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Environmental Assessment for Investigational Use of *Aedes aegypti* OX513A

In support of a proposed field trial of genetically engineered (GE) male *Ae. aegypti* mosquitoes of the line OX513A in Key Haven, Monroe County, Florida under an investigational new animal drug exemption

Month XX, 2016

Prepared by

Center for Veterinary Medicine

United States Food and Drug Administration

Department of Health and Human Services

1 Table of Contents

[TOC \o "1-5" \h \z \u]

Commented [LE1]: Change use of "strain" to "line" throughout.

2 List of Figures

[TOC \h \z \c "Figure"]

3 List of Tables

[TOC \h \z \c "Table"]

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 thuringiensis*
Bti	*Bacillus thuringiensis israelensis*
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
EIP	External incubation period
EST	Expressed Sequence Tag
FAO	Food and Agriculture Organization
FARRP	Food Allergy Research and Resource Program
FASTA	Fast-ALL (DNA and protein sequence format)
FDA	U.S. Food and Drug Administration
FEMA	Federal Emergency Management Agency

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FL	Florida
FL DOH	Florida Department of Health
Ft	feet
KH	Key Haven, Florida
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

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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, U.S. ~~Government~~Office of the Executive
PCR	Polymerase chain reaction
PCT	Patent Cooperation Treaty Countries (148 countries worldwide)
RNA	Ribonucleic ~~acid~~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~~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~~ Comparator Area
UK	United Kingdom
ULV	Ultra-low ~~volume~~Volume
USDA	United States Department of Agriculture
USFWS	United States Fish and Wildlife Service
U.S.	United States of America
VP16	~~Activator sequence~~Viral Protein 16 (tegument protein also called Alpha-TIF from Herpes ~~simplex~~ Simplex ~~virus~~Virus1)
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 ~~line 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

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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 (line ~~strain~~ OX513A), expressing a conditional lethality trait and a fluorescent marker. The ~~strain~~ line was first constructed in 2002, and a publication about it in a peer-reviewed scientific journal in 2007 [ADDIN EN.CITE ADDIN EN.CITE.DATA]. 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.

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The purpose of this proposed 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 recombinant DNA (rDNA) construct, and to determine the efficacy of sustained releases of OX513A mosquitoes for the suppression of a local population of *Ae. aegypti* in the defined release area in Florida Keys, specifically an area known as Key Haven, in Monroe County, which is within the jurisdiction of the Florida Keys Mosquito Control District for mosquito control.

Released adult OX513A mosquitoes are homozygous for an ~~recombinant DNA (~~rDNA~~)~~ construct that confers both late-acting lethality to the ~~strain~~ line 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~~ this 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 and exhibit the associated lethal phenotype). Eggs for the proposed field trial would be ~~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 would be ~~are~~ hatched and reared to pupae, which would be ~~are~~ sorted mechanically to ensure accuracy of the sorting does not exceed a maximum of- 0.2% females ~~>99.9% efficacy (Carvalho et al., 2014; Harris et al., 2013)~~[ADDIN EN.CITE ADDIN EN.CITE.DATA] using the difference in size between male and female pupae (sexual dimorphism). Males, which do not bite, blood feed, or transmit disease, would be ~~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~~

[1] Penetrance is the ~~extent to~~ proportion of the population that~~which~~ carries the conferred trait ~~is present in the resulting population~~and exhibits the phenotype associated with this trait. 95% penetrance means that 95% of the population with the gene (in this case tTAV) also expresses the introduced trait (i.e. also has the tTAV-associated lethality phenotype).

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~~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 suppression 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~~ As a framework for this environmental assessment, we have developed several risk-related questions listed below~~it did?", has been conducted to address the following questions~~:

- What is the likelihood ~~offer~~ inadvertent release of OX513A mosquitoes outside of the proposed trial site~~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 offer~~ establishment of OX513A mosquitoes at the proposed trial site?
- What is the likelihood ~~that OX513A *Ae. aegypti* can reproduce and establish in the environment into which they are released~~of dispersal ~~offer~~ OX513A mosquitoes and their progeny from the proposed trial site?
- What is the likelihood that the rDNA construct could be transferred to humans or other organisms?
- What is the likelihood that release of OX513A mosquitoes would have adverse effects on non-target species at the proposed site?
- What is the likelihood that the rDNA expression products in OX513A mosquitoes would have adverse effects on humans or other animals?
- ~~What are the potential impacts of OX513A *Ae. aegypti* in the environment, including on humans?~~
- What are the likely consequences to, or effects on the environment of the United States associated with the investigational use of OX513A mosquitoes~~for the surrounding environment, should OX513A survive and establish in the environment~~?

In risk assessment, risk (R) may be defined as the joint probability of exposure [P(E)] and the conditional probability of harm (i.e., adverse effects) given that the exposure to a hazard has occurred [P(H|E)]: Risk = P(E) x P(H|E) or Risk = Exposure x Adverse Effect. ~~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-related questions are based on semi-field and field studies, laboratory studies, and published literature.

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The likelihood of escape, survival, and establishment of OX513A would be~~is~~ highly unlikely due to a combination of physical, geophysical, geographic, and biological measures that would be~~are~~ in place during egg production, transport, local rearing, and release. Physical measures would 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 would be~~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 would be ~~is~~ provided by the island location of the proposed release site, which ~~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 would be~~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 not survive to functional adulthood~~die~~ in the absence of tetracycline [ADDIN EN.CITE <EndNote><Cite><Author>Harris</Author><Year>2011</Year><RecNum>92</RecNum><DisplayText>(Harris et al. 2011)</DisplayText><record><rec-number>92</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1455908312">92</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Harris, A. F.</author><author>Nimmo, D.</author><author>McKemey, A. R.</author><author>Kelly, N.</author><author>Scaife, S.</author><author>Donnelly, C. A.</author><author>Beech, C.</author><author>Petrie, W. D.</author><author>Alphey, L.</author></authors></contributors><auth-address>Mosquito Research and Control Unit (MRCU), Grand Cayman, Cayman Islands.</auth-address><titles><title>Field performance of engineered male mosquitoes</title><secondary-title>Nat Biotechnol</secondary-title></titles><periodical><full-title>Nat Biotechnol</full-title></periodical><pages>1034-7</pages><volume>29</volume><number>11</number><keywords><keyword>Aedes/*genetics/virology</keyword><keyword>Animals</keyword><keyword>Animals, Genetically Modified/*genetics</keyword><keyword>Arboviruses/genetics/physiology</keyword><keyword>Dengue/*prevention & control</keyword><keyword>*Dengue Virus</keyword><keyword>Female</keyword><keyword>Humans</keyword><keyword>Infertility, Male/*genetics</keyword><keyword>Male</keyword><keyword>Pest Control, Biological/*methods</keyword><keyword>Reproduction/genetics/physiology</keyword><keyword>Sexual Behavior, Animal</keyword></keywords><dates><year>2011</year><pub-dates><date>Nov</date></pub-dates></dates><isbn>1546-1696 (Electronic)
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[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 [HYPERLINK "http://www.astmh.org/AM/Template.cfm?Section=ACME&Template=/CM/ContentDisplay.cfm&ContentID=1444"][HYPERLINK "http://www.astmh.org/subgroups/acme#arthropod"] (Accessed June 21, 2016).

resource-num>10.1038/nbt.2019</electronic-resource-num></record></Cite></EndNote>], 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 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~~ line OX513A for investigational use in Key Haven, Florida would be~~is~~ unlikely to result in adverse effects on the environment or human health.

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8 Purpose and Need

~~Purpose and Need~~

The U.S. Food and Drug Administration (FDA)'s Center for Veterinary Medicine (CVM, we) has received a proposal for Oxitec's proposed field trial of genetically engineered (GE) male *Ae. aegypti* mosquitoes of the ~~strain~~ line 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 associated with: Zika ~~virus~~, dengue ~~fever~~, and chikungunya viruses. OX513A have been genetically engineered to express a gene that encodes a conditional or repressible lethality trait (also known as self-limiting) (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 would~~ill~~ be carried out in conjunction with the Florida Keys Mosquito Control District (FKMCD) to evaluate the use of male *Ae. aegypti* OX513A ~~strain~~ line 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 eggs from the OX513A line of *Ae. aegypti* mosquitos for a study in Key Haven, Monroe County, Florida~~i~~. In conjunction with the FKMCD, Oxitec ~~is~~ proposes~~ianning~~ 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).

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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[3]. 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. Thus far in 2016, the Florida Department of Health has confirmed one locally-acquired case of dengue in a visitor to Key West, Monroe County.[4] 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 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, including (the Cayman Islands [ADDIN EN.CITE ADDIN EN.CITE.DATA]; ~~and~~ Brazil [ADDIN EN.CITE <EndNote><Cite><Author>Carvalho</Author><Year>2015</Year><RecNum>60</RecNum><DisplayText>(Carvalho et al. 2015)</DisplayText><record><rec-number>60</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1445973125">60</key></foreign-keys><ref-type name="Journal Article">17</ref-

[3] [HYPERLINK "http://www.doh.state.fl.us/Environment/medicine/arboviral/surveillance.htm"] [Accessed June 20, 2016].

[4] [HYPERLINK "http://monroe.floridahealth.gov/newsroom/2016/06/160601-Dengue.html"] [Accessed June 15, 2016].

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

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type><contributors><authors><author>Carvalho, Danilo O.</author><author>McKemey, A.</author><author>Garziera, L.</author><author>Lacroix, R.</author><author>Donnelly, Christl A.</author><author>Alphey, L.</author><author>Malavasi, A.</author><author>Capurro, Margareth L.</author></authors></contributors><titles><title>Supression of a field population of Aedes aegypti in Brazil by sustained release of transgenic male mosquitoes.</title><secondary-title>PLoS Neglected Tropical Diseases</secondary-title></titles><periodical><full-title>PLoS Neglected Tropical Diseases</full-title></periodical><pages>e0003864</pages><volume>9</volume><number>7</number><dates><year>2015</year></dates><urls></urls></record></Cite></EndNote>], where ~~including~~ the Brazilian National Biosafety Commission (CTNBio) determined in 2014 that the Oxitec OX513A mosquito is safe for use in Brazil~~an approval for commercial scale use in Brazil~~[6], and Panama [ADDIN EN.CITE <EndNote><Cite><Author>Gorman</Author><Year>2016</Year><RecNum>249</RecNum><DisplayText>(Gorman et al. 2016)</DisplayText><record><rec-number>249</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1466182262">249</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Gorman, K.</author><author>Young, J.</author><author>Pineda, L.</author><author>Marquez, R.</author><author>Sosa, N.</author><author>Bernal, D.</author><author>Torres, R.</author><author>Soto, Y.</author><author>Lacroix, R.</author><author>Naish, N.</author><author>Kaiser, P.</author><author>Tepedino, K.</author><author>Philips, G.</author><author>Kosmann, C.</author><author>Caceres, L.</author></authors></contributors><auth-address>Oxitec Limited, Abingdon, Oxfordshire, UK.
Gorgas Memorial Institute for Human Health, Ciudad de Panama, Panama.</auth-address><titles><title>Short-term suppression of Aedes aegypti using genetic control does not facilitate Aedes albopictus</title><secondary-title>Pest Manag Sci</secondary-title></titles><periodical><full-title>Pest Manag Sci</full-title></periodical><pages>618-28</pages><volume>72</volume><number>3</number><keywords><keyword>Ox513a</keyword><keyword>Panama</keyword><keyword>chikungunya</keyword><keyword>dengue</keyword><keyword>mosquito</keyword><keyword>transgenic</keyword></keywords><dates><year>2016</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1526-4998 (Electronic)
1526-498X (Linking)</isbn><accession-num>26374668</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/26374668</url></related-urls></urls><electronic-resource-num>10.1002/ps.4151</electronic-resource-num></record></Cite></EndNote>], FKMCD is seeking to assess the utility of the OX513A *Ae. aegypti* mosquito for *Aedes aegypti* vector control in Monroe County.

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[6] [HYPERLINK "http://www.ctnbio.gov.br/index.php/content/view/19522.html"]~~[accessed 14 Jan 2015]~~ http://ctnbio.mcti.gov.br/documents/566529/686098/Thecnical+Report+3964-2014+-+-+Commercial+Release+of+strain+OX513A+of+Aedes+aegypti+-+Process+01200.002919-2013-77/be405f52-a1a5-4c01-97a6-5cd95e058e53 [Aaccessed June 2017, 2016].

Oxitec Ltd. as the Sponsor would conduct the trial in collaboration with FKMCD[7]. This document constitutes the Environmental Assessment (EA) that considers the potential consequences that such an investigational field trial might 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 conduct a field trial in the United States, or they could select another location in the United States to conduct the field trial(s). 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. 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.

In the event of this alternative, the FKMCD would continue to use its existing control measures for the *Ae. aegypti* mosquitoes without also conducting the investigational field trial. (FKMCD will continue its current vector control program whether the field trial proceeds or not.) 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

[7] FKMCD's role in the trial is as a collaborator. They are supplying resources and facilities to Oxitec for the conduct of the field trial.

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products are rotated to avoid prolonged exposure of mosquito larvae to a particular larvicide's 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. aegypti* 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[5] and there is increasing resistance developing to these insecticides [ADDIN EN.CITE <EndNote><Cite><Author>Ranson</Author><Year>2010</Year><RecNum>248</RecNum><DisplayText>(Ranson et al. 2010)</DisplayText><record><rec-number>248</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1466013777">248</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>Ranson, H.</author><author>Burhani, J.</author><author>Lumjuan, N.</author><author>Black IV, W.C.</author></authors></contributors><titles><title>Insecticide resistance in dengue vectors</title><secondary-title>TropIKA</secondary-title></titles><periodical><full-title>TropIKA</full-title></periodical><volume>1</volume><number>1</number><dates><year>2010</year></dates><isbn>2078-8606</isbn><urls><related-urls><url>http://journal.tropika.net/pdf/tropika/v1n1/a03v1n1.pdf</url></related-urls></urls></record></Cite></EndNote>].

[5] [HYPERLINK "http://keysmosquito.org/wp-content/uploads/2015/05/2015-06-23-Reg-Mtg-Minutes.pdf"] [Accessed March 4, 2016]

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 rDNA construct gene from surviving to functional 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-type 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 irradiated insects can reduce the insect population to very low levels. Sterile #Insect tTechniqueIT has been widely used as a successful control tool in plant and animal 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 (tetracycline transcriptional activator variant) 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 tRE DNA in the cell [ADDIN EN.CITE <EndNote><Cite><Author>Gossen</Author><Year>1992</Year><RecNum>12</RecNum><DisplayText>(Gossen and Bujard 1992)</DisplayText><record><rec-number>12</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">12</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Gossen, Manfred</author><author>Bujard, Hermann</author></authors></contributors><titles><title>Tight control of gene expression in mammalian cells by tetracycline-responsive promoters</title><secondary-title>PNAS</secondary-title></titles><periodical><full-title>PNAS</full-title></periodical><pages>5547-5551</pages><volume>89</volume><number>12</number><reprint-edition>Not in File</reprint-edition><dates><year>1992</year><pub-dates><date>1992</date></pub-dates></dates><label>12</label><urls><related-

urls><url>http://www.pnas.org/content/89/12/5547.short</url></related-urls></urls><access-date>3/28/2015</access-date></record></Cite></EndNote>], 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[9]. 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 The 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[10] is an ~~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 [REF _Ref450304935 \h]) 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 [REF _Ref450304935 \h]. 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.

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[9] [HYPERLINK "http://www.tetsystems.com/science-technology/highlighted-publications/"] [Accessed ~~5 June 20 Feb 5,~~ 2016~~5~~].

[10] #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.

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Table [SEQ Table * ARABIC]. Genetic elements, their donor organisms, and function in #OX513.

Genetic Element	Location (bp) in plasmid pOX513	Size (bp)	Originating Donor Organism and	Reference	Function
3' Inverted Terminal Repeat (ITR)	8508-8570	63	*Trichoplusia ni* (Cabbage looper moth)		Short related sequences in reverse orientation at
piggyBac 3'	7524-8507	984	*Trichoplusia ni* (Cabbage looper moth)	[ADDIN EN.CITE ADDIN EN.CITE.DATA]	DNA transposable element with
Non-coding	7484-	40			
Actin5C	4833-7483	2651	*Drosophila melanogaster*		Promoter element driving
Non-coding	4818-	15			
DsRed2	4134-4817	684	*Discosoma* (Coral)	[ADDIN EN.CITE ADDIN EN.CITE.DATA]	Red fluorescent protein marker gene.
Non-coding	4126-	8			
Drosomycin 3' UTR	3340-4125	786	*Drosophila melanogaster*		Terminator region
Non-coding	3301-	39			
tetOx7	3005-3300	296	*Escherichia coli* (bacteria)	[ADDIN EN.CITE <EndNote><Cite><Author>Gossen</Author><Year>1992</Year><RecNum>12</RecNum><DisplayText>(Gossen	Non-coding binding site for
Non-coding	3000-	5			
hsp70	2870-	130	*Drosophila sp.*		Promoter
Non-coding	2858-	12			
adh intron	2788-	70	*Drosophila sp.*		Enhances gene
Non-coding	2780-	8			
tTAV	1766-2779	1014	Synthetic DNA based on a fusion of sequences from *E. coli*	[ADDIN EN.CITE ADDIN EN.CITE.DATA]	Tetracycline repressible transcriptional activator.
Non-coding	1716-	50			
K10 terminator	934-1715	782	*Drosophila sp.* (Vinegar fly)		Terminator region

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Non-coding	830-933	103			
piggyBac 5'	192-829	638	*Trichoplusia ni* (Cabbage	[ADDIN EN.CITE <EndNote><Cite><Author>Cary</Author><Year>1989</Year><RecNum>110</RecNum><DisplayText>(Cary et	DNA transposable
5' ITR	157-191	35	*Trichoplusia ni* (Cabbage looper moth)		Short related sequences in reverse orientation at

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 [ADDIN EN.CITE ADDIN EN.CITE.DATA]. 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 [ADDIN EN.CITE ADDIN EN.CITE.DATA]. [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Arensburger</Author><Year>2011</Year><RecNum>99</RecNum><DisplayTe xt>Arensburger et al. (2011)</DisplayText><record><rec-number>99</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463078554">99</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Arensburger, P.</author><author>Hice, R. H.</author><author>Wright, J. A.</author><author>Craig, N. L.</author><author>Atkinson, P. W.</author></authors></contributors><auth-address>Center for Disease Vector Research, Institute for Integrative Genome Biology, and Department of Entomology, University of California, Riverside, CA 92521, USA.</auth-address><titles><title>The mosquito Aedes aegypti has a large genome size and high transposable element load but contains a low proportion of transposon-specific piRNAs</title><secondary-title>BMC Genomics</secondary-title></titles><periodical><full-title>BMC Genomics</full-title></periodical><pages>606</pages><volume>12</volume><keywords><keyword>Aedes/*genetics</keyword><keyword>Animals</keyword><keyword>*DNA Transposable Elements</keyword><keyword>Gene Silencing</keyword><keyword>*Genome</keyword><keyword>RNA, Small Interfering/*genetics</keyword></keywords><dates><year>2011</year></dates><isbn>1471-2164 (Electronic)
1471-2164 (Linking)</isbn><accession-num>22171608</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/22171608</url></related-urls></urls><custom2>3259105</custom2><electronic-resource-num>10.1186/1471-2164-12-606</electronic-resource-num></record></Cite></EndNote>] 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 see Section [REF _Ref453678163 \r \h]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 [REF _Ref453677804 \r \h]. 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 #OX513 rDNA construct, #OX513A, 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 would use >99.9% male OX513A mosquitoes (sorted to ensure accuracy of the sorting does not exceed a maximum of 0.2% females) 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 (Section [REF _Ref453331762 \r \h]).

9.2.3 Production of the OX513A strain line

StrainThe OX513A line was produced in 2002 [ADDIN EN.CITE ADDIN EN.CITE.DATA] by microinjecting the #OX513 rDNA construct with a transposase helper plasmid (#265) into individual embryos of *Ae. aegypti* from a Rockefeller strain background ([REF _Ref450305641 \h]). 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.

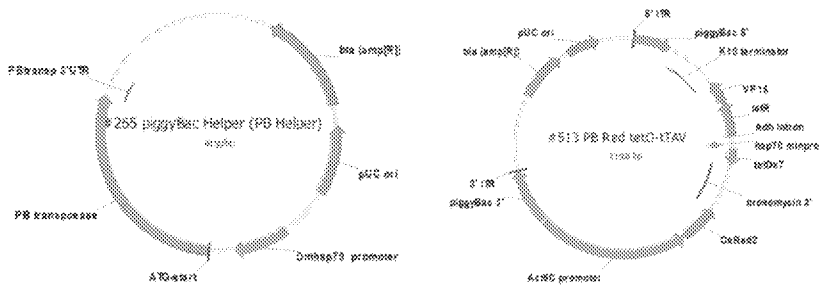

Figure [SEQ Figure * ARABIC]. Map of the vector plasmid pOX513 and the helper plasmid #265.

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 line~~strain~~ designated LA513A in the paper describing transformation [ADDIN EN.CITE ADDIN EN.CITE.DATA] 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 line~~strain~~ has been maintained in culture at Oxitec 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~~ line 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~~ line has been maintained by Oxitec~~Ltd.~~ in a continuously cycling insect colony for the equivalent of over 100 generations.

9.2.4 Molecular characterization ~~of the Band genetic stability of~~ OX513 ~~rDNA constructA~~

Inverse PCR has been used to identify the genomic sequence adjacent to the insertion site of OX513A according to the method of [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Handler</Author><Year>1998</Year><RecNum>139</RecNum><DisplayText>Handler et al. (1998)</DisplayText><record><rec-number>139</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5"

timestamp="1463106826">139</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Handler, Alfred M.</author><author>McCombs, Susan D.</author><author>Fraser, Malcolm J.</author><author>Saul, Stephen H.</author></authors></contributors><titles><title>The lepidopteran transposon vector, piggyBac, mediates germ-line transformation in the Mediterranean fruit fly</title><secondary-title>Proceedings of the National Academy of Sciences</secondary-title></titles><periodical><full-title>Proceedings of the National Academy of Sciences</full-title></periodical><pages>7520-7525</pages><volume>95</volume><number>13</number><dates><year>1998</year><pub-dates><date>1998</date></pub-dates></dates><urls><related-urls><url>http://www.pnas.org/content/95/13/7520.short</url><url>http://www.pnas.org/content/95/13/7520.full.pdf</url></related-urls></urls><remote-database-provider>Google Scholar</remote-database-provider><access-date>2015/03/28/04:10:39</access-date></record></Cite></EndNote>]. ~~Briefly, r~~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 [HYPERLINK "https://www.vectorbase.org"]), transcript and EST databases using the BLAST tool.

The sequence was compared in both orientations at the nucleotide level and at the translated sequence level in all six reading frames with ~~to deposited~~ amino acid sequences in the database. 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 hybridization 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 [REF _Ref450305949 \h].

