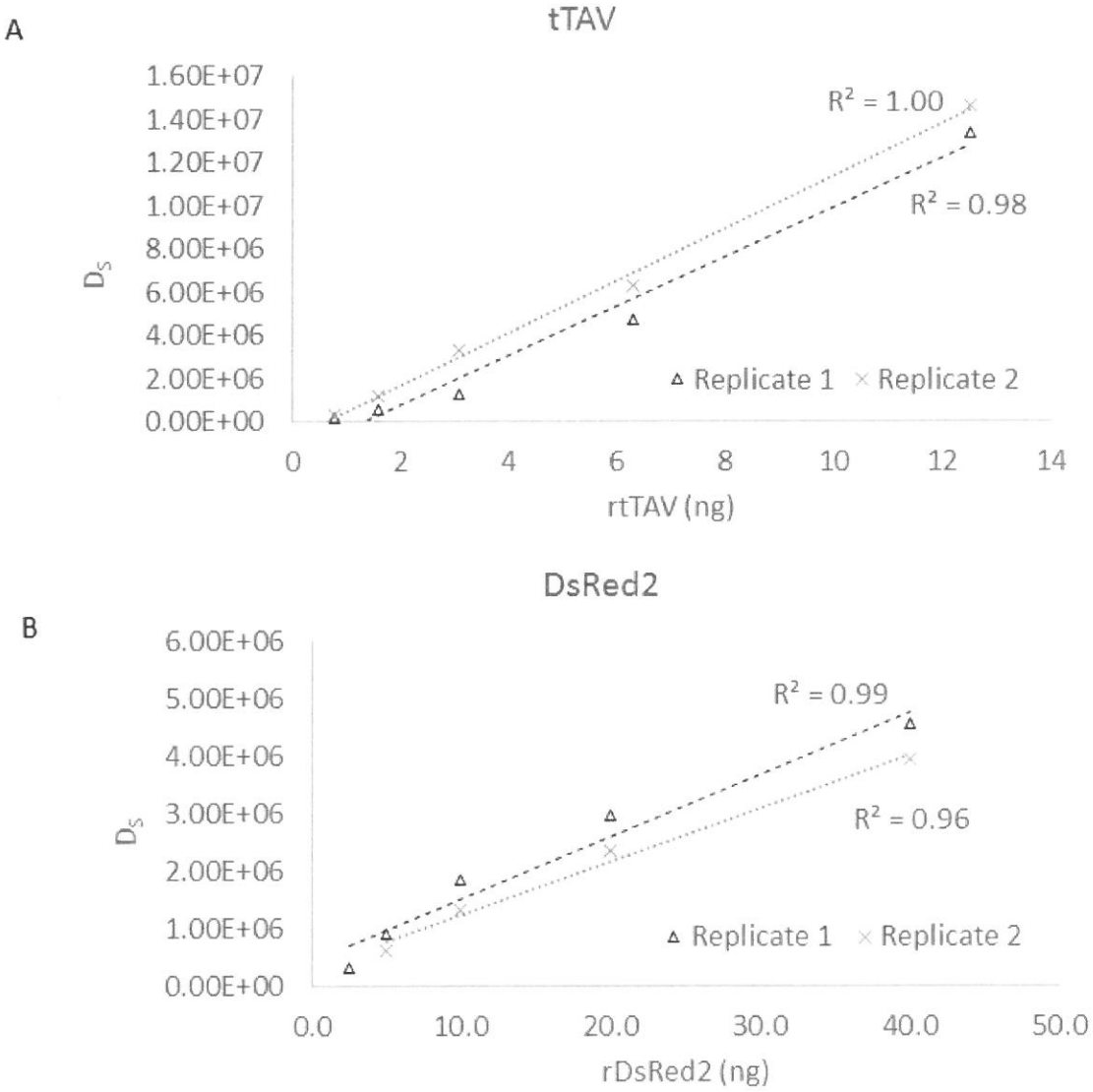

Figure 8: rtTAV and rDsRed2 signals fall within the predictable, linear range for the detection method
D_S values for rtTAV and rDsRed signals from replicate blots for the detection of (A) tTAV and (B) DsRed2 proteins in OX513A saliva were calculated using VisionWorks LS Acquisition and Analysis Software (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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TITLE: Study report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins [CBI deleted]

[5]VisionWorks® LS Image Acquisition and Analysis Software, Installation and User Manual, UVP. ftp://uvp.com/pub/TechSupport/VisionWorks%20PDF%20Manual/UVP%20LS%20Software%20Manual.pdf

[6]SR-00003. Study Report: tTAV Expression and Purification.

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[9]SR-00001. Study Protocol: tTAV Expression and Purification.

[10]SP_00002. Study Protocol: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the detection of tTAV and DsRed2 Proteins.

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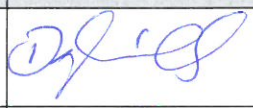
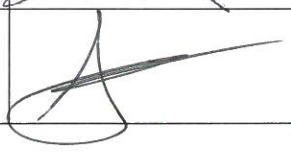
16 APPROVALS

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
Tarig Dafaalla	Senior Scientist, Study Co-ordinator		5 Aug 2015
Lorraine Tomlin	Senior Quality Systems Manager, Report Author		05 AUG 2015
Camilla Beech	Head of Regulatory Affairs, Study Sponsor		5 Aug 2015
Simon Warner	Chief Scientific Officer		5 Aug 2015