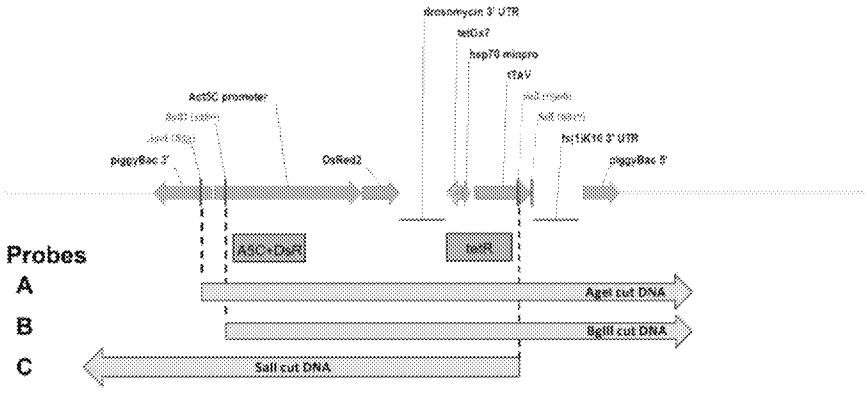

Figure [SEQ Figure * ARABIC]. Schematic of restriction enzyme strategy for ~~the~~ Southern Blots.

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 single discrete genomic integration site.

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

The backbone sequence of the #OX513 ~~plasmid~~ rDNA construct ~~comprises~~ includes 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 G60-64 and G100 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 (Section [REF _Ref453677594 \r \h]).

Therefore, we conclude 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 proposed trial. OX513A eggs would be produced at Oxitec Ltd., UK and shipped by air in multiple shipments to the U.S.[11] for rearing to adults in a specialized facility, known

[11] See Section [REF _Ref453244645 \r \h] for a more complete description of import permits.

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as the Hatching and Rearing Unit (HRU), located in Marathon, FL. Adult male mosquitoes would 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, wild-type populations of *Ae. aegypti* at the specific site identified in Key Haven, Monroe County, FL, although the trial might be concluded earlier if the operational objectives have been met.

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 [REF _Ref450306887 \h] below.

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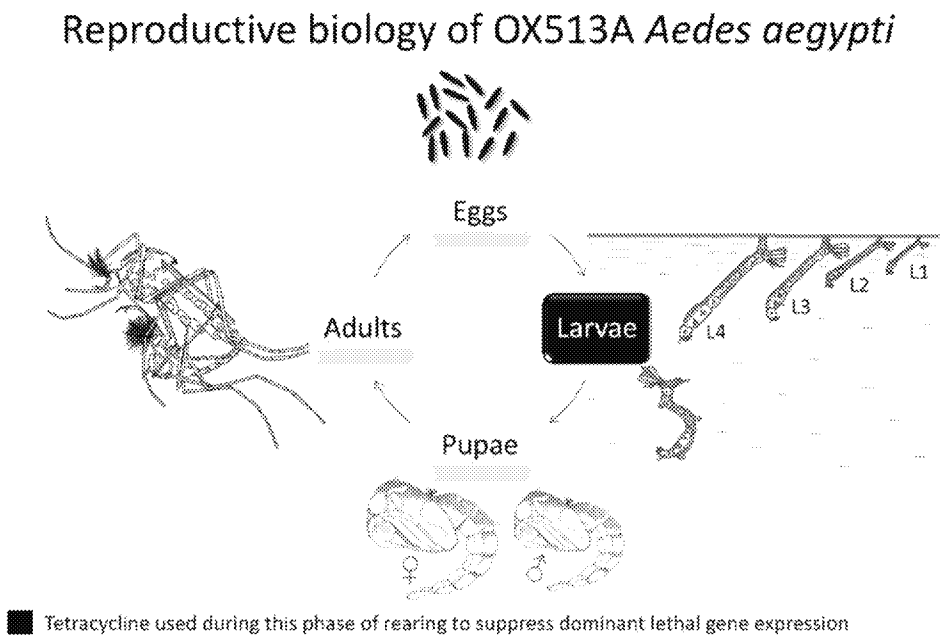

Figure [SEQ Figure * ARABIC]. General overview of the lifecycle of *Ae. aegypti* OX513A.

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. Males 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 rDNA constructgene is inherited by all the offspring creating a true breeding line for the #OX513 rDNA constructgene.

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.

Commented [LE3]: Ashley: Verb tense is present here because this is a description of "general" conditions. Not sure whether this is appropriate.

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 in a ~~as~~ dried state ~~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 w~~ould~~ill be conducted in the HRU in Florida as eggs would ~~are~~ not be produced there; only rearing of ~~the~~ eggs to adults would occur~~s~~ 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 would be ~~are~~ two production sites: a UK-based egg production site to produce *Ae. aegypti* OX513A eggs and a local facility (the HRU) in the U.S. in Marathon, Florida~~, US~~, which would rearing eggs to adults for release. In the UK egg production facility, eggs would ~~are~~ continually be produced from a cycling colony of homozygous OX513A parent mosquitoes. The eggs would ~~will~~ be shipped in multiple shipments throughout the course of the investigation to the HRU facility near the trial site where they would ~~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 [REF _Ref450307413 \h]; the associated process flows for egg production and production of males for release are shown, respectively, in [REF _Ref450307760 \h] and [REF _Ref450308158 \h]).

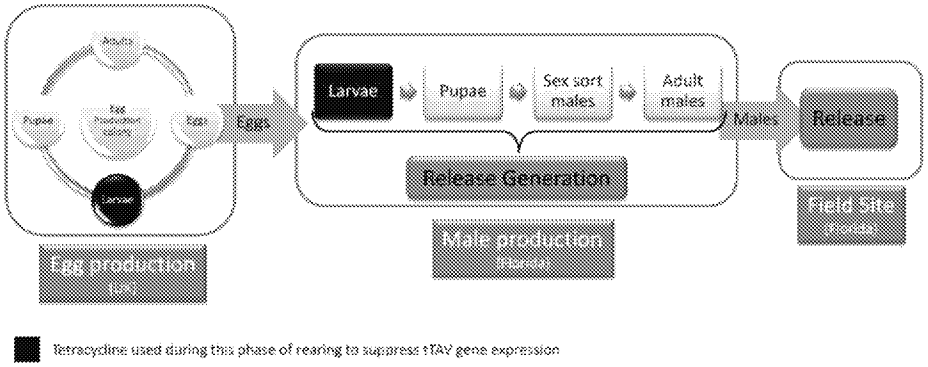

Figure [SEQ Figure * ARABIC]. A schematic of the production process for producing males for release.

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

The process used to produce eggs in the UK is summarized in [REF _Ref450307760 \h].

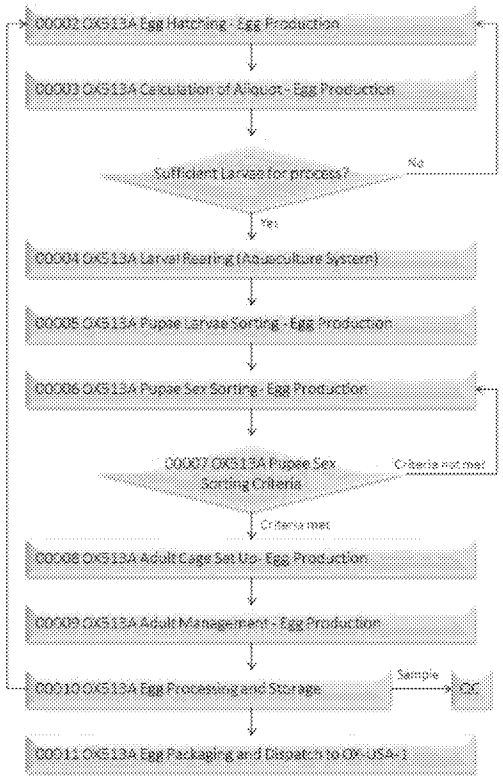

Figure [SEQ Figure * ARABIC]. Process flow for UK egg production.

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 annually 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.~~ HSE conducted the last inspection in March 2016 and, from a verbal close out meeting, no deficiencies were noted.

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

Commented [LE4]: Same question about tense.

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°C/-4°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°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. In the future, mosquito breeding requirements could require testing of blood for arboviruses but at this time ~~T~~the host range of *Aedes aegypti* and *Aedes albopictus* does not extend to the UK [ADDIN EN.CITE ADDIN EN.CITE.DATA][~~12~~] 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.

Commented [LE5]: Same question re: tense.

10.4.1.6 Shipment of eggs to the United States

Shipping from the UK to the U.S. will be conducted in accordance with the requirements of ~~US Federal Regulations~~ 7 CFR Part 340, ~~9 CFR Part 122~~, 42 CFR ~~Part~~ 71.54, ~~9 CFR Part 122~~ and 21 CFR Part 511, including obtaining valid permits from the USDA Animal and Plant Health Inspection Service (APHIS) and the CDC. Oxitec and/or FKMCD would~~ill~~ obtain all necessary permits and make required notifications prior to shipment. Eggs from the UK production facility would~~ill~~ 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 R~~regulations cited above. Boxes would~~ill~~ 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 would~~ill~~ need to occur regularly (probably weekly) prior to and during the investigational use. Shipments would be ~~are~~ labelled with directions ~~as~~ to be kept above 10°C and to only be opened by 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.

~~[12] 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~~

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On receipt by Oxitec or FKMCD, shipments would~~will~~ only be opened by authorized staff and within the designated facility (the HRU). Rearing would~~will~~ be performed as described in Section [REF _Ref453677115 \r \h]~~10.4.2~~ and the associated SOPs. Shipping materials would~~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 [REF _Ref450308158 \h].

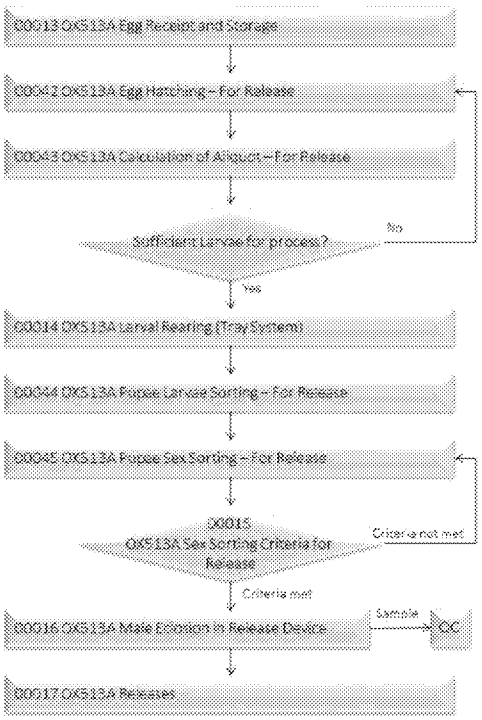

Figure [SEQ Figure * ARABIC]. Process flow for male production for release.

Production of adults in the U.S. would be ~~is proposed~~ in the~~an~~ HRU. This is a dedicated, containment facility for the production of OX513A male adults for release. The proposed HRU is~~will be~~ located within ~~the~~ an existing FKMCD site in Marathon and would~~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 would take place in the UK, the HRU unit would rear to adulthood for release at the trial site eggs produced in the UK and shipped to the HRU to adulthood for release at the trial site. The following procedures would be employed:

Egg hatch

Eggs would 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 [REF _Ref450306887 \h]) would 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 would be counted and aliquoted volumetrically. The larval diet would be added daily. Most of the male larvae wouldwill pupate at Days 7 and 8 post hatching.

Pupal processing

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

Larvae separation from pupae

Pupae would arebe separated from larvae using a proprietary sieve wire sorter device (pending PCT Patent number USPN2015/0008163A1[13]708902WO; http://www.uspto.gov/web/patents/patog/week45/OG/classification/cpcClassGroup_B03.html) 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. The trials in the Cayman Islands and Panama and current operations in Brazil have used this type of wire sorter device, however, Oxitec has improved the device over time, including improvements to the manufacturing accuracy and improvements due to experience of using it with trained staff.

Sex separation of male and female pupae

[13] [HYPERLINK "http://www.uspto.gov/web/patents/patog/week45/OG/classification/cpcClassGroup_B03.html"] [Accessed June 22, 2016].

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Mechanical size separation would~~ill~~ be used to separate sexes as the majority of female pupae are larger than males [ADDIN EN.CITE ADDIN EN.CITE.DATA]. Using the proprietary method above, it is possible to separate males from females with a sorting accuracy of >99.9% ([REF _Ref450308573 \h]) [ADDIN EN.CITE ADDIN EN.CITE.DATA]. Quality control processes would be~~are~~ established to ensure accuracy of the sorting does not exceed a maximum of 0.2% females. Two samples of 500 pupae would be~~are~~ taken for analysis and the number of female pupae in each sample would be counted by trained staff. The sample number would be ~~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 would be~~is~~ re-sorted prior to release to ensure meeting the 0.2% criterion.

Table [SEQ Table * ARABIC]. Average values for ~~presence of females in sorted~~ male pupae in batches from ~~Cayman and Brazilian~~ trials in Cayman, Brazil, and Panama.

Study	Cayman	Brazil	
% Sex sorting efficiency	99.93%	99.98%	
Published reference	[ADDIN EN.CITE <EndNote><Cite><Author>Harris</Author><Year>2012</Year><RecNum>22</RecNum><DisplayText>(Harris et al. 2012)</DisplayText><record><rec-number>22</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">22</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Harris, Angela F.</author><author>McKemey, Andrew R.</author><author>Nimmo, Derric</author><author>Curtis, Zoe</author><author>Black, Isaac</author><author>Morgan, SiÃ¢n A.</author><author>Oviedo, Marco Neira</author><author>Lacroix, Renaud</author><author>Naish, Neil</author><author>Morrison, Neil I.</author><author>Collado, Amandine</author><author>Stevenson, Jessica</author><author>Scaife, Sarah</author><author>Dafa'alla, Tarig</author><author>Fu, Guoliang</author><author>Phillips, Caroline</author><author>Miles, Andrea</author><author>Raduan, Norzahira</author><author>Kelly, Nick</author><author>Beech,	[ADDIN EN.CITE <EndNote><Cite><Author>Carvalho</Author><Year>2014</Year><RecNum>21</RecNum><DisplayText>(Carvalho et al. 2014)</DisplayText><record><rec-number>21</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">21</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Carvalho, Danilo O.</author><author>Nimmo, Derric</author><author>Naish, Neil</author><author>McKemey, Andrew R.</author><author>Gray, Pam</author><author>Wilke, AndrÃ© B.B.</author><author>Marrelli, Mauro T.</author><author>Virginio, Jair F.</author><author>Alphey, Luke</author><author>Capurro, Margareth L.</author></authors></contributors><titles><title>Mass Production of Genetically Modified Aedes aegypti for Field Releases in Brazil</title><secondary-title>J Vis Exp</secondary-title></titles><number>83</number><reprint-edition>Not in File</reprint-edition><keywords><keyword>Aedes</keyword></keywords><dates><year>2014</year><pub-dates><date>2014</date></pub-dates></dates><isbn>1940-087X</isbn><label>22</label><urls><related-	[AD n type> K U 28</p

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	Camilla</author><author>Donnelly, Christl A.</author><author>Petrie, William D.</author><author>Alphey, Luke</author></authors></contributors><titles><title>Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes</title><secondary-title>Nat Biotech</secondary-title></titles><pages>828-830</pages><volume>30</volume><number>9</number><reprint-edition>Not in File</reprint-edition><dates><year>2012</year><pub-dates><date>2012</date></pub-dates></dates><isbn>1087-0156</isbn><label>23</label><urls><related-urls><url>http://www.nature.com/nbt/journal/v30/n9/full/nbt.2350.html</url></related-urls></urls><electronic-resource-num>10.1038/nbt.2350</electronic-resource-num><access-date>4/1/2015</access-date></record></Cite></EndNote>]	urls><url>http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4063546/</url></related-urls></urls><electronic-resource-num>10.3791/3579</electronic-resource-num><access-date>5/14/2015</access-date></record></Cite></EndNote>]	

10.4.2.2 Disposal of female insects

In the HRU~~male production facility,~~ after sorting the male ~~and~~ from the female pupae, ~~have been sorted~~ the female pupae and the larvae would be ~~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 would be~~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 would be~~is~~ aliquoted into release devices volumetrically and water added to a depth of approximately 1 cm. Sugar would be~~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 would be~~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 would be~~are~~ provided with the sugar solution. The release devices would be~~are~~ placed into a double-sealed container, labelled, and transported to the release site. At the appropriate release coordinates, a release device would be ~~is~~ removed from double containment and the lid would be~~is~~ opened to release the mosquitoes. After release, individual release devices would be~~are~~ returned to double containment for transportation back to the rearing facility where they would be ~~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 would~~ll~~ be by vehicle driven by authorized staff from either FKMCD or Oxitec. Release devices for adult release would~~ll~~ be packed in the vehicle. Insects would~~ll~~ be double contained for transport to the field site for release. One level of containment would~~ll~~ be the release device itself and another would~~ll~~ 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~~A chain of custody protocol would require~~such that~~ release devices to be ~~are~~ signed out of the facility, and signed for upon receipt by authorized personnel at the field site. Outer containers would~~ll~~ be labelled "Genetically engineered mosquitoes – only to be opened by FKMCD/Oxitec staff". For transport of release devices back from the field site they would~~ll~~ 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 would~~ll~~ be discarded by incineration via an external contractor.

~~10.4.2.5 Field 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.~~

Commented [EEA6]: Deleted; more detailed info is included in Section 11.

Commented [EEA7]: Moved to Section 11.

~~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 GF wastes and frozen (≤-15°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 Section 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~~

OX513A eggs would~~d~~ be produced by Oxitec in Oxford, UK and shipped to Marathon, Florida~~l~~ for rearing in the specialized HRU at the FKMCD facility. OX513A male mosquitoes reared from these eggs at the HRU in the FKMCD facility would be used for a proposed investigational open release field trial in Key Haven, Monroe County, Florida~~l~~ performed by FKMCD and Oxitec.

The proposed investigational trial has one primary and one secondary goal. The primary goal is comprised of two parts. Part one aims to determine whether released OX513A males mate with local wild-type *Ae. aegypti* females resulting in their progeny inheriting a copy of the #OX513 rDNA construct. Part two aims to determine whether these OX513A progeny inheriting the #OX513 rDNA construct exhibit at least a 2-fold proportional increase in mortality before reaching functional adulthood relative to the local non-GE *Ae. aegypti* progeny at the trial site. The secondary goal aims to determine whether sustained release of OX513A males results in a statistically significant (≥ 50% with 95% Confidence Interval) suppression of the local population

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of *Ae. aegypti* in the treatment area (TA) relative to the untreated comparator area (UCA) (treated and control areas are described in greater detail in Section [REF _Ref453674099 \r \h] of the EA).

OX513A male mosquitoes would~~ill~~ be released in a systematic manner from a pre-determined georeferenced grid of release points up to three times a week to ensure even and consistent coverage of the TA~~area~~. Release points will be spaced approximately 25-70 m apart, with a maximum spacing of 100 m. Release points will be georeferenced using ~~the~~ Global Positioning System (GPS) coordinates and the area that is mapped with the spatial data will be incorporated into an appropriate Geographical Information System (GIS).

The number of OX513A mosquitoes released would~~ill~~ be proportional to the local population of *Ae. aegypti*, human population density at the proposed trial site, and the initial rate determined from ~~the~~ Phase II of the trial in order to achieve a mating fraction of ≥ 0.5. The mating fraction would~~ill~~ be determined and monitored via ovitraps as the proportion of fluorescent (OX513A) and non-fluorescent (wild-type) eggs collected from ovitraps. The overall number of mosquitoes to be released depends on multiple factors including seasonality, egg banks, time of year, and rainfall and would be based on the estimates obtained from the initial six to eight week range finding phase (Phase II) (see below). The number of mosquitoes released will likely decrease over time if suppression is achieved due to adaptive management of releases. Notwithstanding the variability in the number of OX513A that would be released based on these factors, we have estimated the minimum number of OX513A mosquitoes that might be released. The estimate is for either a six-week or an eight-week duration for the range finding phase and the maximum proposed period of 96 weeks (22 months) for the suppression phase (see below) (although, thus far, all of the trials conducted in other countries have achieved their suppression goals in less than 96 weeks). We have estimated the number of mosquitoes that would be released based on 460 human residents in the TA (assuming four residents for each of approximately 115 houses within the designated TA). Because this is a residential area with no commercial properties, population flux during the day is not expected to be substantial enough to alter the total number of humans in the TA significantly. Based on these assumptions, the total number of OX513A mosquitoes that would be released is estimated at 14,352,000 over the 104 week period (estimates for eight week range finding phase plus 96 week suppression phase under high initial infestation conditions and not accounting for adaptive management adjustments in number of mosquitoes released during the suppression phase). The result is less than 58 female mosquitoes released per person in the TA over a total of 104 weeks or 0.6 female mosquitoes per person per week at the highest initial infestation levels.

Commented [KJ8]: This is an odd expression females per person. I guess I understand the PR reason but it might be better to also include a per acre or household value .

~~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 suppression 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.~~

The trial will be divided into three phases:

- Phase I (Preparation phase) would be used by Oxitec and the FKMCD to evaluate the initial density of the *Ae. aegypti* mosquito population at the proposed trial~~field~~ site and optimize the OX513A mosquito rearing methodology to local conditions in Florida. This phase of the investigational trial is expected to last 8 to 16 weeks.
- Phase II (Range finding phase) would be used to address the two parts of the primary ~~primary~~ goal of the trial: "do released OX513A males mate with local wild-type *Ae. aegypti* females resulting in their progeny inheriting a copy of the #OX513 rDNA construct" 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." During this phase, OX513A males would be released up to

three times a week at a constant release rate. This phase would~~is planned to~~ last from six ~~up~~ to eight weeks.

* Phase III (Suppression phase) would be used to evaluate the secondary goal of the trial: "does sustained release of OX513A result in statistically significant suppression (≥ 50% with 95% CI) of the local *Ae. aegypti* population relative to the comparator area that is not treated with the released OX513A". During this phase, OX513A male mosquitoes would~~will~~ be released up to three times a week at a rate that might~~may~~ be changed in accordance with changes in *Ae. aegypti* population. This phase of the investigational trial would~~is expected to~~ last up to 22 months (approximately 96 weeks).

Commented [KJ9]: I expect this value to also reflect the Ae. aegypti population values but that is not seen here. Isn't there some target of males to wild type female ratio that is the goal?

FKMCD would~~will~~ continue its standard mosquito abatement procedures, including insecticide use, at the proposed investigational site during the entire duration of the trial.

11.2 Data collection

All data obtained during the investigational trial would~~will~~ be collected using ovitraps (eggs) and BG-Sentinel (Biogents, Germany) (adults) traps.

An ovitrap is a device that mimics the preferred breeding site for container breeding mosquitoes such as *Ae. aegypti* and is routinely used to monitor the presence/absence of mosquitoes in an area of interest [ADDIN EN.CITE <EndNote><Cite><Author>Silver</Author><Year>2008</Year><RecNum>59</RecNum><DisplayText>(Silver 2008)</DisplayText><record><rec-number>59</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1445971835">59</key></foreign-keys><ref-type name="Book">6</ref-type><contributors><authors><author>Silver, J.B.</author></authors></contributors><titles><title>Mosquito Ecology</title></titles><edition>3rd</edition><dates><year>2008</year></dates><pub-location>New York</pub-location><publisher>Springer</publisher><urls></urls></record></Cite></EndNote>]. Oxitec states that a minimum of 60 ovitraps each would be used in the TA and the UCA respectively, with a trap density of 3-4 traps/ha. All trapping locations would~~will~~ receive a unique number and would~~will~~ be georeferenced using GPS coordinates. Oxitec or FKMCD employees would~~will~~ check traps every 6-8 days and collect the oviposition substrate for further analysis in the FKMCD laboratory in~~at~~ Marathon, Florida.

BG-Sentinel traps are designed to directly capture adults [ADDIN EN.CITE <EndNote><Cite><Author>Krockel</Author><Year>2006</Year><RecNum>51</RecNum><DisplayText>(Krockel et al. 2006)</DisplayText><record><rec-number>51</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1445368697">51</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Krockel, U.</author><author>Rose, A.</author><author>Eiras, A. E.</author><author>Geier, M.</author></authors></contributors><auth-address>Institut fur Zoologie, University of Regensburg, Universitatstrasse 31, 93040 Regensburg, Germany.</auth-

address><titles><title>New tools for surveillance of adult yellow fever mosquitoes: comparison of trap catches with human landing rates in an urban environment</title><secondary-title>J Am Mosq Control Assoc</secondary-title></titles><periodical><full-title>J Am Mosq Control Assoc</full-title></periodical><pages>229-38</pages><volume>22</volume><number>2</number><keywords><keyword>Aedes/virology</keyword><keyword>Animals</keyword><keyword>Brazil</keyword><keyword>*Culicidae</keyword><keyword>Female</keyword><keyword>Humans</keyword><keyword>Male</keyword><keyword>Population Surveillance</keyword><keyword>Urban Population</keyword><keyword>Yellow fever virus</keyword></keywords><dates><year>2006</year><pub-dates><date>Jun</date></pub-dates></dates><isbn>8756-971X (Print)
8756-971X (Linking)</isbn><accession-num>17019768</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/17019768</url></related-urls></urls><electronic-resource-num>10.2987/8756-971X(2006)22[229:NTFSOA]2.0.CO;2</electronic-resource-num></record></Cite></EndNote>]. These traps are routinely used for monitoring *Ae. aegypti* populations and provide an indirect measure of *Ae. aegypti* abundance in the area [ADDIN EN.CITE ADDIN EN.CITE.DATA]. Oxitec's protocol states that a minimum of 20 different locations would be sampled in the TA and the UCA respectively, in parallel, once every week. All trapping locations would~~ill~~ receive a unique number and would~~ill~~ be georeferenced using GPS coordinates. These traps would~~ill~~ be deployed overnight with the trap catch recovered the following day in both the TA and the UCA. The number of female *Ae. aegypti* captured from each BG-Sentinel trap would~~ill~~ be recorded.

11.3 Sample Analysis

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

The samples returned from the field would~~ill~~ be analyzed in a variety of ways:

11.3.1 Ovitrap analysis

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

11.3.2 Adult analysis

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

11.3.3 Testing of functional adult mortality

Eggs from ovitraps, representing the progeny of matings with OX513A in the treatment area wouldill 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 wouldill also be shipped to the UK for analysis to confirm their genotype by PCR methods. The mosquitoes caught in the traps would be are expected to be either hemizygous for the #OX513 rDNA construct or without any copies of the #OX513 rDNA construct i.e., local wild-type *Ae. aegypti* or other non-*Ae. aegypti* mosquito species. It would beis possible that some mosquitoes homozygous for the #OX513 rDNA construct wouldill be detected. These would likely be derived from matings between the small number of females (≤0.2%) that might may be co-released and with the male OX513A mosquitoes they are released with (Section [REF _Ref453764606 \r \h]). Any co-released OX513A female wouldwill live no longer than a wild-type *Ae. aegypti* and, becauseas there are insufficient sources of tetracycline in the environment, progeny resulting from any matings of these females she makes wouldill die as described in Section [REF _Ref453330318 \r \h].

Commented [KJ10]: Not clear here how these would be detected and distinguished. Is it possible to include a short explanation?

11.3.4 Estimating *Ae. aegypti* suppression at the proposed trial site

Oxitec plans to estimate the suppression of *Ae. aegypti* at the investigational trial site by calculating relative ovitrap and relative adult density indices based on the data collected from ovitraps and BG-Sentinel adult traps.

Ovitraps are a useful and effective tool for demonstrating the presence or absence of *Ae. aegypti* in the area of interest and a good indicator of changes in mosquito population [ADDIN EN.CITE <EndNote><Cite><Author>Silver</Author><Year>2008</Year><RecNum>59</RecNum><DisplayText>(Silver 2008)</DisplayText><record><rec-number>59</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1445971835">59</key></foreign-keys><ref-type name="Book">6</ref-type><contributors><authors><author>Silver, J.B.</author></authors></contributors><titles><title>Mosquito Ecology</title></titles><edition>3rd</edition><dates><year>2008</year></dates><pub-location>New York</pub-location><publisher>Springer</publisher><urls></urls></record></Cite></EndNote>]. As the size of the adult *Ae. aegypti* population decreases, the number of positive ovitraps and the number of

eggs per ovitrap decreases as well[14] [ADDIN EN.CITE <EndNote><Cite><Author>Dibo</Author><Year>2008</Year><RecNum>61</RecNum><DisplayText>(Dibo et al. 2008)</DisplayText><record><rec-number>61</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1447351792">61</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Dibo, M.R.;</author><author>Chierotti, A.P.;</author><author>Ferrari, M.S.;</author><author>Mendonca, A.L.;</author><author>Neto, F.C.</author></authors></contributors><titles><title>Study of the relationship between Aedes (Stegomyia) aegypti egg and adult densities, dengue fever and climate in Mirassol, state of Sao Paulo, Brazil.</title><secondary-title>Mem Inst Oswaldo Cruz</secondary-title></titles><periodical><full-title>Mem Inst Oswaldo Cruz</full-title></periodical><pages>554-560</pages><volume>103</volume><number>6</number><dates><year>2008</year></dates><urls></urls></record></Cite></EndNote>]. Therefore, changes in the ovitrap index[15] over time and between the sites would be a good indicator of changes in the relative population of *Ae. aegypti* or relative population densities in compared areas [ADDIN EN.CITE ADDIN EN.CITE.DATA]. Evaluation of the change in ~~the~~ size of the *Ae. aegypti* population using relative ovitrap index[16] was also used by Oxitec in previous studies that were published in ~~a~~ peer-reviewed scientific literature [ADDIN EN.CITE ADDIN EN.CITE.DATA].

Using the same approach as for ovitrap data, relative abundance of adult density in the TA and UCA before and after the suppression phase also would~~ill~~ be ~~also~~ used as a measure of suppression of local wild-type *Ae.aegypti*.

12 Environmental Risk Analysis

12.1 Accessible environments

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

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[14] This relationship does not hold in studies/programs that involve removal of *Ae. aegypti* natural oviposition sites as decrease in available sites may lead to increase in the number of positive ovitraps and/or the number of eggs per ovitrap.

[15] The ovitrap index (OI) is a measure of mosquito abundance in the TA and the UCA. For a single time point of trap collection, the OI is defined as $OI = \frac{L}{T}$ where L is the number traps from which one or more eggs positively identified as *Ae. aegypti* after hatching [fluorescent or non-fluorescent] and T is the total traps recovered.

[16] Relative ovitrap index (ROI) is defined as $ROI = \frac{OI\ in\ TA}{OI\ in\ UCA}$.

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12.1.1 *Aedes aegypti* habitat

Ae. aegypti mosquitoes are a non-native mosquito species introduced into the United States with human migrations and international trade (Tabachnik, 1991, Gubler *et al.*, 2001, Slosek, 1986). *Ae. aegypti* ~~is~~ has limited interactions with ecological systems outside domestic settings in this habitat, although a sylvatic subspecies of *Ae. aegypti, Ae. aegypti formosa* has been found in tree holes and more sylvan or rural settings in its native Africa [ADDIN EN.CITE ADDIN EN.CITE.DATA]~~(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 [ADDIN EN.CITE
<EndNote><Cite><Author>Ramasamy</Author><Year>2011</Year><RecNum>168</RecNum><Display Text>(Ramasamy et al. 2011)</DisplayText><record><rec-number>168</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106826">168</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Ramasamy, Ranjan</author><author>Surendran, Sinnathamby N.</author><author>Jude, Pavilupillai J.</author><author>Dharshini, Sangaralingam</author><author>Vinobaba, Muthuladchumy</author></authors><secondary-authors><author>Barrera, Roberto</author></secondary-authors></contributors><titles><title>Larval Development of Aedes aegypti and Aedes albopictus in Peri-Urban Brackish Water and Its Implications for Transmission of Arboviral Diseases</title><secondary-title>PLoS Neglected Tropical Diseases</secondary-title></titles><periodical><full-title>PLoS Neglected Tropical Diseases</full-title></periodical><pages>e1369</pages><volume>5</volume><number>11</number><dates><year>2011</year><pub-dates><date>2011/11/22/</date></pub-dates></dates><isbn>1935-2735</isbn><urls><related-urls><url>http://dx.plos.org/10.1371/journal.pntd.0001369</url><url>http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3222631/pdf/pntd.0001369.pdf</url></related-urls></urls><electronic-resource-num>10.1371/journal.pntd.0001369</electronic-resource-num><remote-database-provider>CrossRef</remote-database-provider><language>en</language><access-date>2015/03/28/04:18:33</access-date></record></Cite></EndNote>]. *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 [ADDIN EN.CITE

<EndNote><Cite><Author>Clark</Author><Year>2004</Year><RecNum>115</RecNum><DisplayText>(Clark et al. 2004)</DisplayText><record><rec-number>115</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463104275">115</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Clark, T. M.</author><author>Flis, B. J.</author><author>Remold, S. K.</author></authors></contributors><auth-address>Department of Biological Sciences, Indiana University, South Bend, IN 46634-1700, USA. tclark2@iusb.edu</auth-address><titles><title>Differences in the effects of salinity on larval growth and developmental programs of a freshwater and a euryhaline mosquito species (Insecta: Diptera, Culicidae)</title><secondary-title>J Exp Biol</secondary-title></titles><periodical><full-title>J Exp Biol</full-title></periodical><pages>2289-95</pages><volume>207</volume><number>Pt 13</number><keywords><keyword>Aedes/*growth & development</keyword><keyword>Animals</keyword><keyword>Body Weight</keyword><keyword>Female</keyword><keyword>Fresh Water</keyword><keyword>Larva/growth & development</keyword><keyword>Linear Models</keyword><keyword>Male</keyword><keyword>Ochlerotatus/*growth & development</keyword><keyword>*Phenotype</keyword><keyword>Seawater</keyword><keyword>Sex Factors</keyword><keyword>Sodium Chloride/*analysis</keyword><keyword>Species Specificity</keyword></keywords><dates><year>2004</year><pub-dates><date>Jun</date></pub-dates></dates><isbn>0022-0949 (Print)
0022-0949 (Linking)</isbn><accession-num>15159433</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/15159433</url></related-urls></urls></record></Cite></EndNote>]. 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 [ADDIN EN.CITE ADDIN EN.CITE.DATA]. As best described by [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Burke</Author><Year>2010</Year><RecNum>108</RecNum><DisplayText>Burke et al. (2010)</DisplayText><record><rec-number>108</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463103818">108</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Burke, R.</author><author>Barrera, R.</author><author>Lewis, M.</author><author>Kluchinsky, T.</author><author>Claborn, D.</author></authors></contributors><auth-address>Armed Forces Health Surveillance Center, Silver Spring, MD 20910, USA. ronald.l.burke@amedd.army.mil</auth-address><titles><title>Septic tanks as larval habitats for the mosquitoes Aedes aegypti and Culex quinquefasciatus in Playa-Playita, Puerto Rico</title><secondary-title>Med Vet Entomol</secondary-title></titles><periodical><full-title>Med Vet Entomol</full-title></periodical><pages>117-23</pages><volume>24</volume><number>2</number><keywords><keyword>Aedes/*physiology</keyword><keyword>Animals</keyword><keyword>Culex/*physiology</keyword><keyword>*Ecosystem</keyword><keyword>Insect Vectors/*physiology</keyword><keyword>Population Density</keyword><keyword>Puerto

Rico</keyword><keyword>*Sewage/chemistry</keyword></keywords><dates><year>2010</year><pub-dates><date>Jun</date></pub-dates></dates><isbn>1365-2915 (Electronic)
0269-283X (Linking)</isbn><accession-num>20374477</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/20374477</url></related-urls></urls><electronic-resource-num>10.1111/j.1365-2915.2010.00864.x</electronic-resource-num></record></Cite></EndNote>] and [ADDIN EN.CITE ADDIN EN.CITE.DATA], septic tanks were more productive breeding habitats 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 [ADDIN EN.CITE <EndNote><Cite><Author>Hribar</Author><Year>2001</Year><RecNum>47</RecNum><DisplayText>(Hribar et al. 2001)</DisplayText><record><rec-number>47</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432063934">47</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Hribar, L.J.</author><author>Smith, J.M.</author><author>Vlach, J.J.</author><author>Verna, T.N.</author></authors></contributors><titles><title>Survey of Containter-Feeding Mosquitoes from the Florida Keys, Monroe County, Florida.</title><secondary-title>J Am Mosquito Contr Association</secondary-title></titles><periodical><full-title>J Am Mosquito Contr Association</full-title></periodical><pages>245-248</pages><volume>17</volume><number>4</number><dates><year>2001</year></dates><urls></urls></record></Cite></EndNote>] 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~~ Desiccated eggs that survive to hatch in the following season form the egg bank.

12.1.1.2 Terrestrial habitats

Adult *Ae. aegypti* occupies terrestrial (land-based) habitats. Male adults require three kinds of resources to survive and propagate: a) access to plant sugars for food, b) mates, and c) resting sites. Female adults require the same three resources as well as ~~a~~ sources of blood meals 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~~ the human environment and rarely flies spontaneously for distances greater than 200 meters, as described in Section [REF _Ref453246258 \r \h] of ~~this document~~ the EA.

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 106~~94.7~~ million visitors to Florida in 2015~~3~~, an increase of 8~~3.5~~ percent over 2014~~2~~[17]. 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 is comprise~~d~~s of sea-based biodiversity resources encompassing the majority of the Keys). Monroe County has a sub-tropical climate, ~~with a~~Average monthly temperatures ranged~~ing~~ from 68.5 °F to 86.4 °F ~~a mean daily temperature of 83.4°F (range max 87.4 – min 79.4°F)~~ and rarely fell below 65°F at night between ~~during~~ January 2014 – March 2016 ~~period~~ ([REF _Ref453775979 \h][REF _Ref453775979 \h]).[18] During the same period ~~P~~the amount of ~~p~~recipitation ~~varies throughout the year ranging from~~varied between 0.31~~.51~~ ~~inches in Feb 2014 to~~ and 6.79~~5.45~~ inches ~~in Sept of the same year (NOAA[18]) with a relative humidity of around 76%. Climate data is summarized in~~ [REF _Ref450309634 \h].

Commented [KJ11]: Monthly?

[17] [HYPERLINK "http://www.flgov.com/2014/02/14/gov-rick-scott-another-record-year-for-florida-tourism/"] http://www.visitfloridamediablog.com/home/florida-facts/research/ [accessed ~~14~~ June 15, 2016~~6~~]

[18] [REF _Ref453775979 \h * MERGEFORMAT] summarizes temperature and precipitation data from the weather station located at the Key West International Airport provided by the NOAA's National Centers for Environmental Information [HYPERLINK "https://www.ncdc.noaa.gov/cdo-web/datatools"] [Accessed June 15, 2016].

~~[18] http://(Accessed 27 Sept 27, 2012)~~

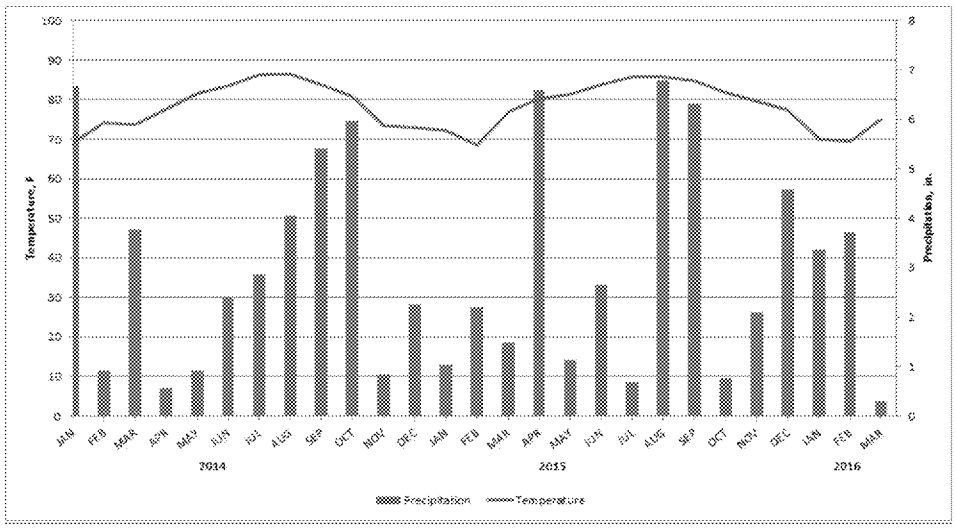
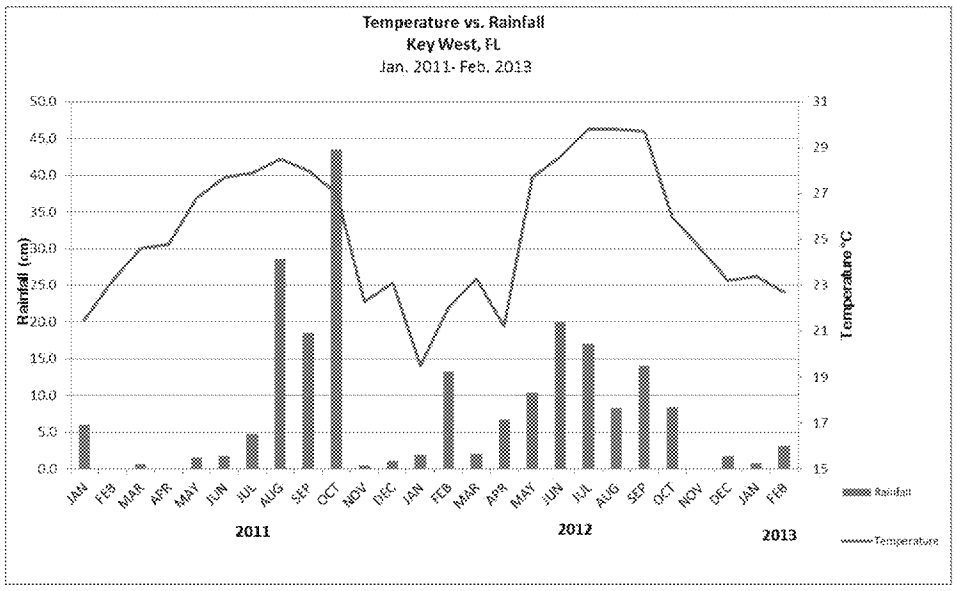


Figure [SEQ Figure * ARABIC]**. Temperature vs Precipitation, Key West, FL. Jan 2014 - Mar 2016.**



~~Figure~~ [SEQ Figure * ARABIC] ~~Temperature vs Rainfall, Key West, FL, Jan 2011-Feb 2013.~~

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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 or 2 hurricane passing within 50~~75~~ nautical miles of ~~the Florida~~ Key West~~s~~ every eight years [ADDIN EN.CITE <EndNote><Cite><Author>Blake</Author><Year>2011</Year><RecNum>247</RecNum><DisplayText>(Blake et al. 2011)</DisplayText><record><rec-number>247</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1466011860">247</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Blake, E.S.</author><author>Landesea, C.W.</author><author>Gibney, E.J.</author></authors></contributors><titles><title>The deadliest, costliest, and most intense United States tropical cyclones from 1851 to 2010)and other frequently requested hurricane facts)</title><secondary-title>NOAA Technical Memorandum NWS NHC-6.</secondary-title></titles><periodical><full-title>NOAA Technical Memorandum NWS NHC-6.</full-title></periodical><dates><year>2011</year></dates><urls></urls></record></Cite></EndNote>], ~~4.5 years~~[26]. The historical average for a Category 3 storm and higher passing within 50~~75~~ nautical miles of the Keys, which requires mandatory resident evacuation, is every ~~nine~~ 18 years [ADDIN EN.CITE <EndNote><Cite><Author>Blake</Author><Year>2011</Year><RecNum>247</RecNum><DisplayText>(

[26] [HYPERLINK "http://www.nhc.noaa.gov/HAW2/english/basics/images/cat1_gulf.gif.%20"] ~~(Accessed 27 Sept 22, 2012)~~

Blake et al. 2011)</DisplayText><record><rec-number>247</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1466011860">247</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Blake, E.S.</author><author>Landesea, C.W.</author><author>Gibney, E.J.</author></authors></contributors><titles><title>The deadliest, costliest, and most intense United States tropical cyclones from 1851 to 2010)and other frequently requested hurricane facts)</title><secondary-title>NOAA Technical Memorandum NWS NHC-6.</secondary-title></titles><periodical><full-title>NOAA Technical Memorandum NWS NHC-6.</full-title></periodical><dates><year>2011</year></dates><urls></urls></record></Cite></EndNote>]. Hurricane season extends from June to November with most of the hurricanes that ~~making~~ make landfall in the Keys occurring in the month of September[21]. 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 ([REF _Ref453674565 \h]). Key Haven was flooded following Hurricane Wilma in 2005 as were most of the "Lower Keys"[22]. There are more up-to-date FEMA interactive maps[23] available for storm surge impacts 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 would~~ill~~ 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 might~~ev~~ need to be extended to allow sufficient sustained releases of OX513A to suppress the local population of *Ae. aegypti* or the investigational field trial would~~ill~~ be abandoned, depending on the severity of the disruption encountered.

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[21] [HYPERLINK "http://www.aoml.noaa.gov/hrd/tcfaq/E20.html" \h] [Accessed ~~27 June 15Sept 27,~~ 2016~~12~~]

[22] [HYPERLINK "http://www.srh.noaa.gov/key/?n=wilma"] [Accessed June 15, 2016].

[23] [HYPERLINK " https://msc.fema.gov/portal/search?AddressQuery=key%20west%2C%20FL#searchresultsanchor "][Accessed ~~3 June 20Oct 3,~~ 2016~~3~~].

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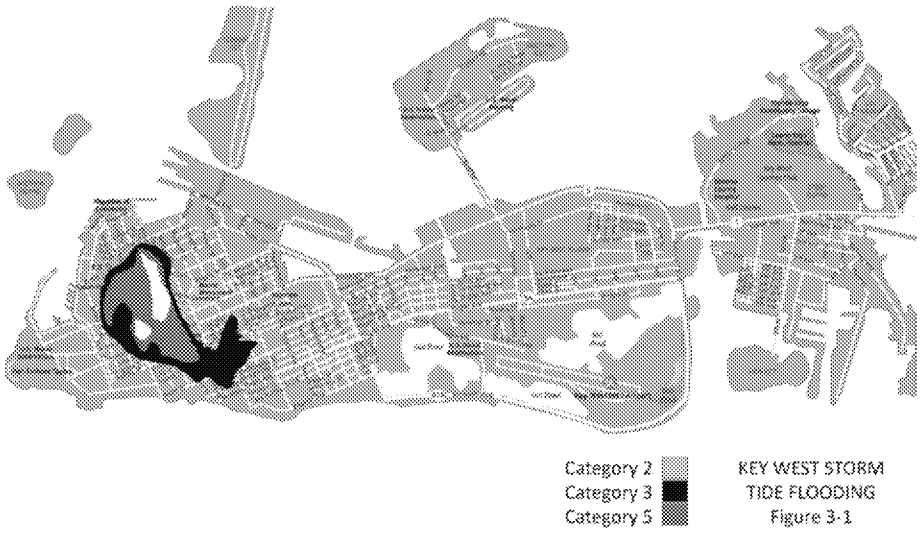

Figure [SEQ Figure * ARABIC]. Storm surge flooding map for Key West.

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 (*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.[24] There was no ~~habitat~~ overlap between the threatened and endangered species' ~~with their~~ habitat and the domestic or peri-domestic environment of *Ae. aegypti* in Key Haven. The Stock Island Tree Snail is the only species found in the physical vicinity of the proposed trial site. An assessment has

[24] Based on the search of the ECOS Environmental Conservation Online System maintained by the USFWS [HYPERLINK "http://ecos.fws.gov/tess_public/reports/species-by-current-range-county?fips=12087"] [Accessed June 23, 2015].

been conducted according the United States Fish and Wildlife Service (USFWS) criteria[25] 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 OX513A mosquitoes would not occupy the same habitat as these threatened and endangered species.

There would be no impact on the additional 42 threatened or endangered species' habitats because they are located farther than the 200 m OX513A are capable of flying. While individual OX513A mosquitoes could migrate in a car, boat, or other conveyance, they would die within 2-3 days in the absence of tetracycline and, consequently, such individual mosquitoes would not impact the habitat of any threatened or 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 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

[25][HYPERLINK "http://www.fws.gov/verobeach/ConservationinKeysPDFs/20130729_updated%20Stock%20Island%20Tree%20Snail%20Assessment%20Guide.pdf"] [Accessed June 9, 2016].

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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 [ADDIN EN.CITE <EndNote><Cite><Author>Leal</Author><Year>2010</Year><RecNum>151</RecNum><DisplayText>(Leal and Hribar 2010)</DisplayText><record><rec-number>151</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106826">151</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Leal, Andrea L.</author><author>Hribar, Lawrence J.</author></authors></contributors><titles><title>Mosquito Fauna of Wilderness Islands Within the National Key Deer Refuge and the Great White Heron National Wildlife Refuge, Monroe County, Florida</title><secondary-title>Journal of the American Mosquito Control Association</secondary-title></titles><periodical><full-title>Journal of the American Mosquito Control Association</full-title></periodical><pages>141-147</pages><volume>26</volume><number>2</number><dates><year>2010</year><pub-dates><date>2010</date></pub-dates></dates><urls><related-urls><url>http://www.bioone.org/doi/abs/10.2987/09-5927.1</url><url>http://www.bioone.org/doi/10.2987/09-5927.1?url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&rfr_dat=cr_pub%3Dpubmed&</url></related-urls></urls><remote-database-provider>Google Scholar</remote-database-provider><access-date>2015/03/28/04:12:35</access-date></record></Cite></EndNote>].

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

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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 [ADDIN EN.CITE ADDIN EN.CITE.DATA].

Commented [KJ12]: Shouldn't this be "an attractive habitat"?

12.1.2.2.3 Conclusion

FDA ~~It is therefore~~ conclude~~s~~ that release of OX513A w~~ould~~ill not affect threatened and endangered species or their habitats in Monroe County as there is no habitat overlap between the Key Haven release site for OX513A 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 [REF _Ref450310557 \h])[26]. The release site is an island ~~peninsula 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 compromise the success of the investigational trial. The Key Haven site has been monitored for *Ae. aegypti* since 2012, ~~with~~using both ovitraps and adult traps. FKMCD indicates that ~~with~~ all the current control measures (source reduction, larviciding, and adult insecticide) used over the whole of the Florida Keys achieve at best only 50% ~~that~~ control of *Ae. aegypti* ~~is only up to 50%~~ [27] ~~effective (FKMCD 2014, personal communication).~~

The proposed site for evaluation of OX513A w~~ould~~ill be divided into two areas of similar size separated by a buffer zone ([REF _Ref450310557 \h]). The area to receive releases of OX513A mosquitoes is identified as the Treatment Area (TA). The Untreated Comparator Area (UCA) is also identified in [REF _Ref450310557 \h]~~Figure 11~~, below. At its narrowest point, the buffer area is approximately 500 meters wide, which is sufficient to preclude the migration of the released OX513A mosquitoes from the ~~treated area~~TA into the ~~untreated comparator area~~UCA because *Ae. aegypti* rarely ~~travel~~fly more than 200 meters. ~~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).~~

[26] 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.

[27] [HYPERLINK "http://keysmosquito.org/wp-content/uploads/2015/05/2015-06-23-Reg-Mtg-Minutes.pdf"] [Accessed March 4, 2016]

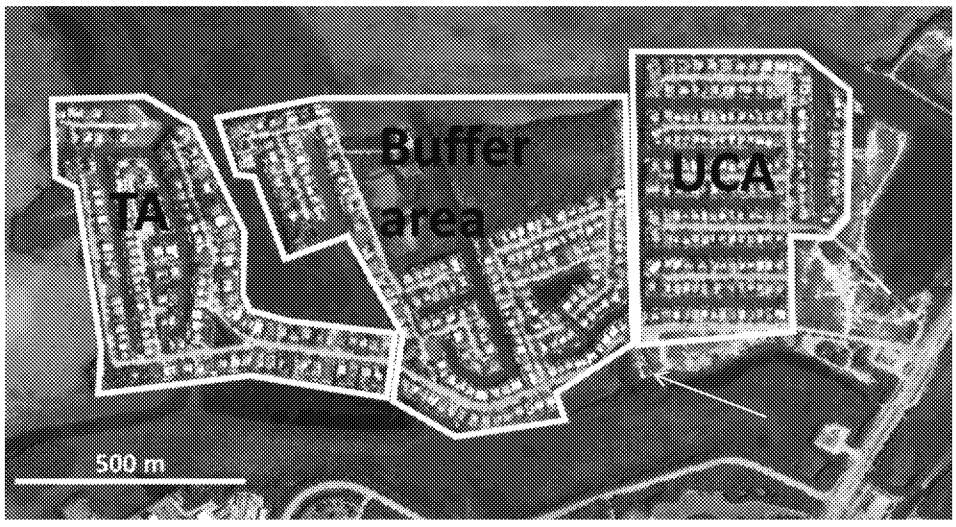

Figure [SEQ Figure * ARABIC]. Proposed trial area on Key Haven.

Proposed site for investigational release of OX513A mosquitoes. Areas identified are Treated (TA), Buffer, and Untreated ~~Control Comparator~~ Areas (UCA), respectively. The location of the Waste Water Treatment Plant servicing Key Haven residents is marked with an arrow.

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~~ [HYPERLINK "http://www.monroecounty-fl.gov/DocumentCenter/Home/View/1291"].

According to the Monroe County Master Plan for Future Development on Stock Island and Key Haven,[28] ~~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

[28] [HYPERLINK "http://www.monroecounty-fl.gov/DocumentCenter/Home/View/1291"] [Accessed June 16, 2016].

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[29]; 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[30]). 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[31] designates suitable habitat as hammock and beach berm. The USFWS species assessment guide has been utilized to determine if the proposed ~~project trial~~ could have an impact on the Stock Island Tree Snail (see Section [REF _Ref453244060 \r \h]).

The Monroe County Planning Department brought in tiered land characterization in 2002 (Goal 105)[32][NOTEREF _Ref453854847 \f \h] 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 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[33].

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

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[29] [HYPERLINK "http://myfwc.com/wildlifehabitats/managed/american-crocodile/"] ~~http://myfwc.com/wildlifehabitats/managed/american-crocodile/ [Accessed June 9, 2016accessed 26 Mar 2015]~~

[30][HYPERLINK "http://www.fws.gov/verobeach/MSRPPDFs/StockIslandTreeSnail.pdf"] [Accessed June 9, 2016]

[31][HYPERLINK "http://www.fws.gov/verobeach/ConservationinKeysPDFs/20130729_updated%20Stock%20Island%20Tree%20Snail%20Assessment%20Guide.pdf"] [~~Accessed 23 Sept 2013~~Accessed June 9, 2016]

[32][HYPERLINK "http://www.monroecounty-fl.gov/DocumentCenter/Home/View/1291"].

[33]" 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",

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water. The Monroe County Commissioners Resolution 426-2007[34] 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 Key Haven WWTP is in Monroe County are shown in [REF _Ref450310557 \h][REF _Ref450310960 \h], 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. Key Haven WWTP is currently operational and services the residents of Key Haven. According to FKAA, Key Haven WWTP is expected to be decommissioned within the next 2-3 years.[35]

[34] [HYPERLINK "http://www.minutes-monroe-clerk.com/WebLink8/DocView.aspx?id=131444&page=18&dbid=0"] [Accessed 6 Jan 6June 20, 2016].

[35] Phone conversation with FKAA, May 27, 2016.

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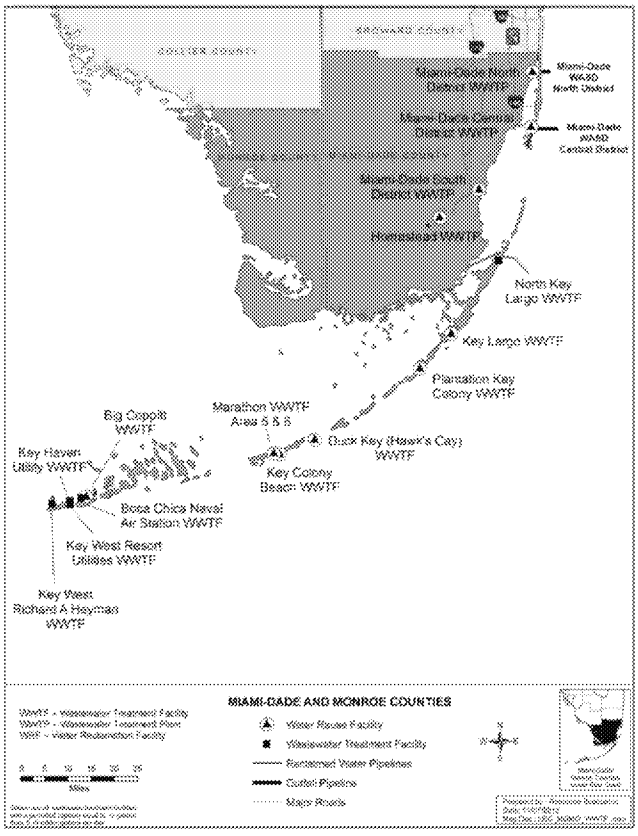

~~Figure~~ [SEQ Figure * ARABIC] ~~Locations of the Wastewater Treatment Facilities (WWTFs) in Monroe County, including Key Haven WWTF.~~

12.1.2.3.3 ~~Facilities~~The HRU site

The HRU is ~~proposed to be~~ located in Marathon ([REF _Ref450311508 \h]). The relationship between the HRU in Marathon and the proposed release site is shown in [REF _Ref450311519 \h].

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[36]. Marathon has piped potable water and a centralized

[36] [HYPERLINK "http://cityofm.tikilive.com/download/download.php?id=795"] [Accessed June 20, 2016].

sewerage system. The site is in sub-area 2 identified on the Marathon Master Plan[37], 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 would be~~are~~ shipped via international air carrier and then once cleared through customs and border protection at a major port would be ~~are sent~~~~couriered~~ by air to Marathon. This is further described in Section [REF _Ref453244645 \r \h].

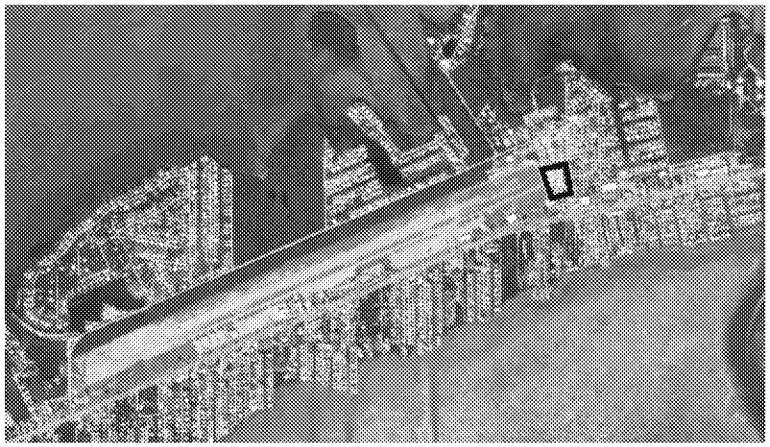

Figure [SEQ Figure * ARABIC]. The HRU site at the FKMCD Marathon base.

[37] [HYPERLINK "http://cityofm.tikilive.com/download/download.php?id=2826" \h] (Accessed June 20, 2016).

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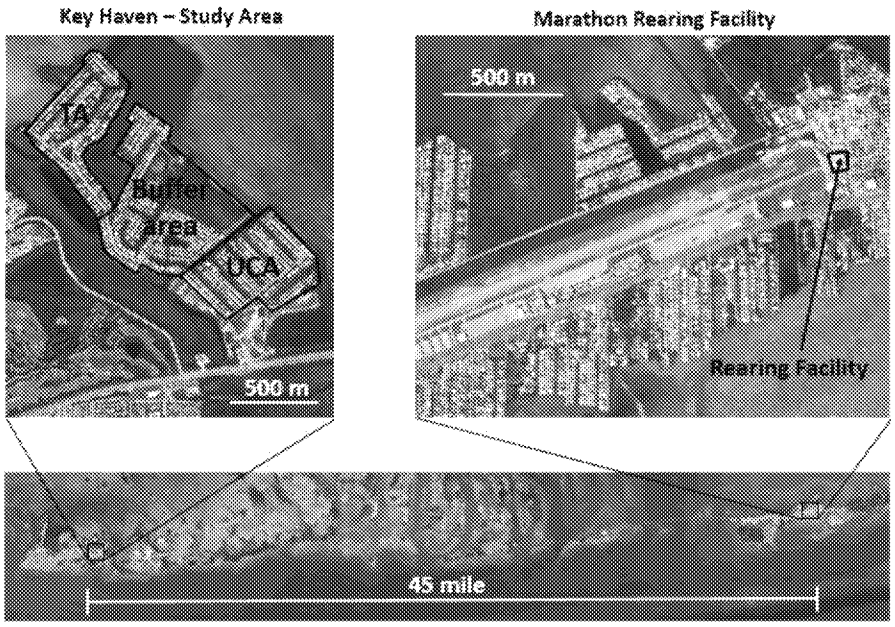

Figure [SEQ Figure * ARABIC]. Relationships between the proposed site of the HRU and the field trial location.

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 [illegible]. 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 [ADDIN EN.CITE ADDIN EN.CITE.DATA]. 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 [ADDIN EN.CITE <EndNote><Cite><Author>Lacroix</Author><Year>2012</Year><RecNum>43</RecNum><DisplayText>(Lacroix et al. 2012)</DisplayText><record><rec-number>43</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">43</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>Lacroix, R.</author><author>McKemey, A.R.</author><author>Raduan, N.</author><author>Kwee Wee, L.</author><author>Nordin, O.</author></authors></contributors><titles><title>Open Field Release of

Genetically Engineered Sterile Male Aedes aegypti in Malasia</title><secondary-title>PLoS ONE</secondary-title></titles><periodical><full-title>PLoS ONE</full-title></periodical><pages>e42771</pages><volume>7</volume><number>8</number><reprint-edition>Not in File</reprint-edition><keywords><keyword>Aedes</keyword><keyword>Aedes aegypti</keyword></keywords><dates><year>2012</year><pub-dates><date>2012</date></pub-dates></dates><label>44</label><urls></urls></record></Cite></EndNote>] and the Cayman Islands [ADDIN EN.CITE ADDIN EN.CITE.DATA] 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 line~~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 line~~strain~~ to tetracycline under a variety of scenarios (*Appendix C*). The OX513A ~~strain~~ line 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 [REF _Ref453675631 \r \h] 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~~ line to a range of temperatures, including those outside the known isothermic range of the insect (the isothermal range is reported as between 10°C - 30°C (50°F - 86°F), with optimal survival at 25-27 °C (77°F - 81°F) [ADDIN EN.CITE ADDIN EN.CITE.DATA] 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 (*Appendix D*).

~~Tolerance~~ Resistance to current insecticides is a further potential factor that could impact not only on the survivability of the OX513A ~~strain~~line, but also if the ~~strain~~ line 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 line~~strain~~ to a range of current chemical control methods, using a standardized insecticide testing regime from the World Health Organization[38] as well as using literature information. The results showed that the OX513A ~~strain~~ line 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 (*Appendix E*). A further study was conducted with the OX513A line~~strain~~ in Malaysia [ADDIN EN.CITE <EndNote><Cite><Author>Nazni</Author><Year>2009</Year><RecNum>82</RecNum><DisplayText>(Nazni et al. 2009b)</DisplayText><record><rec-number>82</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1451933589">82</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Nazni, W.A.</author><author>Selvi, S.</author><author>Lee, H.L.</author><author>Sadiyah, I.</author><author>Azahari, A.H.</author><author>Derric, N.</author><author>Vasan, S.</author></authors></contributors><titles><title>Susceptibility status of transgenic Aedes aegypti (L.) against insecticides.</title><secondary-title>Dengue Bulletin</secondary-title></titles><periodical><full-title>Dengue Bulletin</full-title></periodical><pages>124-129</pages><volume>33</volume><dates><year>2009</year></dates><urls></urls></record></Cite></EndNote>] which reported that the OX513A was susceptible to the current insecticides in use in vector control programs.

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 line~~strain~~. In order to determine the response of the OX513A line~~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~~ line was examined for longevity without tetracycline in the diet, as the length of time the ~~strain~~ line survives in the environment contributes to overall survivability potential (*Appendix F*). Furthermore, the ~~strain~~ line 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.

[38] [HYPERLINK "http://whqlibdoc.who.int/hq/1998/WHO_CDS_CPC_MAL_98.12.pdf?ua=1"] (Accessed June 15, 2016).

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12.2.1.1.1 Dose-response study to tetracycline

The response of the OX513A ~~strain~~ line 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 10 pg/mL to 1 µg/mL. Oxitec determined that concentrations of 3 ng/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 [REF _Ref450311899 \h]). Therefore the no observable effect level (NOEL) was determined to be 1 ng/mL.

[REF _Ref450311899 \h] 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 [SEQ Table * ARABIC]. Dose-response of hemizygous OX513A larvae to different concentrations of tetracycline[39].

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%)

[39] Rows do not add up to 100% as dead larvae are not recorded in this table.

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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 tetracyclines from field sites around the world as follows: tetracyclines 0.096 ng mL^{-1} to 1.3 ng mL^{-1} (e.g., chlortetracycline 0.04 ng mL^{-1} to 0.97 ng mL^{-1}, oxytetracycline 0.7 ng mL^{-1} to 1.34 ng mL^{-1} and doxycycline 0.07 ng mL^{-1} to 0.4 ng mL^{-1})[ADDIN EN.CITE ADDIN EN.CITE.DATA].

A review of environmental antibiotic degradation indicated that, in general, the highest sources of environmental tetracyclines (in the µg/L range) were from hospitals and municipal wastewater, whereas surface waters, and sea and ground waters were in the ng/L range [ADDIN EN.CITE <EndNote><Cite><Author>Homem</Author><Year>2011</Year><RecNum>224</RecNum><DisplayTex t>(Homem and Santos 2011)</DisplayText><record><rec-number>224</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463109848">224</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Homem, V.</author><author>Santos, L.</author></authors></contributors><auth-address>LEPAE, Departamento de Engenharia Quimica, Faculdade de Engenharia da Universidade do Porto, Rua Dr, Roberto Frias, 4200-465 Porto, Portugal.</auth-address><titles><title>Degradation and removal methods of antibiotics from aqueous matrices--a review</title><secondary-title>J Environ Manage</secondary-title></titles><periodical><full-title>J Environ Manage</full-title></periodical><pages>2304-47</pages><volume>92</volume><number>10</number><keywords><keyword>*Anti-Bacterial Agents</keyword><keyword>Humans</keyword><keyword>Refuse Disposal</keyword><keyword>Water/chemistry</keyword><keyword>*Water Pollutants, Chemical</keyword><keyword>Water Pollution, Chemical/*prevention & control</keyword><keyword>Water Purification/*methods</keyword></keywords><dates><year>2011</year><pub-dates><date>Oct</date></pub-dates></dates><isbn>1095-8630 (Electronic)
0301-4797 (Linking)</isbn><accession-num>21680081</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/21680081</url></related-urls></urls><electronic-resource-num>10.1016/j.jenvman.2011.05.023</electronic-resource-num></record></Cite></EndNote>]~~(Homem and Santos, 2011)~~. Key Haven residencats are serviced by

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the Key Haven Waste Water Treatment Plant (WWTP)~~Waste water drains from Key Haven and Key West via sewerage, and there are two local water treatment plants~~ (Section [REF _Ref453245045 \r \h] and [REF _Ref450310557 \h][REF _Ref450310960 \h]), which ~~that~~ could hypothetically hold waters with residues of tetracyclines. Tetracyclines 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 [ADDIN EN.CITE <EndNote><Cite><Author>Bautitz</Author><Year>2007</Year><RecNum>9</RecNum><DisplayText>(Bautitz and Nogueira 2007)</DisplayText><record><rec-number>9</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">9</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Bautitz, Ivonete Rossi</author><author>Nogueira, Raquel F.P.</author></authors></contributors><titles><title>Degradation of tetracycline by photo-Fenton process - Solar irradiation and matrix effects</title><secondary-title>J Photochem. Photobiol A: Chemistry</secondary-title></titles><pages>33-39</pages><volume>187</volume><number>1</number><reprint-edition>Not in File</reprint-edition><dates><year>2007</year><pub-dates><date>2007</date></pub-dates></dates><isbn>10106030</isbn><label>9</label><urls><related-urls><url>http://linkinghub.elsevier.com/retrieve/pii/S1010603006005053</url></related-urls></urls><electronic-resource-num>10.1016/j.jphotochem.2006.09.009</electronic-resource-num><access-date>3/28/2015</access-date></record></Cite></EndNote>]. 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). [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Homem</Author><Year>2011</Year><RecNum>224</RecNum><DisplayText>Homem and Santos (2011)</DisplayText><record><rec-number>224</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463109848">224</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Homem, V.</author><author>Santos, L.</author></authors></contributors><auth-address>LEPAE, Departamento de Engenharia Quimica, Faculdade de Engenharia da Universidade do Porto, Rua Dr, Roberto Frias, 4200-465 Porto, Portugal.</auth-address><titles><title>Degradation and removal methods of antibiotics from aqueous matrices--a review</title><secondary-title>J Environ Manage</secondary-title></titles><periodical><full-title>J Environ Manage</full-title></periodical><pages>2304-47</pages><volume>92</volume><number>10</number><keywords><keyword>*Anti-Bacterial Agents</keyword><keyword>Humans</keyword><keyword>Refuse Disposal</keyword><keyword>Water/chemistry</keyword><keyword>*Water Pollutants, Chemical</keyword><keyword>Water Pollution, Chemical/*prevention & control</keyword><keyword>Water Purification/*methods</keyword></keywords><dates><year>2011</year><pub-dates><date>Oct</date></pub-dates></dates><isbn>1095-8630 (Electronic)
0301-4797 (Linking)</isbn><accession-num>21680081</accession-num><urls><related-

urls><url>http://www.ncbi.nlm.nih.gov/pubmed/21680081</url></related-urls></urls><electronic-resource-num>10.1016/j.jenvman.2011.05.023</electronic-resource-num></record></Cite></EndNote>] report that with tetracyclines 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 tetracyclines during treatment. This is likely an overestimate for *Ae. aegypti* as waste water treatment environments are not typical *Ae. aegypti* larval habitats. Typical environments ~~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 [REF _Ref453245105 \r \h]), there are no apparent sources of high concentrations of environmental tetracyclines, as there are no commercial farming (land based or marine) enterprises, including citrus groves, 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 comprised~~ing~~ 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 [ADDIN EN.CITE ADDIN EN.CITE.DATA], especially as there are/would~~ld~~ be sufficient breeding sites (ovitraps) in the release site, so the male mosquitoes would not~~don't~~ need to fly far to find ~~the~~ females with which to mate.

Potential sources of tetracycline in and around residences in the TA would be highly unlikely to affect OX513A survival. Pet or human food from animal-derived sources with potential tetracycline residues would not affect survival for several reasons. First, animal-derived food products must have residue levels below established tolerance levels of 2 ppm in muscle, 6 ppm in liver, and 12 ppm in fat and kidney (21 CFR 556.720), which is not sufficiently high to affect OX513A because levels of tetracycline would likely have to be close to 1 µg/ml or higher in water to have an effect on eclosion and adult OX513A survival. To achieve µg/ml tetracycline in water from a tetracycline residue at the tolerance level, all the tetracycline in the pet or human food would have to leach out into the water so that 50%, 16.7%, and 8.33% of the drinking water was muscle, liver, and fat/kidney respectively. Secondly, A~~a~~ny tetracycline or tetracycline derivative in the food would also be subject to photodegradation by exposure to light resulting in lower effective concentrations in any potential mosquito habitat. Additionally, such animal-derived food would have to be left out continuously for 5-7 days and the container holding such food would need to contain sufficient fresh water throughout this time for the aquatic phase of the mosquito life cycle to be completed, allowing adults to eclose. The combined probability of all these events occurring at once is very low and, therefore, the risk of GE females surviving, mating, laying eggs that survive and eclose, and resulting in the trait persisting in the environment is negligible.

Commented [KJ13]: The tolerance levels for oxytetracycline is 0.35 ppm on apples peaches and pears. 40CFR180.337

Commented [LE14]: EPA: Please add crop residue tolerances as applicable.

The dose-response study presented in [REF _Ref450311899 \h] 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 2,500 times greater than the maximum value we found in the literature for environmental tetracyclines.

12.2.1.1.2 Conclusion

***Tetracycline concentrations above the rescue level of 1 ng/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 proposed release site that have the potential to provide sufficient levels of tetracycline residues. Data from the literature regarding environmental presence of tetracyclines and the data reported in Table 2 [REF _Ref453245194 \h * MERGEFORMAT] 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 an OX513A female were to mated with an OX513A male and lays her eggs in water with tetracycline and some adults were to may emerge depending on the tetracycline concentration, then these males would 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)[ADDIN EN.CITE <EndNote><Cite><Author>Harris</Author><Year>2011</Year><RecNum>92</RecNum><DisplayText>(Harris et al. 2011)</DisplayText><record><rec-number>92</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1455908312">92</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Harris, A. F.</author><author>Nimmo, D.</author><author>McKemey, A. R.</author><author>Kelly, N.</author><author>Scaife, S.</author><author>Donnelly, C. A.</author><author>Beech, C.</author><author>Petrie, W. D.</author><author>Alphey, L.</author></authors></contributors><auth-address>Mosquito Research and Control Unit (MRCU), Grand Cayman, Cayman Islands.</auth-address><titles><title>Field performance of engineered male mosquitoes</title><secondary-title>Nat Biotechnol</secondary-title></titles><periodical><full-title>Nat Biotechnol</full-title></periodical><pages>1034-7</pages><volume>29</volume><number>11</number><keywords><keyword>Aedes/*genetics/virology</keyword><keyword>Animals</keyword><keyword>Animals, Genetically Modified/*genetics</keyword><keyword>Arboviruses/genetics/physiology</keyword><keyword>Dengue/*prevention & control</keyword><keyword>*Dengue Virus</keyword><keyword>Female</keyword><keyword>Humans</keyword><keyword>Infertility, Male/*genetics</keyword><keyword>Male</keyword><keyword>Pest Control, Biological/*methods</keyword><keyword>Reproduction/genetics/physiology</keyword><keyword>Sexual Behavior, Animal</keyword></keywords><dates><year>2011</year><pub-dates><date>Nov</date></pub-dates></dates><isbn>1546-1696 (Electronic)
1087-0156***

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*(Linking)</isbn><accession-num>22037376</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/22037376</url></related-urls></urls><electronic-resource-num>10.1038/nbt.2019</electronic-resource-num></record></Cite></EndNote>]. As Ae. aegypti prefers to lay eggs in different containers (a phenomenon known as skip oviposition [ADDIN EN.CITE <EndNote><Cite><Author>Rey</Author><Year>2014</Year><RecNum>204</RecNum><DisplayText>(Rey and O'Connell 2014)</DisplayText><record><rec-number>204</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463108514">204</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Rey, J. R.</author><author>O'Connell, S. M.</author></authors></contributors><auth-address>University of Florida - IFAS, Florida Medical Entomology Laboratory and Department of Entomology and Nematology, Vero Beach, FL 32962, U.S.A.. jrey@ufl.edu.</auth-address><titles><title>Oviposition by Aedes aegypti and Aedes albopictus: influence of congeners and of oviposition site characteristics</title><secondary-title>J Vector Ecol</secondary-title></titles><periodical><full-title>J Vector Ecol</full-title></periodical><pages>190-6</pages><volume>39</volume><number>1</number><keywords><keyword>Aedes/*physiology</keyword><keyword>Animals</keyword><keyword>Female</keyword><keyword>Florida</keyword><keyword>Oviposition/*physiology</keyword><keyword>Aedes aegypti</keyword><keyword>Aedes albopictus</keyword><keyword>mosquito</keyword><keyword>oviposition</keyword><keyword>site selection</keyword></keywords><dates><year>2014</year><pub-dates><date>Jun</date></pub-dates></dates><isbn>1948-7134 (Electronic)
1081-1710 (Linking)</isbn><accession-num>24820572</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24820572</url></related-urls></urls><electronic-resource-num>10.1111/j.1948-7134.2014.12086.x</electronic-resource-num></record></Cite></EndNote>]) the probability that ~~they would~~ all the oviposition containers 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~~ line (adult males and females) has been evaluated in the laboratory. The homozygous OX513A ~~strain~~ line 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 1 µg/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.

Commented [KJ15]: I would change to mosquitoes just to be clearer about what you're talking about.

These experiments therefore examine the longevity of the two types of OX513A female that most plausibly would be 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 lethality 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 in *Appendix F*. Environmental factors are known to reduce daily survival compared to in the laboratory [ADDIN EN.CITE <EndNote><Cite><Author>Joy</Author><Year>2012</Year><RecNum>90</RecNum><DisplayText>(Joy et al. 2012)</DisplayText><record><rec-number>90</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1455821800">90</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Joy, T. K.</author><author>Jeffrey Gutierrez, E. H.</author><author>Ernst, K.</author><author>Walker, K. R.</author><author>Carriere, Y.</author><author>Torabi, M.</author><author>Riehle, M. A.</author></authors></contributors><auth-address>Department of Entomology, University of Arizona, Tucson, Arizona, USA.</auth-address><titles><title>Aging field collected Aedes aegypti to determine their capacity for dengue transmission in the southwestern United States</title><secondary-title>PLoS One</secondary-title></titles><periodical><full-title>PLoS ONE</full-title></periodical><pages>e46946</pages><volume>7</volume><number>10</number><keywords><keyword>Aedes/genetics/*virology</keyword><keyword>Aging</keyword><keyword>Animals</keyword><keyword>Calcium-Binding Proteins/*genetics</keyword><keyword>Dengue/*transmission</keyword><keyword>Dengue Virus/*pathogenicity</keyword><keyword>Female</keyword><keyword>Gene Expression Regulation, Developmental</keyword><keyword>Genes, Insect</keyword><keyword>Humans</keyword><keyword>Insect Proteins/*genetics</keyword><keyword>Insect Vectors/genetics/*virology</keyword><keyword>Southwestern United States</keyword></keywords><dates><year>2012</year></dates><isbn>1932-6203 (Electronic)
1932-6203 (Linking)</isbn><accession-num>23077536</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23077536</url></related-urls></urls><custom2>3470585</custom2><electronic-resource-num>10.1371/journal.pone.0046946</electronic-resource-num></record></Cite></EndNote>] and from previous trials with OX513A [ADDIN EN.CITE ADDIN EN.CITE.DATA].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.

Commented [KJ16]: Just a short statement as to lab versus field values would be significant for public understanding.

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 [ADDIN EN.CITE <EndNote><Cite><Author>Agwuh</Author><Year>2006</Year><RecNum>96</RecNum><DisplayText>(Agwuh and MacGowan 2006)</DisplayText><record><rec-number>96</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463078298">96</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Agwuh, K. N.</author><author>MacGowan, A.</author></authors></contributors><auth-address>Department of Medical Microbiology, Old Medical School, Leeds General Infirmary Great George Street, Leeds LS1 2EX, UK.</auth-address><titles><title>Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines</title><secondary-title>J Antimicrob Chemother</secondary-title></titles><periodical><full-title>J Antimicrob Chemother</full-title></periodical><pages>256-65</pages><volume>58</volume><number>2</number><keywords><keyword>Humans</keyword><keyword>Tetracyclines/blood/*pharmacokinetics/*pharmacology</keyword></keywords><dates><year>2006</year><pub-dates><date>Aug</date></pub-dates></dates><isbn>0305-7453 (Print)
0305-7453 (Linking)</isbn><accession-num>16816396</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/16816396</url></related-urls></urls><electronic-resource-num>10.1093/jac/dkl224</electronic-resource-num></record></Cite></EndNote>]. In both humans and livestock, the peak concentration of tetracycline in blood (plasma) following standard therapeutic doses normally remains below 10 µg/ml [ADDIN EN.CITE ADDIN EN.CITE.DATA]. 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) [ADDIN EN.CITE ADDIN EN.CITE.DATA]. 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 ([REF _Ref450312496 \h]).

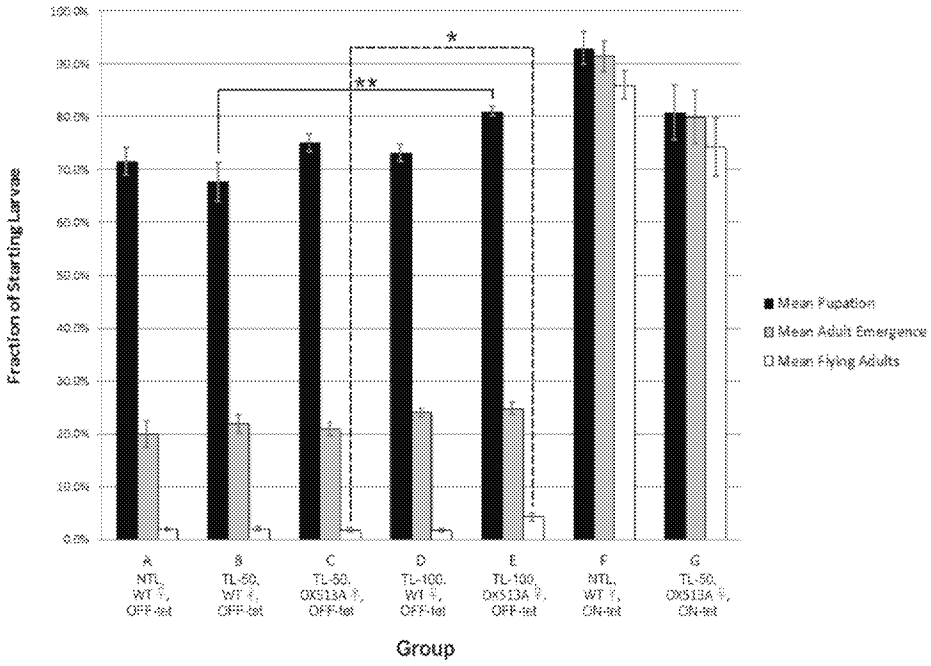

Figure [SEQ Figure * ARABIC]. Summary of results of tetracycline-loaded blood study.

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 and G) are shown for reference. NTL: Non tet-loaded. TL-50: Tetracycline loaded, 50 µg/mL. TL-100: Tetracycline loaded, 100 µg/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 ([REF _Ref450312496 \h]) 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 that~~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 would be ~~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

Based on the results of this tetracycline-loaded blood study, Taken together with the longevity data in 12.2.1.2, FDA concludes these results support the assertion that the ability of the strain OX513A line 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 line from the environment, and standard mosquito control would continue to be used, as necessary, during the duration of the proposed field trial (see Section [REF _Ref453245461 \r \h]). 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 (*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 line 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[40] 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 [REF _Ref453245495 \h].

Table [SEQ Table * ARABIC]. Mosquito mortality recorded 24 hours after exposure to insecticide.

Insecticide	Dose	OX513A No. tested	OX513A No. alive	OX513A No. dead	OX513A % mort.	NEW ORLEANS No. tested	NEW ORLEANS No. alive	NEW ORLEANS No. dead	NEW ORLEANS % mort.

[40] [HYPERLINK "http://whqlibdoc.who.int/hq/2006/WHO_CDS_NTD_WHOPES_GCDPP_2006.3_eng.pdf" \h][Accessed June 15, 2015~~8th May 2014~~].

ED_006741_00009696-00075

temephos	0.012 mg/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~~ line 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.

Commented [KJ17]: "...and the original colony was established by the CDC."

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.

Commented [KJ18]: ...old pesticide solutions."

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 line ~~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~~ line.

A separate study was conducted in Malaysia by [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Nazni</Author><Year>2009</Year><RecNum>82</RecNum><DisplayText>Nazni et al. (2009b)</DisplayText><record><rec-number>82</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1451933589">82</key></foreign-

keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Nazni, W.A.</author><author>Selvi, S.</author><author>Lee, H.L.</author><author>Sadiyah, I.</author><author>Azahari, A.H.</author><author>Derric, N.</author><author>Vasan, S.</author></authors></contributors><titles><title>Susceptibility status of transgenic Aedes aegypti (L.) against insecticides.</title><secondary-title>Dengue Bulletin</secondary-title></titles><periodical><full-title>Dengue Bulletin</full-title></periodical><pages>124-129</pages><volume>33</volume><dates><year>2009</year></dates><urls></urls></record></Cite></EndNote>]. This study compared the susceptibility of the ~~line~~strain MyRIDL-513A[41] and the laboratory ~~line~~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 line~~strain ~~is susceptible to the currently used insecticides (as described in Section~~ []~~) 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 [ADDIN EN.CITE <EndNote><Cite><Author>Couret</Author><Year>2014</Year><RecNum>116</RecNum><DisplayText>(Couret et al. 2014)</DisplayText><record><rec-number>116</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463104428">116</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Couret, J.</author><author>Dotson, E.</author><author>Benedict, M. Q.</author></authors></contributors><auth-address>Department of Biology, Emory University, Atlanta, Georgia, United States of America.
Division of Parasitic Diseases and Malaria, Center for Global Health, Centers for Disease Control and Prevention, Atlanta, Georgia, United States of America.
Dipartimento di Medicina Sperimentale e Scienze Biochimiche,

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

Universita di Perugia, Perugia, Italy.</auth-address><titles><title>Temperature, larval diet, and density effects on development rate and survival of Aedes aegypti (Diptera: Culicidae)</title><secondary-title>PLoS One</secondary-title></titles><periodical><full-title>PLoS ONE</full-title></periodical><pages>e87468</pages><volume>9</volume><number>2</number><keywords><keyword>Aedes/*physiology/virology</keyword><keyword>Analysis of Variance</keyword><keyword>Animals</keyword><keyword>Dengue/transmission</keyword><keyword>Diet</keyword><keyword>Feeding Behavior/*physiology</keyword><keyword>Humans</keyword><keyword>Insect Vectors/*physiology/virology</keyword><keyword>Larva/physiology</keyword><keyword>Population Density</keyword><keyword>Survival Analysis</keyword><keyword>*Temperature</keyword><keyword>Yellow Fever/transmission</keyword></keywords><dates><year>2014</year></dates><isbn>1932-6203 (Electronic)
1932-6203 (Linking)</isbn><accession-num>24498328</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24498328</url></related-urls></urls><custom2>3911954</custom2><electronic-resource-num>10.1371/journal.pone.0087468</electronic-resource-num></record></Cite></EndNote>].

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° F] [ADDIN EN.CITE ADDIN EN.CITE.DATA]. 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°C [104°F]) are unlikely to limit the species but low temperatures are limiting with the threshold being around the 15°C [59°F] isotherm. At temperatures lower than 15°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.

Commented [KJ19]: (except in East Africa where it is native)

Global historical collections and laboratory experiments on this well-studied vector have suggested its distribution is limited by the 10°C [~50°F] winter isotherm[42] (Christophers, 1960), while a more recent and complex stochastic population dynamics model analysis suggests the temperature's limiting value to be more towards the 15°C[~59 °F] yearly isotherm [ADDIN EN.CITE <EndNote><Cite><Author>Otero</Author><Year>2006</Year><RecNum>165</RecNum><DisplayText>(Otero et al. 2006)</DisplayText><record><rec-number>165</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106826">165</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Otero, Marcelo</author><author>Solari, Hernán G.</author><author>Schweigmann, Nicolás</author></authors></contributors><titles><title>A

[42] 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.

Stochastic Population Dynamics Model for Aedes Aegypti: Formulation and Application to a City with Temperate Climate</title><secondary-title>Bulletin of Mathematical Biology</secondary-title><short-title>A Stochastic Population Dynamics Model for Aedes Aegypti</short-title></titles><periodical><full-title>Bulletin of Mathematical Biology</full-title></periodical><pages>1945-1974</pages><volume>68</volume><number>8</number><dates><year>2006</year><pub-dates><date>2006/11/03/</date></pub-dates></dates><isbn>0092-8240, 1522-9602</isbn><urls><related-urls><url>http://link.springer.com/10.1007/s11538-006-9067-y</url><url>http://download.springer.com/static/pdf/969/art%253A10.1007%252Fs11538-006-9067-y.pdf?originUrl=http%3A%2F%2Flink.springer.com%2Farticle%2F10.1007%2Fs11538-006-9067-y&token2=exp=1463108106~acl=%2Fstatic%2Fpdf%2F969%2Fart%25253A10.1007%25252Fs11538-006-9067-y.pdf%3ForiginUrl%3Dhttp%253A%252F%252Flink.springer.com%252Farticle%252F10.1007%252Fs11538-006-9067-y*~hmac=5e87fa0f036684db97970c67e8b144a810272698fb9e42e33a8aea2d8038d265</url></related-urls></urls><electronic-resource-num>10.1007/s11538-006-9067-y</electronic-resource-num><remote-database-provider>CrossRef</remote-database-provider><language>en</language><access-date>2015/03/28/04:18:00</access-date></record></Cite></EndNote>]. 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. [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Scholte</Author><Year>2010</Year><RecNum>173</RecNum><DisplayText>Scholte et al. (2010)</DisplayText><record><rec-number>173</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106827">173</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Scholte, E. J.</author><author>Hartog, W. D.</author><author>Dik, M.</author><author>Schoelitsz, B.</author><author>Brooks, M.</author><author>Schaffner, F.</author><author>Foussadier, R.</author><author>Braks, M.</author><author>Beeuwkes, J.</author></authors></contributors><titles><title>Introduction and control of three invasive mosquito species in the Netherlands, July-October 2010</title><secondary-title>Euro Surveill</secondary-title></titles><periodical><full-title>Euro Surveill</full-title></periodical><pages>1-4</pages><volume>15</volume><number>45</number><dates><year>2010</year><pub-dates><date>2010/11/11/</date></pub-dates></dates><urls><related-urls><url>http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19710</url></related-urls></urls><language>en</language><access-date>2015/03/31/14:46:44</access-date></record></Cite></EndNote>] indicated that *Ae. aegypti* could not survive winter temperatures in Northern Europe. In a recent study, [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Thomas</Author><Year>2012</Year><RecNum>183</RecNum><DisplayText>Thomas et al. (2012)</DisplayText><record><rec-number>183</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106827">183</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Thomas, Stephanie Margarete</author><author>Obermayr, Ulla</author><author>Fischer, Dominik</author><author>Kreyling,

Juergen</author><author>Beierkuhnlein, Carl</author></authors></contributors><titles><title>Low-temperature threshold for egg survival of a post-diapause and non-diapause European aedine strain, Aedes albopictus (Diptera: Culicidae)</title><secondary-title>Parasit Vectors</secondary-title><short-title>Low-temperature threshold for egg survival of a post-diapause and non-diapause European aedine strain, Aedes albopictus (Diptera</short-title></titles><periodical><full-title>Parasit Vectors</full-title></periodical><pages>1-7</pages><volume>5</volume><number>1</number><dates><year>2012</year><pub-dates><date>2012</date></pub-dates></dates><urls><related-urls><url>http://www.biomedcentral.com/content/pdf/1756-3305-5-100.pdf</url><url>http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3259067/pdf/1756-3305-5-1.pdf</url></related-urls></urls><remote-database-provider>Google Scholar</remote-database-provider><access-date>2015/03/28/04:22:18</access-date></record></Cite></EndNote>] 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 Florida Keys.

12.2.1.4.1 Study on the temperature response of OX513A

The temperature response of the OX513A line ~~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~~line, 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 (*Appendix D*).

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 would be~~are~~ likely to encounter in the field and that they would~~ill~~ be extremely unlikely to expand the habitable geographic range of *Ae. aegypti*.

The geophysical containment of the species is also discussed in Section [REF _Ref453245747 \r \h].

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~~ line is impacted by sensitivity to temperature, the antibiotic tetracycline and its analogues,

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used to control the repressible lethality of the strain line, 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 line 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).***

Commented [KJ20]: I thought tTAV was constitutively expressed. Its activity of repressing fatal transcriptional activation was inhibited by tetracycline binding. This needs to be reworded.

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 [ADDIN EN.CITE <EndNote><Cite><Author>Hartberg</Author><Year>1971</Year><RecNum>142</RecNum><DisplayText>(Hartberg 1971)</DisplayText><record><rec-number>142</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106826">142</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Hartberg, W. K.</author></authors></contributors><titles><title>Observations on the mating behaviour of Aedes aegypti in nature</title><secondary-title>Bulletin of the World Health Organization</secondary-title></titles><periodical><full-title>Bulletin of the World Health Organization</full-title></periodical><pages>847</pages><volume>45</volume><number>6</number><dates><year>1971</year><pub-dates><date>1971</date></pub-dates></dates><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2428001/</url><url>http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2428001/pdf/bullwho00199-0153.pdf</url></related-urls></urls><remote-database-provider>Google Scholar</remote-database-provider><access-date>2015/03/28/04:11:14</access-date></record></Cite></EndNote>]. These aggregations are primarily composed of males, with females entering the swarm singly. Pheromones are also involved in swarming behavior [ADDIN EN.CITE

<EndNote><Cite><Author>Fawaz</Author><Year>2014</Year><RecNum>124</RecNum><DisplayText>(Fawaz et al. 2014)</DisplayText><record><rec-number>124</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463105130">124</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Fawaz, E. Y.</author><author>Allan, S. A.</author><author>Bernier, U. R.</author><author>Obenauer, P. J.</author><author>Diclaro, J. W., 2nd</author></authors></contributors><auth-address>Vector Biology Research Program, U.S. Naval Medical Research Unit No. 3, Abbassia, Cairo, Egypt. Emadeldin.yehia@gmail.com.</auth-address><titles><title>Swarming mechanisms in the yellow fever mosquito: aggregation pheromones are involved in the mating behavior of Aedes aegypti</title><secondary-title>J Vector Ecol</secondary-title></titles><periodical><full-title>J Vector Ecol</full-title></periodical><pages>347-54</pages><volume>39</volume><number>2</number><keywords><keyword>Aedes/*metabolism/*physiology</keyword><keyword>Animals</keyword><keyword>Female</keyword><keyword>Male</keyword><keyword>Pheromones/*metabolism</keyword><keyword>Sexual Behavior, Animal/physiology</keyword><keyword>Yellow Fever/transmission</keyword><keyword>Aedes aegypti</keyword><keyword>aggregation pheromones</keyword><keyword>mating behavior</keyword><keyword>swarm formation</keyword></keywords><dates><year>2014</year><pub-dates><date>Dec</date></pub-dates></dates><isbn>1948-7134 (Electronic)
1081-1710 (Linking)</isbn><accession-num>25424264</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25424264</url></related-urls></urls><electronic-resource-num>10.1111/jvec.12110</electronic-resource-num></record></Cite></EndNote>]. Mating occurs in flight, where males and females meet, form a "copula" in mid-air, and mate in a matter of seconds [ADDIN EN.CITE ADDIN EN.CITE.DATA]. 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 [ADDIN EN.CITE ADDIN EN.CITE.DATA].

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 or eggs, which are then fertilized and oviposited (laid). Although females may go through several gonotrophic cycles in their lifespan, once ~~as~~ inseminated, females store enough spermatozoa to fertilize a number of egg batches. ~~,~~ ~~t~~They are therefore largely regarded to mate only once during their lifetime [ADDIN EN.CITE <EndNote><Cite><Author>Pascini</Author><Year>2012</Year><RecNum>207</RecNum><DisplayText>(Pascini et al. 2012)</DisplayText><record><rec-number>207</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463108700">207</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pascini, T. V.</author><author>Ramalho-Ortigao,

Commented [KJ21]: I made a few changes here to make the sentences flow. Take it or leave it.

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M.</author><author>Martins, G. F.</author></authors></contributors><auth-address>Departamento de Biologia Geral, Universidade Federal de Vicosa, Vicosa, MG, Brasil.</auth-address><titles><title>Morphological and morphometrical assessment of spermathecae of Aedes aegypti females</title><secondary-title>Mem Inst Oswaldo Cruz</secondary-title></titles><periodical><full-title>Mem Inst Oswaldo Cruz</full-title></periodical><pages>705-12</pages><volume>107</volume><number>6</number><keywords><keyword>Aedes/physiology/*ultrastructure</keyword><keyword>Animals</keyword><keyword>Exocrine Glands/physiology/secretion/*ultrastructure</keyword><keyword>Female</keyword><keyword>Histocytochemistry</keyword><keyword>Insemination/*physiology</keyword><keyword>Male</keyword><keyword>Microscopy, Electron</keyword><keyword>Oviducts/anatomy & histology</keyword><keyword>Sperm Transport</keyword><keyword>Spermatozoa/*physiology</keyword></keywords><dates><year>2012</year><pub-dates><date>Sep</date></pub-dates></dates><isbn>1678-8060 (Electronic)
0074-0276 (Linking)</isbn><accession-num>22990957</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/22990957</url></related-urls></urls></record></Cite></EndNote>]. ~~, as s~~ Seminal fluid proteins ~~are~~ transferred with that original mating ~~, which~~ render females unreceptive and more refractory to further copulation [ADDIN EN.CITE ADDIN EN.CITE.DATA].

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 [ADDIN EN.CITE <EndNote><Cite><Author>Helinski</Author><Year>2011</Year><RecNum>144</RecNum><DisplayText>(Helinski and Harrington 2011)</DisplayText><record><rec-number>144</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106826">144</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Helinski, Michelle E. H.</author><author>Harrington, Laura C.</author></authors></contributors><titles><title>Male Mating History and Body Size Influence Female Fecundity and Longevity of the Dengue Vector Aedes aegypti</title><secondary-title>Journal of Medical Entomology</secondary-title></titles><periodical><full-title>Journal of Medical Entomology</full-title></periodical><pages>202-211</pages><volume>48</volume><number>2</number><dates><year>2011</year><pub-dates><date>2011/03/01/</date></pub-dates></dates><isbn>00222585, 00222585</isbn><urls><related-urls><url>http://jme.oxfordjournals.org/cgi/doi/10.1603/ME10071</url><url>http://jme.oxfordjournals.org/content/jmedent/48/2/202.full.pdf</url></related-urls></urls><electronic-resource-num>10.1603/ME10071</electronic-resource-num><remote-database-provider>CrossRef</remote-database-provider><language>en</language><access-date>2015/03/28/04:11:33</access-date></record></Cite></EndNote>]. Nonetheless, even small

ED_006741_00009696-00083

males appear to transfer sufficient seminal fluid proteins to prevent further mating of the female [ADDIN EN.CITE <EndNote><Cite><Author>Dickinson</Author><Year>1997</Year><RecNum>122</RecNum><DisplayText>(Dickinson and Klowden 1997)</DisplayText><record><rec-number>122</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463104732">122</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Dickinson, J. M.</author><author>Klowden, M. J.</author></authors></contributors><auth-address>Division of Entomology, University of Idaho, Moscow 83844-233, USA.</auth-address><titles><title>Reduced transfer of male accessory gland proteins and monandry in female Aedes aegypti mosquitoes</title><secondary-title>J Vector Ecol</secondary-title></titles><periodical><full-title>J Vector Ecol</full-title></periodical><pages>95-8</pages><volume>22</volume><number>1</number><keywords><keyword>Aedes/*metabolism</keyword><keyword>Animals</keyword><keyword>*Drosophila Proteins</keyword><keyword>Female</keyword><keyword>Larva</keyword><keyword>Male</keyword><keyword>Peptides/*metabolism</keyword><keyword>Sexual Behavior, Animal</keyword></keywords><dates><year>1997</year><pub-dates><date>Jun</date></pub-dates></dates><isbn>1081-1710 (Print)
1081-1710 (Linking)</isbn><accession-num>9221745</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/9221745</url></related-urls></urls></record></Cite></EndNote>].

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 ([ADDIN EN.CITE <EndNote><Cite><Author>Bargielowski</Author><Year>2011</Year><RecNum>8</RecNum><DisplayText>(Bargielowski et al. 2011a)</DisplayText><record><rec-number>8</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">8</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>Bargielowski, Irka</author><author>Alphey, Luke</author><author>Koella, Jacob C.</author></authors><secondary-authors><author>Langsley, Gordon</author></secondary-authors></contributors><titles><title>Cost of Mating and Insemination Capacity of a Genetically Modified Mosquito Aedes aegypti OX513A Compared to Its Wild Type Counterpart</title><secondary-title>PLoS ONE</secondary-title></titles><periodical><full-title>PLoS ONE</full-title></periodical><pages>e26086</pages><volume>6</volume><number>10</number><reprint-edition>Not in File</reprint-edition><keywords><keyword>Aedes</keyword></keywords><dates><year>2011</year><pub-dates><date>2011</date></pub-dates></dates><isbn>1932-6203</isbn><label>8</label><urls><related-urls><url>http://dx.plos.org/10.1371/journal.pone.0026086</url></related-urls></urls><electronic-

resource-num>10.1371/journal.pone.0026086</electronic-resource-num><access-date>3/28/2015</access-date></record></Cite></EndNote>] [REF _Ref450313211 \h]).

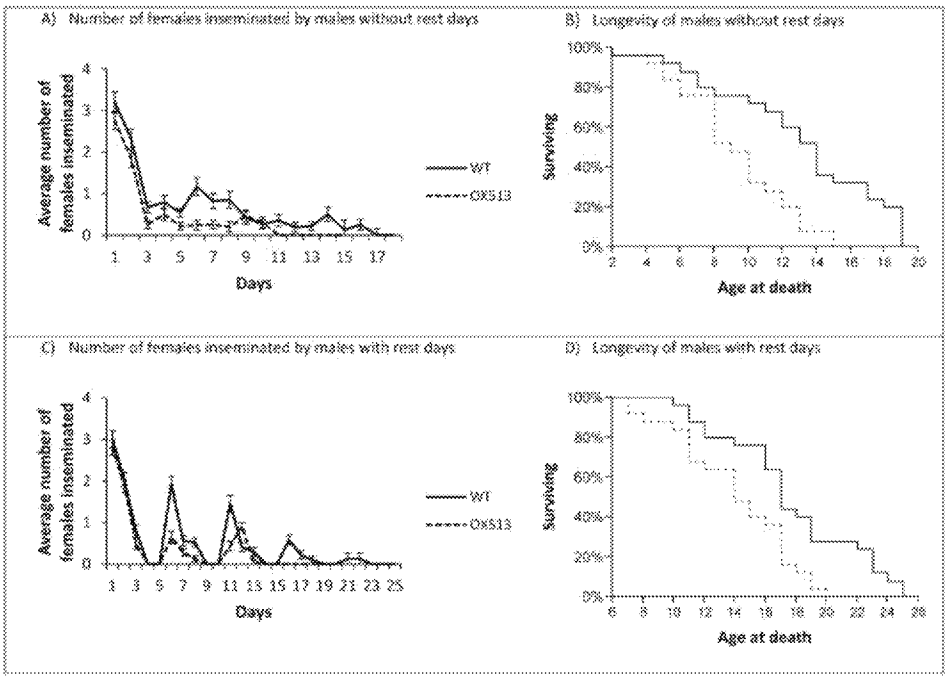

Figure [SEQ Figure * ARABIC]. Insemination capacity of OX513A males (from Bargielowski *et al.*, 2011a).

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 [ADDIN EN.CITE ADDIN EN.CITE.DATA]~~(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 [ADDIN EN.CITE

<EndNote><Cite><Author>Cator</Author><Year>2009</Year><RecNum>111</RecNum><DisplayT
ext>(Cator et al. 2009)</DisplayText><record><rec-number>111</rec-number><foreign-keys><key
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timestamp="1463104033">111</key></foreign-keys><ref-type name="Journal Article">17</ref-
type><contributors><authors><author>Cator, L. J.</author><author>Arthur, B.
J.</author><author>Harrington, L. C.</author><author>Hoy, R.
R.</author></authors></contributors><auth-address>Department of Entomology, Cornell
University, Ithaca, NY 14853, USA.</auth-address><titles><title>Harmonic convergence in the love
songs of the dengue vector mosquito</title><secondary-title>Science</secondary-
title></titles><periodical><full-title>Science</full-title></periodical><pages>1077-
9</pages><volume>323</volume><number>5917</number><keywords><keyword>Aedes/*physi
ology</keyword><keyword>*Animal
Communication</keyword><keyword>Animals</keyword><keywo rd>Auditory
Perception</keyword><keyword>Dengue/transmission</keyword><keyword>Evoked
Potentials</keyword><keyword>Female</keyword><keyword>Flight,
Animal</keyword><keyword>Hearing</keyword><keyword>Insect
Vectors/*physiology</keyword><keyword>Male</keyword><keyword>Pitch
Perception</keyword><keyword>Sense Organs/physiology</keyword><keyword>*Sexual
Behavior, Animal</keyword><keyword>Wings,
Animal/physiology</keyword></keywords><dates><year>2009</year><pub-dates><date>Feb
20</date></pub-dates></dates><isbn>1095-9203 (Electronic)
0036-8075
(Linking)</isbn><accession-num>19131593</accession-num><urls><related-
urls><url>http://www.ncbi.nlm.nih.gov/pubmed/19131593</url></related-
urls></urls><custom2>2847473</custom2><electronic-resource-
num>10.1126/science.1166541</electronic-resource-num></record></Cite></EndNote>]. 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 line~~ 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 [ADDIN EN.CITE ADDIN EN.CITE.DATA] and Brazil [ADDIN EN.CITE <EndNote><Cite><Author>Carvalho</Author><Year>2015</Year><RecNum>60</RecNum><DisplayText>(C
arvalho et al. 2015)</DisplayText><record><rec-number>60</rec-number><foreign-keys><key app="EN"
db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1445973125">60</key></foreign-keys><ref-
type name="Journal Article">17</ref-type><contributors><authors><author>Carvalho, Danilo
O.</author><author>McKemey, A.</author><author>Garziera, L.</author><author>Lacroix,
R.</author><author>Donnelly, Christl A.</author><author>Alphey, L.</author><author>Malavasi,
A.
/author><author>Capurro, Margareth L.</author></authors></contributors><titles><title>Supression of
a field population of Aedes aegypti in Brazil by sustained release of transgenic male
mosquitoes.</title><secondary-title>PLoS Neglected Tropical Diseases</secondary-
title></titles><periodical><full-title>PLoS Neglected Tropical Diseases</full-

title></periodical><pages>e0003864</pages><volume>9</volume><number>7</number><dates><year>2015</year></dates><urls></urls></record></Cite></EndNote>].

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 ([REF _Ref453830164 \h]). The OX513A ~~line 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).

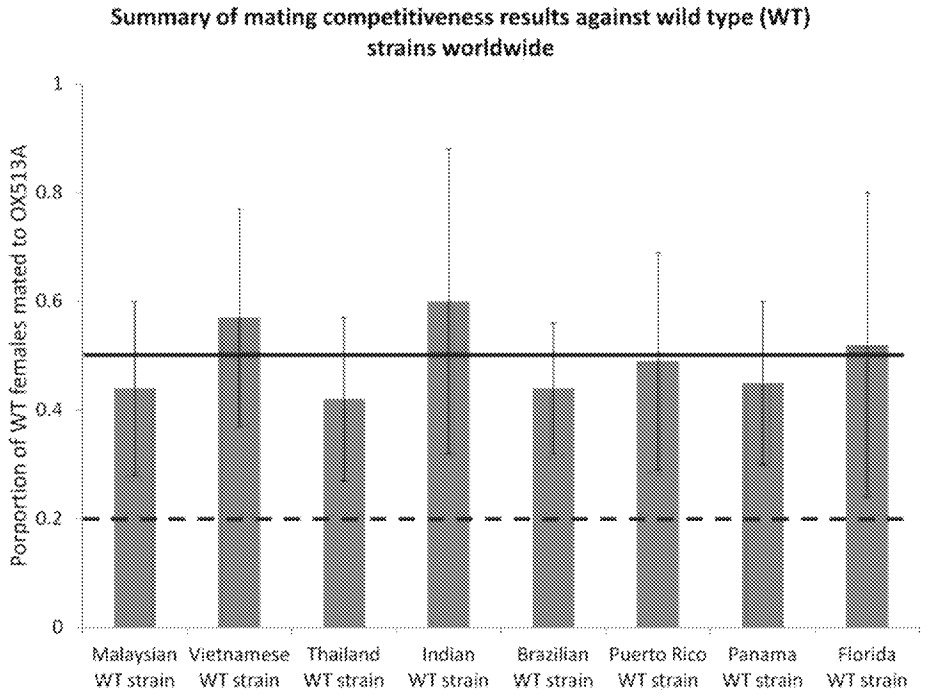

Figure [SEQ Figure * ARABIC]. Summary of mating competitiveness results against wild-type *Ae. aegypti* strains worldwide in the laboratory.

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 aimed ~~to at demonstrate~~ demonstrating the proof of principle that Oxitec could produce competitive males, used low rearing densities which gave a mating competitiveness estimate of 0.56 (95% CI: 0.032-1.97, [ADDIN EN.CITE <EndNote><Cite><Author>Harris</Author><Year>2011</Year><RecNum>92</RecNum><DisplayText>(Harris et al. 2011)</DisplayText><record><rec-number>92</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1455908312">92</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Harris, A. F.</author><author>Nimmo, D.</author><author>McKemey, A. R.</author><author>Kelly, N.</author><author>Scaife, S.</author><author>Donnelly, C. A.</author><author>Beech, C.</author><author>Petrie, W. D.</author><author>Alphey, L.</author></authors></contributors><auth-address>Mosquito Research and Control Unit (MRCU), Grand Cayman, Cayman Islands.</auth-address><titles><title>Field performance of engineered male mosquitoes</title><secondary-title>Nat Biotechnol</secondary-title></titles><periodical><full-title>Nat Biotechnol</full-title></periodical><pages>1034-7</pages><volume>29</volume><number>11</number><keywords><keyword>Aedes/*genetics/virology</keyword><keyword>Animals</keyword><keyword>Animals, Genetically Modified/*genetics</keyword><keyword>Arboviruses/genetics/physiology</keyword><keyword>Dengue/*prevention & control</keyword><keyword>*Dengue Virus</keyword><keyword>Female</keyword><keyword>Humans</keyword><keyword>Infertility, Male/*genetics</keyword><keyword>Male</keyword><keyword>Pest Control, Biological/*methods</keyword><keyword>Reproduction/genetics/physiology</keyword><keyword>Sexual Behavior, Animal</keyword></keywords><dates><year>2011</year><pub-dates><date>Nov</date></pub-dates></dates><isbn>1546-1696 (Electronic)
1087-0156 (Linking)</isbn><accession-num>22037376</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/22037376</url></related-urls></urls><electronic-resource-num>10.1038/nbt.2019</electronic-resource-num></record></Cite></EndNote>]). 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 [ADDIN EN.CITE ADDIN EN.CITE.DATA]. This range is not unexpected given that mating competitiveness as measured by this approach includes any effect of mass rearing, handling and distribution, and in 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~~ where the local *Ae. aegypti* population had already been suppressed during that period [ADDIN EN.CITE <EndNote><Cite><Author>Carvalho</Author><Year>2015</Year><RecNum>60</RecNum><DisplayText>(Carvalho et al. 2015)</DisplayText><record><rec-number>60</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1445973125">60</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Carvalho, Danilo O.</author><author>McKemey, A.</author><author>Garziera, L.</author><author>Lacroix, R.</author><author>Donnelly, Christl A.</author><author>Alphey, L.</author><author>Malavasi, A.</author><author>Capurro, Margareth L.</author></authors></contributors><titles><title>Supression of a field population of Aedes aegypti in Brazil by sustained release of transgenic male mosquitoes.</title><secondary-title>PLoS Neglected Tropical Diseases</secondary-title></titles><periodical><full-title>PLoS Neglected Tropical Diseases</full-title></periodical><pages>e0003864</pages><volume>9</volume><number>7</number><dates><year>2015</year></dates><urls></urls></record></Cite></EndNote>].

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*) [ADDIN EN.CITE ADDIN EN.CITE.DATA]. Therefore the mating competitiveness range seen over a variety of different environments with OX513A is predominantly within the reported 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 [SEQ Table * ARABIC]. Summary of mating competitiveness evaluation of the Oxitec OX513A males in the wild.

Cayman Islands 2009	
Cayman Islands 2010	
Itaberaba, Brazil 2011-2012	
Itaberaba, Brazil 2011-2012	
Itaberaba, Brazil 2011-2012	
Itaberaba, Brazil 2011-2012	
Itaberaba, Brazil 2011-2012	
Itaberaba, Brazil 2011-2012	
Itaberaba, Brazil 2011-2012	
Itaberaba, Brazil 2011-2012	
Itaberaba, Brazil 2011-2012	
Itaberaba, Brazil 2011-2012	
Itaberaba, Brazil 2011-2012	
Itaberaba, Brazil 2011-2012	
Mandacaru, Brazil 2012	

ED_006741_00009696-00089

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

The data provided in [REF _Ref453240613 \h * MERGEFORMAT] are from three different types of typical environments for *Ae. aegypti.* The Cayman Islands data [ADDIN EN.CITE ADDIN EN.CITE.DATA] represent a site that was isolated and untreated with conventional insect control measures; the Brazilian Itaberaba site data [ADDIN EN.CITE <EndNote><Cite><Author>Carvalho</Author><Year>2015</Year><RecNum>60</RecNum><DisplayText>(Carvalho et al. 2015)</DisplayText><record><rec-number>60</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1445973125">60</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Carvalho, Danilo O.</author><author>McKemey, A.</author><author>Garziera, L.</author><author>Lacroix, R.</author><author>Donnelly, Christl A.</author><author>Alphey, L.</author><author>Malavasi, A.</author><author>Capurro, Margareth L.</author></authors></contributors><titles><title>Supression of a field population of Aedes aegypti in Brazil by sustained release of transgenic male mosquitoes.</title><secondary-title>PLoS Neglected Tropical Diseases</secondary-title></titles><periodical><full-title>PLoS Neglected Tropical Diseases</full-title></periodical><pages>e0003864</pages><volume>9</volume><number>7</number><dates><year>2015</year></dates><urls></urls></record></Cite></EndNote>]~~(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 Conclusions

The successful mating ~~competitiveness~~ of OX513A with wild-type Ae. aegypti ~~from~~ under different ~~backgrounds~~ conditions and in different housing densities suggests~~implies~~ that the insertion of tTAV and DsRed2 at the insertion site in the OX513A line does not ~~appear to~~ exert ~~any~~ positional effects including alterations in the ability of OX513A to react to specific mating signals from wild-type Ae. aegypti i.e., the mating competitiveness of OX513A. This leads us to conclude ~~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~~ carry a repressible lethality trait and consequently would die before reaching functional 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 are~~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 U.S. (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 400 m 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 feet likely to be limiting to the species in the tropics and even lower ~~level~~elevations ~~s~~ 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* at any location over 2,000 m. The slope of the elevation could also be an influencing factor, with plateaus being more preferable as habitat ~~-~~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[43].

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 c~~Conversely in parts of these arid 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).

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[43] [HYPERLINK "https://www.google.com/maps/place/Raccoon+Key/@24.5747095,-81.7357574,3591m/data=!3m1!1e3!4m5!3m4!1s0x88d1b18002287e7b:0x445a14e3e77aff8!8m2!3d24.5818125!4d-81.7348125"]

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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~~ line 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~~ lines were used, OX513A back-crossed from the original Rockefeller strain into the My1 strain for 5 generations (OX513A-My1) and ~~a~~ the My1 wild-type laboratory strain (Jinjang, Malaysia), 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~~ by the OX513A line~~strain~~ was lower (52 vs. 100 m) than that for the wild-type comparator used. 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~~is similar in terms of its ~~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 local wild-type 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 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 [REF _Ref450314108 \h].

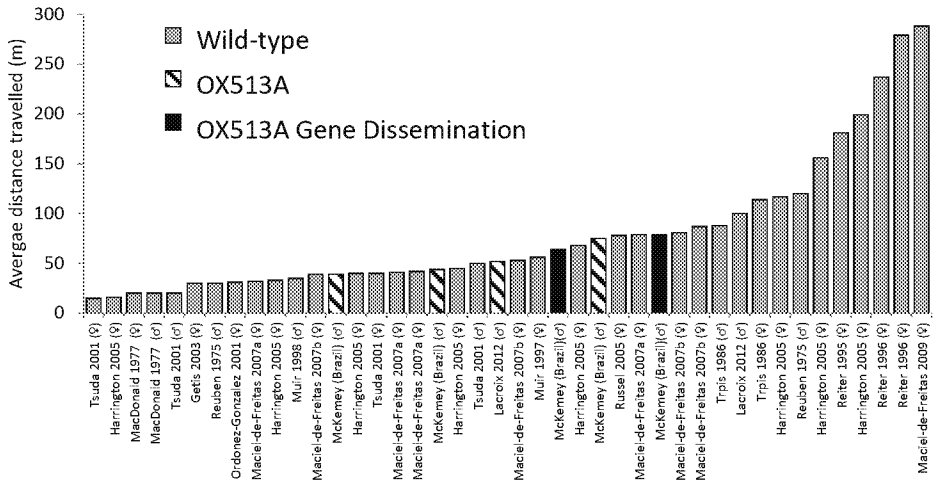

Figure [SEQ Figure * ARABIC]. Review of reported mean distance travelled (m) for wild-type and OX513A *Ae. aegypti* and observed dissemination of ~~§~~OX513~~A~~ ~~rDNA constructgene~~ from male release.

References for [REF _Ref450314108 \h * MERGEFORMAT]: 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~~ line shows a similar dispersion pattern to the ~~wild-type~~unmodified comparator strain in dispersal experiments and falls within the midrange of the ~~reported~~ flight distances of Ae. aegypti ~~flight from~~reported in the scientific 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.

13 Evaluation of Potential Impacts

This environmental assessment addresses the potential for significant environmental impacts as the result of the conduct of the proposed field trial. The~~se~~ 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-GEgenetically engineered, wild-type comparators; i.e., the existing mosquito control measures and their consequences on the environment as well as the existing wild-type *Ae. aegypti* mosquito population and its consequences for on human health impacts.

13.1 Likelihood of Impacts occurring

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

13.213.1 What is the Lilikelihood for inadvertent release of OX513A mosquitoes outside of the proposed trial site?escape

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

13.2.113.1.1 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.

TEggs of the OX513A line of *Ae. aegypti* wouldill be hatched and reared to adulthood at the HRU facility (see Section [REF _Ref453677115 \r \h]). There wouldill be life stages for both female and male mosquitoes in the HRU, although the females wouldill be sorted to ensure accuracy of the sorting does not exceed a maximum of 0.2% females and sorted females would be killed at the larvae/pupae separation stage, which would beis conducted in the containment facility, and t Therefore, the chances of their all life stages of OX513A mosquitoesfemales escapinge isare extremely low. Male The OX513A mosquitoes wouldill be maintained with multiplea minimum of two levels of physical containment (primary rearing containers, the HRU, and the building housing the HRU) in accordance with ACL2 requirements and those of the U.S. agencies (CDC and USDA APHIS) permitting the import (see Sections [REF _Ref453333982 \r \h] and [REF _Ref453334011 \r \h]). Every effort would be is made to avoid inadvertent release by following established procedures and implementing staff training. FDA verified physical and procedural cContainment and procedures implemented at the HRU were verified during anthe Bioresearch Monitoring inspection. FDA inspectors were performed by the FDA and accompanied

by a ~~ying~~ subject matter expert from CDC. No Form-483[46] was issued at the conclusion of the inspection.

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 an inadvertent release. The building housing the HRU is a Category 4 hurricane-protected building. In the case of a hurricane, there is a hurricane preparedness policy for the HRU that aims to minimize inadvertent release. ~~,~~ The policy calls for killing mosquitoes~~where insects will be killed~~ within 36 hours of a hurricane strike warning issued by the U.S. National Weather Service~~forecast~~. The decision to implement these measures would~~ill~~ be made~~taken~~ by the FKMCD program manager and the study director, in accordance with the hurricane management plan.

Oxitec performed an analysis of the likelihood of ~~has conducted An assessment of the~~ potential impacts during transport of their GE ~~insects~~ mosquitoes ~~has been conducted by Oxitec~~ along with potential control measures. The results of the analysis are ~~and is~~ summarized in [REF _Ref450334009 \h] below. Potential impacts are categorized as being "low", "moderate," or "likely."

Table [SEQ Table * ARABIC]. 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 would~~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 would~~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 would~~ill~~ be in FKMCD or Oxitec staff custody throughout journey, any loss of boxes would~~ill~~ be reported immediately, and every effort would~~ill~~ be made to recover them boxes/mosquitoes. A chain of custody would be~~is~~ in place for all transport. Even if not located~~found~~, insects cannot establish in the environment due to intrinsic biological containment (reliance on presence of tetracycline).	LOW

[46] FDA issues a Form 483 to firm management at the conclusion of an inspection when an investigator(s) has observed any conditions that in their judgement may constitute violations of the FD&C Act and related Acts. ~~to communicate to the sponsor deficiencies noted during a facility inspection that need to be corrected/addressed.~~

Boxes dropped ~~during while~~ loading for transport.	GE mosquito released to environment.	~~Use s~~Secure, shatterproof double containers ~~would~~ be used 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 w~~ould~~ be accompanied by FKMCD or Oxitec staff at all times. Any loss of boxes w~~ould~~ be reported immediately and appropriate authorities w~~ould~~ 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 w~~ould~~ 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

13.1.2 Question conclusions

Based on our evaluation of the physical containment measures and procedures implemented for the rearing and transportation of OX513A mosquitoes, FDA concludes~~determines~~ that the likelihood that OX513A mosquitoes would be inadvertently released outside of the intended field trial site, should the field trial proceed, is ~~very~~ low.

~~13.3~~13.2 What is the l~~L~~ikelihood for establishment of OX513A mosquitoes at the proposed trial site?

If ~~For~~ a GE animal ~~to make a significant impact on the environment it must~~ spread~~s~~ and establish~~es~~ in the environment ~~community~~ in which it is released, it would have a significant impact on that environment. The National Academy of Sciences (NAS) ~~therefore~~ defines exposure as the establishment of the GE animal in the community. [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>NRC</Author><Year>2002</Year><RecNum>44</RecNum><DisplayText>NRC (2002)</DisplayText><record><rec-number>44</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">44</key></foreign-keys><ref-type name="Book">6</ref-type><contributors><authors><author>NRC</author></authors></contributors><titles><title>Animal biotechnology: science-based concerns</title></titles><reprint-edition>Not in File</reprint-edition><dates><year>2002</year><pub-dates><date>2002</date></pub-dates></dates><pub-location>Washington, DC</pub-

Commented [KJ22]: Unless this is a quote, I suggest "could" to indicate potential, not "would" indicating surety.

location><publisher>The National Academic Press</publisher><isbn>0-309-08439-3</isbn><label>138</label><urls></urls></record></Cite></EndNote>] 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 the OX513A ~~strain~~ mosquito in comparison to a wild–type control strain mosquito contribute to assessment of the overall fitness of the ~~strain~~OX513A line. Fitness of ~~the~~ OX513A mosquitoes should be considered within the context that the intended effect of the expression of the rDNA construct is to confer dominant conditional lethality to the ~~strain~~line, i.e., a competitive disadvantage, and the ~~strain~~ line will die without access to the tetracycline antidote in its diet.

This section focuses on the fitness of the ~~strain~~ line as the ability of OX513A mosquitoes to escape and disperse into diverse communities is covered in Section [REF _Ref453246258 \r \h]. The stability and resiliency of the receiving environment is described in Section [REF _Ref453246150 \r \h] on accessible environments.

Fitness is comprised of reproductive potential, mating success, and survival. Of these components, survival has been evaluated in Section [REF _Ref453246198 \r \h] and will not be addressed here further.

~~13.3.1~~13.2.1 Lifecycle parameters

The lifecycle parameters of the OX513A *Ae. aegypti* have been examined in a study by [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Lee</Author><Year>2009</Year><RecNum>244</RecNum><DisplayText>Lee et al. (2009b)</DisplayText><record><rec-number>244</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1465585323">244</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lee, H.L.</author><author>Joko, H.</author><author>Nazni, W.A.</author><author>Vasan, S.</author></authors></contributors><titles><title>Comparative life parameters of transgenic and wild strain of Aedes aegypti in the laboratory </title><secondary-title>Dengue Bulletin</secondary-title></titles><periodical><full-title>Dengue Bulletin</full-title></periodical><pages>103-114</pages><volume>33</volume><dates><year>2009</year></dates><urls></urls></record></Cite></EndNote>]. 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.

Commented [KJ23]: I assume this study was done in the presence of tetracycline. Otherwise how could the OX513A line survive? Probably needs to be mentioned here.

[ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Bargielowski</Author><Year>2011</Year><RecNum>101</RecNum><DisplayText>Bargielowski et al. (2011b)</DisplayText><record><rec-number>101</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463078672">101</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Bargielowski, I.</author><author>Nimmo, D.</author><author>Alphey, L.</author><author>Koella, J. C.</author></authors></contributors><auth-address>Division of Biology, Imperial College London, London, United Kingdom. irka.bargielowski06@imperial.ac.uk</auth-address><titles><title>Comparison of life history characteristics of the genetically modified OX513A line and a wild type strain of Aedes aegypti</title><secondary-title>PLoS One</secondary-title></titles><periodical><full-title>PLoS ONE</full-title></periodical><pages>e20699</pages><volume>6</volume><number>6</number><keywords><keyword>Aedes/genetics/*physiology</keyword><keyword>Animals</keyword><keyword>Female</keyword><keyword>Life Cycle Stages</keyword><keyword>Longevity</keyword><keyword>Male</keyword></keywords><dates><year>2011</year></dates><isbn>1932-6203 (Electronic)
1932-6203 (Linking)</isbn><accession-num>21698096</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/21698096</url></related-urls></urls><custom2>3117796</custom2><electronic-resource-num>10.1371/journal.pone.0020699</electronic-resource-num></record></Cite></EndNote>] compared the life history characteristics of the OX513A line and a wild-type strain of *Ae. aegypti* to the effects of increasing larval rearing density ~~using~~ in the presence of a constant amount of food per larva. Parameters examined were larval mortality, developmental rate (i.e., time to pupation), adult size, and longevity under permissive conditions (i.e., in the presence of tetracycline). Only two statistically significant differences were found between the strains: the OX513A *Ae. aegypti* larval survival was 5% lower than that of the wild-type and ~~there was a reduced~~ OX513A adult longevity was lower than that seen in the wild-type (20 days OX513A vs 24 days ~~WT~~ wild-type mean lifespan). The OX513A line pupated approximately one day sooner than ~~the WT~~ wild-type *Ae. aegypti* resulting in smaller adults than the unmodified line. This effect was more pronounced in females than in males.

Commented [KJ24]: Does this complicate their male sorting strategy?

These life-cycle characterization studies between the investigational 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~~ line and the wild-type *Ae. aegypti* mosquito is the expression of the intended proteins (tTAV and DsRed2) and a small fitness disadvantage~~penalty~~.

~~13.3.~~13.2.2 Mating competitiveness

Mating competitiveness is a key parameter in the assessment of the fitness of the OX513A mosquito~~insect~~. Data in [REF _Ref453830164 \h] indicate that in the laboratory, the GE mosquitoes performed as well as the WT, and none of the mating competitiveness estimates differ significantly from 0.5.[45] As a point of comparison, the International Atomic Energy Agency considers a mating competitiveness of 0.2 to be acceptable for a successful SIT program (Section [REF _Ref453831364 \r \h]).

[ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Bargielowski</Author><Year>2011</Year><RecNum>8</RecNum><DisplayText>Bargielowski et al. (2011a)</DisplayText><record><rec-number>8</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">8</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>Bargielowski, Irka</author><author>Alphey, Luke</author><author>Koella, Jacob C.</author></authors><secondary-authors><author>Langsley, Gordon</author></secondary-authors></contributors><titles><title>Cost of Mating and Insemination Capacity of a Genetically Modified Mosquito Aedes aegypti OX513A Compared to Its Wild Type Counterpart</title><secondary-title>PLoS ONE</secondary-title></titles><periodical><full-title>PLoS ONE</full-title></periodical><pages>e26086</pages><volume>6</volume><number>10</number><reprint-edition>Not in File</reprint-edition><keywords><keyword>Aedes</keyword></keywords><dates><year>2011</year><pub-dates><date>2011</date></pub-dates></dates><isbn>1932-6203</isbn><label>8</label><urls><related-urls><url>http://dx.plos.org/10.1371/journal.pone.0026086</url></related-urls></urls><electronic-resource-num>10.1371/journal.pone.0026086</electronic-resource-num><access-date>3/28/2015</access-date></record></Cite></EndNote>]. ([REF _Ref450313211 \h]), however, indicated that the insemination capacity of OX513A males was significantly reduced in OX513A versus~~s~~ the wild-type Malaysian strain of *Ae. aegypti* used as a comparator in this study. The authors hypothesized that the reduced insemination capacity of OX513A males may be potentially attributed to a slight loss of fitness in the GE mosquito compared to the wild-type, likely due to the effects of mass-rearing on the GE mosquitoes (Section [REF _Ref454435190 \r \h]).

It is not clear whether the difference in results described ~~between~~in the [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Bargielowski</Author><Year>2011</Year><RecNum>8</RecNum><DisplayTe

[45] For OX513A males, ~~the~~ mating competitiveness of 0.5 indicates that wild-type *Ae. aegypti* females are equally attracted to OX513A and wild-type *Ae. aegypti* males.

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xt>Bargielowski et al. (2011a)</DisplayText><record><rec-number>8</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">8</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>Bargielowski, Irka</author><author>Alphey, Luke</author><author>Koella, Jacob C.</author></authors><secondary-authors><author>Langsley, Gordon</author></secondary-authors></contributors><titles><title>Cost of Mating and Insemination Capacity of a Genetically Modified Mosquito Aedes aegypti OX513A Compared to Its Wild Type Counterpart</title><secondary-title>PLoS ONE</secondary-title></titles><periodical><full-title>PLoS ONE</full-title></periodical><pages>e26086</pages><volume>6</volume><number>10</number><reprint-edition>Not in File</reprint-edition><keywords><keyword>Aedes</keyword></keywords><dates><year>2011</year><pub-dates><date>2011</date></pub-dates></dates><isbn>1932-6203</isbn><label>8</label><urls><related-urls><url>http://dx.plos.org/10.1371/journal.pone.0026086</url></related-urls></urls><electronic-resource-num>10.1371/journal.pone.0026086</electronic-resource-num><access-date>3/28/2015</access-date></record></Cite></EndNote>] study and the survey of strains illustrated in [REF _Ref453830164 \h] is due to differences in the measured endpoint (mating competitiveness vs. insemination capacity), or some other factor. Nonetheless, the weight of evidence seems to indicate that there are no biologically relevant differences in the relative ability of OX513A males to mate with WT *Ae. aegypti* females in the laboratory and The successful mating competitiveness of OX513A compared with wild-type *Ae. aegypti* suggestsimplies 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 in prior to field studies being conducted in the Cayman Islands [ADDIN EN.CITE ADDIN EN.CITE.DATA] and Brazil [ADDIN EN.CITE <EndNote><Cite><Author>Carvalho</Author><Year>2015</Year><RecNum>60</RecNum><DisplayText>(Carvalho et al. 2015)</DisplayText><record><rec-number>60</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1445973125">60</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Carvalho, Danilo O.</author><author>McKemey, A.</author><author>Garziera, L.</author><author>Lacroix, R.</author><author>Donnelly, Christl A.</author><author>Alphey, L.</author><author>Malavasi, A.</author><author>Capurro, Margareth L.</author></authors></contributors><titles><title>Supression of a field population of Aedes aegypti in Brazil by sustained release of transgenic male mosquitoes.</title><secondary-title>PLoS Neglected Tropical Diseases</secondary-title></titles><periodical><full-title>PLoS Neglected Tropical Diseases</full-title></periodical><pages>e0003864</pages><volume>9</volume><number>7</number><dates><year>2015</year></dates><urls></urls></record></Cite></EndNote>]. 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 [REF _Ref411865223 \r \h * MERGEFORMAT]),

~~which suggests~~ and demonstrated that there are unlikely to be differences in mating behaviors of OX513A mosquitoes compared with the local wild-type population of *Ae. aegypti* across different backgrounds and environments.

~~13.3.3~~13.2.3 Question conclusions~~Conclusion on the likelihood for establishment in the environment~~

The OX513A line of *Ae. aegypti* mosquitoes carries a repressible dominant lethality trait that prevents progeny inheriting the ~~causes death of~~ #OX513A ~~gener~~DNA construct from surviving to functional adulthood in the absence ~~mosquito progeny at the early pupal or larval stage unless reared in the presence~~ of tetracycline. Although it appears that the introduced lethality trait did not affect mating competitiveness of OX513A males, data demonstrating hemizygous females reared without tetracycline have a median lifespan of two days relative to a wild-type median lifespan of 68 indicating a ~~we conclude that it had a significant impact on survival of OX513A mosquitoes dramatically reducing their lifespan. The lack of exogenous tetracycline and its derivatives in the environment would~~ further reduc~~e~~tion in the likelihood of survival of OX513A mosquitoes and their progeny. FDA therefore concludes that it is highly unlikely that OX513A mosquitoes and their progeny would be able to establish ~~or persist~~ at the proposed ~~investigational~~ trial site ~~*Lifecycle parameters, fitness and mating competitiveness have all been assessed in laboratory studies and, in the case of mating competiveness in the field, have also been assessed as well during previous releases in the Cayman Islands and Brazil. The ability to survive has been addressed in Section* **[REF _Ref411529408 \r \h * MERGEFORMAT]**, *which concluded that the response of OX513A mosquitoes to abiotic factors is likely to be the same as the response of a wild-typenon-genetically-engineered Ae. aegypti and survival was unlikely to be increased. Based on a*~~All the available data, we conclude~~ ~~*indicates that it is extremely unlikely for there to be differences between OX513A and wild-type Ae. aegypti that would advantage OX513A and affect change the likelihood of its for establishment in the environment*~~.

~~13.4~~13.3 What is the L**ikelihood** ~~for of spread~~dispersal of OX513A mosquitoes and their progeny from the proposed trial site?

The effect of the introduced traits on the dispersal ability of OX513A mosquitoes is discussed in Section [REF _Ref453246258 \r \h]. ~~The dispersal ability of OX513A mosquitoes was also evaluated by~~ [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Bargielowski</Author><Year>2012</Year><RecNum>36</RecNum><DisplayText>Bargielowski et al. (2012)</DisplayText><record><rec-number>36</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">36</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Bargielowski, Irka</author><author>Kaufmann, Christian</author><author>Alphey, Luke</author><author>Reiter, Paul</author><author>Koella, Jacob</author></authors></contributors><titles><title>Flight Performance and Teneral Energy Reserves of Two Genetically-Modified and One Wild-Type Strain of the Yellow Fever Mosquito Aedes aegypti</title><secondary-title>Vector-Borne and Zoonotic Diseases</secondary-title></titles><pages>1053-1058</pages><volume>12</volume><number>12</number><reprint-

edition>Not in File</reprint-edition><keywords><keyword>Aedes</keyword><keyword>Aedes aegypti</keyword><keyword>larval diet</keyword></keywords><dates><year>2012</year><pub-dates><date>2012</date></pub-dates></dates><isbn>1530-3667</isbn><label>37</label><urls><related-urls><url>http://online.liebertpub.com/doi/abs/10.1089/vbz.2012.0994</url></related-urls></urls><electronic-resource-num>10.1089/vbz.2012.0994</electronic-resource-num><access-date>5/14/2015</access-date></record></Cite></EndNote>] ~~who reported that OX513A male mosquitoes reared in the presence of tetracycline covered 38% less distance than their wild-type comparators. These data suggest that the introduced trait may have affected the fitness of OX513A mosquitoes with respect to their dispersion.~~ In a ~~another~~ study of OX513A mosquitoes in Malaysia, [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Lacroix</Author><Year>2012</Year><RecNum>43</RecNum><DisplayText>Lacroix et al. (2012)</DisplayText><record><rec-number>43</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">43</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>Lacroix, R.</author><author>McKemey, A.R.</author><author>Raduan, N.</author><author>Kwee Wee, L.</author><author>Nordin, O.</author></authors></contributors><titles><title>Open Field Release of Genetically Engineered Sterile Male Aedes aegypti in Malasia</title><secondary-title>PLoS ONE</secondary-title></titles><periodical><full-title>PLoS ONE</full-title></periodical><pages>e42771</pages><volume>7</volume><number>8</number><reprint-edition>Not in File</reprint-edition><keywords><keyword>Aedes</keyword><keyword>Aedes aegypti</keyword></keywords><dates><year>2012</year><pub-dates><date>2012</date></pub-dates></dates><label>44</label><urls></urls></record></Cite></EndNote>] ~~showed that the *mean distance traveled (MDT)* for the OX513A-My1 line of mosquitoes was significantly lower that of their wild-type comparator, My1 line (52 m and 100 m respectively) (Section~~ [REF _Ref453837077 \r \h]~~).~~

~~Nonetheless, the *maximum distance traveled* was similar for both lines of mosquitoes. In a recent study carried out in Brazil, Oxitec determined that the MDT for OX513A mosquitoes in an urban environment was 52.8 m.~~ [ADDIN EN.CITE <EndNote><Cite><Author>Winskill</Author><Year>2015</Year><RecNum>81</RecNum><DisplayText>(Winskill et al. 2015)</DisplayText><record><rec-number>81</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1451885303">81</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Winskill, P.</author><author>Carvalho, D. O.</author><author>Capurro, M. L.</author><author>Alphey, L.</author><author>Donnelly, C. A.</author><author>McKemey, A. R.</author></authors></contributors><auth-address>Medical Research Council Centre for Outbreak Analysis and Modelling, Department of Infectious Disease Epidemiology, School of Public Health, Faculty of Medicine, Imperial College London, St Mary's Campus, London, United Kingdom.
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Department of Zoology, University of Oxford, Oxford, United Kingdom.</auth-address><titles><title>Dispersal of Engineered Male Aedes aegypti Mosquitoes</title><secondary-title>PLoS Negl Trop Dis</secondary-title></titles><periodical><full-title>PLoS Negl Trop Dis</full-title></periodical><pages>e0004156</pages><volume>9</volume><number>11</number><dates><year>2015</year><pub-dates><date>Nov</date></pub-dates></dates><isbn>1935-2735 (Electronic)
1935-2727 (Linking)</isbn><accession-num>26554922</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/26554922</url></related-urls></urls><custom2>4640874</custom2><electronic-resource-num>10.1371/journal.pntd.0004156</electronic-resource-num></record></Cite></EndNote>]. ~~Although the data presented in~~ [REF _Ref450314108 \h] ~~do demonstrate that similarity between the OX513A and wild-type *Ae. aegypti*'s have similar *MDT*, they have to be compared with a caution because these studies more likely were performed using different methodology in different environments (e.g., urban, rural, or semi-rural) under a variety of environmental conditions making such comparisons more difficult. Therefore, we consider that more weight should be given to studies that were performed using appropriate comparators in the same environmental setting (i.e.,~~ [ADDIN EN.CITE ADDIN EN.CITE.DATA] ~~). Considering uncertainties regarding the *MDT* for *Ae. aegypti* in existing scientific literature and studies demonstrating a significant effect of the introduced trait on the *MDT* for OX513A mosquitoes,~~ [ADDIN EN.CITE ADDIN EN.CITE.DATA] ~~, we conclude that the dispersal ability of OX513A mosquitoes appears to be adversely affected by the introduced trait and in general, the population of OX513A mosquitoes is not expected to exhibit dispersion greater than wild-type *Ae. aegypti*. 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* (Maxine *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.~~

The location of the proposed field trial site would further limit the spread of released OX513A mosquitoes and their progeny due to various barriers to movement including roads, dense vegetation, and other natural obstacles [ADDIN EN.CITE ADDIN EN.CITE.DATA]. The proposed field site is located

in Key Haven~~, Raccoon Island~~, which ~~a peninsulat that and~~ is surrounded by salt water on three sides ([REF _Ref450310557 \h]). The closest island is located more than 250 m away. This will considerably limit the spread of mosquitoes from the proposed field trial site because the *MDT* for OX513A mosquitoes is approximately 200 m [ADDIN EN.CITE ADDIN EN.CITE.DATA]. The shoreline of both islands is covered with dense vegetation and will further limit the spread of mosquitoes. Although mosquitoes can potentially be dispersed passively by boats and cars, the likelihood that large numbers of mosquitoes would be dispersed in this way is low. The island is connected to Highway 1 via a single road, with no through traffic passing through the area especially the TA. Additionally, the TA is located at the end of the peninsula that is furthest away from Highway 1. ~~This also~~These factors considerably limit~~s~~ the spread of mosquitoes from the proposed field study site.

During the trial, FKMCD would continue ~~their~~ standard mosquito control practice~~treatment program~~ at the proposed trial site. T~~hese~~ practices ~~program~~ includes container dumping and removal (source reduction), container treatment with larvicides, and adulticide treatment. The description of FKMCD activities and chemicals used to control mosquitoes is included in Section [REF _Ref453245565 \r \h] ~~of the EA~~.

~~13.3.1 Question conclusions~~

Based on our analysis of data available in the literature, we consider that dispersal of OX513A mosquitoes appears to be adversely affected as measured by *MDT*, but not by *maximum distance traveled*, indicating, that in, general, the population of OX513A mosquitoes is not expected to exhibit dispersion greater than wild-type *Ae. aegypti*. The location of the proposed trial site and mosquito control measures implemented by FKMCD would considerably limit the dispersion of OX513A mosquitoes as well. FDA therefore concludes that it is highly unlikely that OX513A mosquitoes and their progeny would be able to~~o assist~~ spread beyond boundaries of the proposed field trial site, should the trial proceed.

~~13.5~~13.4 What is the l~~L~~ikelihood ~~of~~ that the ~~the~~ rDNA construct could be ~~spreading~~ transferred to humans or other organisms?

~~13.5.1~~13.4.1 Likelihood of sexual transfer of ~~the~~ rDNA construct~~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 [REF _Ref453837077 \r \h].

~~13.5.2~~13.4.2 Likelihood of non-sexual transfer of ~~the rDNA construct 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[46]. 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 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 the OX513A mosquito, it has been shown (Section [REF _Ref453246620 \r \h]) 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 an animal is~~has also been~~ assessed below~~in the following sections~~:

~~13.5.2.1~~13.4.2.1 Acquisition of genes through oral ingestion or biting~~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. It is highly unlikely that the rDNA construct could be transferred to humans or other animals by biting. As discussed in Section~~ [REF _Ref453332132 \r \h]~~, the rDNA construct is stably integrated into the mosquito genome and is not capable of re-mobilization due to altered ITR sequences even when treated with appropriate transposases.~~ [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Nordin</Author><Year>2013</Year><RecNum>18</RecNum><DisplayText>Nordin et al. (2013)</DisplayText><record><rec-number>18</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">18</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>Nordin,

[46] 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.

Oreenaiza</author><author>Donald, Wesley</author><author>Ming, Wong Hong</author><author>Ney, Teoh Guat</author><author>Mohamed, Khairul Asuad</author><author>Halim, Nor Azlina Abdul</author><author>Winskill, Peter</author><author>Hadi, Azahari Abdul</author><author>Muhammad, Zulkamal Safi'in</author><author>Lacroix, Renaud</author><author>Scaife, Sarah</author><author>McKemey, Andrew Robert</author><author>Beech, Camilla</author><author>Shahnaz, Murad</author><author>Alphey, Luke</author><author>Nimmo, Derric David</author><author>Nazni, Wasi Ahmed</author><author>Lee, Han Lim</author></authors></contributors><titles><title>Oral Ingestion of Transgenic RIDL Ae. aegypti Larvae Has No Negative Effect on Two Predator Toxorhynchites Species</title><secondary-title>PLoS ONE</secondary-title></titles><periodical><full-title>PLoS ONE</full-title></periodical><pages>e58805</pages><volume>8</volume><number>3</number><reprint-edition>Not in File</reprint-edition><dates><year>2013</year><pub-dates><date>2013</date></pub-dates></dates><isbn>1932-6203</isbn><label>18</label><urls><related-urls><url>http://dx.plos.org/10.1371/journal.pone.0058805</url></related-urls></urls><electronic-resource-num>10.1371/journal.pone.0058805</electronic-resource-num><access-date>3/28/2015</access-date></record></Cite></EndNote>]Nordlin et al. 2013 did not find evidence of non-sexual transfer of the engineered traits through oral ingestion in *Toxorhynchites* fed solely on a diet of OX513A larvae (raised both on and off-tetracycline) and assayed using PCR (approximately 52,000 events in 121 adults tested). Further, Mmosquitoes 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 bitingblood 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 [ADDIN EN.CITE <EndNote><Cite><Year>1992</Year><RecNum>569</RecNum><DisplayText>(1992)</DisplayText><record><rec-number>569</rec-number><foreign-keys><key app="EN" db-id="sfpa9es0see0t5earrr5e2phrxsx0psxprf2">569</key></foreign-keys><ref-type name="Legal Rule or Regulation">50</ref-type><contributors><secondary-authors><author>USFDA</author></secondary-authors></contributors><titles><title>Statement of Policy: Foods Derived from New Plant Varieties</title></titles><dates><year>1992</year></dates><isbn>57 Federal Register 22984</isbn><urls><related-urls><url>http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/Biotechnology/ucm096095.htm</url></related-urls></urls></record></Cite></EndNote>]. 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 [ADDIN EN.CITE <EndNote><Cite><Author>Klotz</Author><Year>2002</Year><RecNum>147</RecNum><DisplayText>(Klotz et al. 2002)</DisplayText><record><rec-number>147</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106826">147</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Klotz, Andreas</author><author>Mayer, Johann</author><author>Einspanier, Ralf</author></authors></contributors><titles><title>Degradation and possible carry over of feed DNA monitored in pigs and poultry</title><secondary-title>European Food Research and Technology</secondary-title></titles><periodical><full-title>European Food Research and Technology</full-title></periodical><pages>271-275</pages><volume>214</volume><number>4</number><dates><year>2002</year><pub-dates><date>2002/04/01/</date></pub-dates></dates><isbn>1438-2377, 1438-2385</isbn><urls><related-urls><url>http://link.springer.com/10.1007/s00217-001-0444-3</url><url>http://download.springer.com/static/pdf/127/art%253A10.1007%252Fs00217-001-0444-3.pdf?originUrl=http%3A%2F%2Flink.springer.com%2Farticle%2F10.1007%2Fs00217-001-0444-3&token2=exp=1463108071~acl=%2Fstatic%2Fpdf%2F127%2Fart%25253A10.1007%25252Fs00217-001-0444-3.pdf%3ForiginUrl%3Dhttp%253A%252F%252Flink.springer.com%252Farticle%252F10.1007%252Fs00217-001-0444-3*~hmac=b869bb5cb471ca4de427071492ccc657d8a84bd550de16118e75b27c84ce6e0f</url></related-urls></urls><electronic-resource-num>10.1007/s00217-001-0444-3</electronic-resource-num><remote-database-provider>CrossRef</remote-database-provider><access-date>2015/03/28/04:11:45</access-date></record></Cite></EndNote>], 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 feed derived from GEgenetically engineered crops, [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Alexander</Author><Year>2007</Year><RecNum>127</RecNum><DisplayText>Alexander et al. (2007)</DisplayText><record><rec-number>127</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106826">127</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Alexander, Trevor W.</author><author>Reuter, Tim</author><author>Aulrich, Karen</author><author>Sharma, Ranjana</author><author>Okine, Erasmus K.</author><author>Dixon, Walter T.</author><author>McAllister, Tim A.</author></authors></contributors><titles><title>A review of the detection and fate of novel plant molecules derived from biotechnology in livestock production</title><secondary-title>Animal Feed

Science and Technology</secondary-title></titles><periodical><full-title>Animal Feed Science and Technology</full-title></periodical><pages>31-62</pages><volume>133</volume><number>1-2</number><dates><year>2007</year><pub-dates><date>2007/02//</date></pub-dates></dates><isbn>03778401</isbn><urls><related-urls><url>http://linkinghub.elsevier.com/retrieve/pii/S0377840106003051</url><url>http://ac.els-cdn.com/S0377840106003051/1-s2.0-S0377840106003051-main.pdf?_tid=2642f462-18b3-11e6-ba06-00000aab0f01&acdnat=1463107020_03d5a73a1b5306ae60a112279083d3e5</url></related-urls></urls><electronic-resource-num>10.1016/j.anifeedsci.2006.08.003</electronic-resource-num><remote-database-provider>CrossRef</remote-database-provider><language>en</language><access-date>2015/03/28/04:03:34</access-date></record></Cite></EndNote>], [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Flachowsky</Author><Year>2012</Year><RecNum>135</RecNum><DisplayText>Flachowsky et al. (2012)</DisplayText><record><rec-number>135</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106826">135</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Flachowsky, Gerhard</author><author>Schafft, Helmut</author><author>Meyer, Ulrich</author></authors></contributors><titles><title>Animal feeding studies for nutritional and safety assessments of feeds from genetically modified plants: a review</title><secondary-title>Journal für Verbraucherschutz und Lebensmittelsicherheit</secondary-title><short-title>Animal feeding studies for nutritional and safety assessments of feeds from genetically modified plants</short-title></titles><periodical><full-title>Journal für Verbraucherschutz und Lebensmittelsicherheit</full-title></periodical><pages>179-194</pages><volume>7</volume><number>3</number><dates><year>2012</year><pub-dates><date>2012/09//</date></pub-dates></dates><isbn>1661-5751, 1661-5867</isbn><urls><related-urls><url>http://link.springer.com/10.1007/s00003-012-0777-9</url><url>http://download.springer.com/static/pdf/418/art%253A10.1007%252Fs00003-012-0777-9.pdf?originUrl=http%3A%2F%2Flink.springer.com%2Farticle%2F10.1007%2Fs00003-012-0777-9&token2=exp=1463108050~acl=%2Fstatic%2Fpdf%2F418%2Fart%25253A10.1007%25252Fs00003-012-0777-9.pdf%3ForiginUrl%3Dhttp%253A%252F%252Flink.springer.com%252Farticle%252F10.1007%252Fs00003-012-0777-9*~hmac=6b18d229b0e1199c546584c5d7b33b91c21ca41fb29bb40370677c345849f86c</url></related-urls></urls><electronic-resource-num>10.1007/s00003-012-0777-9</electronic-resource-num><remote-database-provider>CrossRef</remote-database-provider><language>en</language><access-date>2015/03/28/04:09:44</access-date></record></Cite></EndNote>], and [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Van Eenennaam</Author><Year>2014</Year><RecNum>256</RecNum><DisplayText>Van Eenennaam and Young (2014)</DisplayText><record><rec-number>256</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1466540176">256</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Van

Eenennaam, A. L.</author><author>Young, A. E.</author></authors></contributors><auth-address>Department of Animal Science, University of California, Davis 95616 alvaneenennaam@ucdavis.edu.
Department of Animal Science, University of California, Davis 95616.</auth-address><titles><title>Prevalence and impacts of genetically engineered feedstuffs on livestock populations</title><secondary-title>J Anim Sci</secondary-title></titles><periodical><full-title>J Anim Sci</full-title></periodical><pages>4255-78</pages><volume>92</volume><number>10</number><keywords><keyword>Animal Feed/*analysis</keyword><keyword>Animals</keyword><keyword>*Breeding</keyword><keyword>DNA, Plant/analysis/genetics</keyword><keyword>Edible Grain/chemistry/*genetics</keyword><keyword>Eggs/analysis</keyword><keyword>Female</keyword><keyword>*Genetic Engineering</keyword><keyword>Livestock/*physiology</keyword><keyword>Meat/analysis</keyword><keyword>Milk/chemistry</keyword><keyword>Pregnancy</keyword><keyword>*Pregnancy Rate</keyword><keyword>Prevalence</keyword><keyword>genetic engineering</keyword><keyword>genetically modified organisms</keyword><keyword>livestock feed</keyword><keyword>safety</keyword></keywords><dates><year>2014</year><pub-dates><date>Oct</date></pub-dates></dates><isbn>1525-3163 (Electronic)
0021-8812 (Linking)</isbn><accession-num>25184846</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25184846</url></related-urls></urls><electronic-resource-num>10.2527/jas.2014-8124</electronic-resource-num></record></Cite></EndNote>] concluded that there were no safety concerns for livestock consuming feedbeing 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 establish (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 [ADDIN EN.CITE <EndNote><Cite><Author>Thomas</Author><Year>2005</Year><RecNum>3</RecNum><DisplayText>(Thomas and Nielsen 2005)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">3</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Thomas, C., M.</author><author>Nielsen, K., M.</author></authors></contributors><titles><title>Mechanisms of, and Barriers to, Horizontal Gene Transfer between Bacteria</title><secondary-title>Nature Reviews Microbiology</secondary-title></titles><periodical><full-title>Nature Reviews Microbiology</full-title></periodical><pages>711-721</pages><volume>3</volume><number>9</number><reprint-edition>Not in File</reprint-edition><dates><year>2005</year><pub-dates><date>2005</date></pub-dates></dates><isbn>1740-1526, 1740-1534</isbn><label>3</label><urls><related-

urls><url>http://www.nature.com/doifinder/10.1038/nrmicro1234</url></related-urls></urls><electronic-resource-num>10.1038/nrmicro1234</electronic-resource-num><access-date>4/30/2015</access-date></record></Cite></EndNote>]. The occurrence of NST between prokaryotes and eukaryotes is more controversial, very difficult to detect, and likely happens on an evolutionary time scale only [ADDIN EN.CITE ADDIN EN.CITE.DATA]. In a recent study, [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Crisp</Author><Year>2015</Year><RecNum>117</RecNum><DisplayText>Crisp et al. (2015)</DisplayText><record><rec-number>117</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463104467">117</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Crisp, A.</author><author>Boschetti, C.</author><author>Perry, M.</author><author>Tunnacliffe, A.</author><author>Micklem, G.</author></authors></contributors><titles><title>Expression of multiple horizontally acquired genes is a hallmark of both vertebrate and invertebrate genomes</title><secondary-title>Genome Biol</secondary-title></titles><periodical><full-title>Genome Biol</full-title></periodical><pages>50</pages><volume>16</volume><keywords><keyword>Animals</keyword><keyword>Bacteria/genetics</keyword><keyword>*Evolution, Molecular</keyword><keyword>Gene Expression/*genetics</keyword><keyword>Gene Transfer, Horizontal/*genetics</keyword><keyword>*Genome</keyword><keyword>Humans</keyword><keyword>Invertebrates/genetics</keyword><keyword>Nematoda</keyword><keyword>Phylogeny</keyword><keyword>Vertebrates/genetics</keyword></keywords><dates><year>2015</year></dates><isbn>1474-760X (Electronic)
1474-7596 (Linking)</isbn><accession-num>25785303</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25785303</url></related-urls></urls><custom2>4358723</custom2><electronic-resource-num>10.1186/s13059-015-0607-3</electronic-resource-num></record></Cite></EndNote>] 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 mosquitoes 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 [ADDIN EN.CITE ADDIN EN.CITE.DATA]. 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.

It is also highly unlikely that the rDNA construct could be transferred to microorganisms (e.g., bacteria in the intestine of OX513A mosquitoes, humans, or other animals; bacteria present in soil and involved in decomposition of organic matter). Every organism has a number of physical, biochemical, and genetic barriers to restrict non-sexual horizontal gene transfer [ADDIN EN.CITE <EndNote><Cite><Author>Thomas</Author><Year>2005</Year><RecNum>3</RecNum><DisplayText>(Thomas and Nielsen 2005)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">3</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Thomas, C., M.</author><author>Nielsen, K., M.</author></authors></contributors><titles><title>Mechanisms of, and Barriers to, Horizontal Gene Transfer between Bacteria</title><secondary-title>Nature Reviews Microbiology</secondary-title></titles><periodical><full-title>Nature Reviews Microbiology</full-title></periodical><pages>711-721</pages><volume>3</volume><number>9</number><reprint-edition>Not in File</reprint-edition><dates><year>2005</year><pub-dates><date>2005</date></pub-dates></dates><isbn>1740-1526, 1740-1534</isbn><label>3</label><urls><related-urls><url>http://www.nature.com/doifinder/10.1038/nrmicro1234</url></related-urls></urls><electronic-resource-num>10.1038/nrmicro1234</electronic-resource-num><access-date>4/30/2015</access-date></record></Cite></EndNote>]. Despite the fact that prokaryotes are exposed to ~~the~~an abundance of genetic material from eukaryotic organisms, the presence of eukaryotic genes in the genome of prokaryotes is extremely limited and suggests the existence of functional and selective barriers that limit the acquisition of eukaryotic genes by bacteria [ADDIN EN.CITE <EndNote><Cite><Author>Andersson</Author><Year>2005</Year><RecNum>4</RecNum><DisplayText>(Andersson 2005)</DisplayText><record><rec-number>4</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">4</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Andersson, J.O.</author></authors></contributors><titles><title>Lateral gene transfer in eukaryotes</title><secondary-title>Cell. Mol. Life Sci</secondary-title></titles><pages>1182-1197</pages><volume>62</volume><number>11</number><reprint-edition>Not in File</reprint-edition><keywords><keyword>Biochemistry,general</keyword><keyword>Biomedicine general</keyword><keyword>Cell Biology</keyword><keyword>endosymbiotic gene transfer</keyword><keyword>eukaryote phylogeny</keyword><keyword>Horizontal gene transfer</keyword><keyword>lateral gene transfer</keyword><keyword>Life Sciences,general</keyword><keyword>origin of eukaryotes</keyword><keyword>phagotrophy</keyword><keyword>phylogeny</keyword></keywords><dates><year>2005</year><pub-dates><date>2005</date></pub-dates></dates><isbn>1420-682X, 1420-9071</isbn><label>4</label><urls><related-urls><url>http://link.springer.com/article/10.1007/s00018-005-4539-z</url></related-urls></urls><electronic-resource-num>10.1007/s00018-005-4539-z</electronic-resource-num><access-date>5/4/2015</access-date></record></Cite></EndNote>].

13.4.3 Question conclusions

Based on evaluation of data and information submitted by Oxitec, FDA determined that the #OX513 rDNA construct is stably integrated in the OX513A mosquito genome and completely refractory to remobilization, even when deliberately re-exposed to the *piggyBac* transposase used for insertion into the mosquito genome. Should the proposed field trial proceed, FDA considers that it is highly unlikely that the #OX513 rDNA construct could be transmitted to other closely related species by inter-breeding, as *Ae. aegypti* mating behavior is highly species-specific. Horizontal or non-sexual transfer of the rDNA construct to humans and other animals is also highly unlikely due to complexity of the process. Mosquitoes have been feeding on humans and other animals for millennia with no evidence of DNA transfer between humans and mosquitoes.

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 [REF _Ref411865378 \r \h * MERGEFORMAT].

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 (Whitcutt 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.

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

Commented [EEA25]: Not relevant here as the section focuses on the transfer of the rDNA construct not the safety of the tTAV and DsRed2 proteins. Toxicity of these proteins is covered in Section 13.6.

~~13.613.5 What is the likelihood that release of OX513A mosquitoes would have an adverse effect on non-target species at the proposed trial site? Interactions with other organisms~~

Ae. aegypti is considered uniquely domestic amongst the mosquito species, being closely associated with human habitations. It is a non-native species in the U.S. present predominantly in the Gulf Coast States [ADDIN EN.CITE <EndNote><Cite><Author>Lounibos</Author><Year>2002</Year><RecNum>220</RecNum><DisplayText>(Lounibos 2002)</DisplayText><record><rec-number>220</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463109433">220</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lounibos, L. P.</author></authors></contributors><auth-address>Florida Medical Entomology Laboratory, University of Florida, Vero Beach, Florida 32962, USA. Lounibos@ufl.edu</auth-address><titles><title>Invasions by insect vectors of human disease</title><secondary-title>Annu Rev Entomol</secondary-title></titles><periodical><full-title>Annu Rev Entomol</full-title></periodical><pages>233-66</pages><volume>47</volume><keywords><keyword>Aedes</keyword><keyword>Aircraft</keyword><keyword>Animal Migration</keyword><keyword>Animals</keyword><keyword>Culicidae</keyword><keyword>*Disease</keyword><keyword>Disease Outbreaks</keyword><keyword>Ecology</keyword><keyword>Forecasting</keyword><keyword>Humans</keyword><keyword>Insect Control</keyword><keyword>*Insect Vectors/physiology</keyword><keyword>Insects/physiology</keyword><keyword>Pacific Islands</keyword></keywords><dates><year>2002</year></dates><isbn>0066-4170 (Print)
0066-4170 (Linking)</isbn><accession-num>11729075</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/11729075</url></related-urls></urls><electronic-resource-num>10.1146/annurev.ento.47.091201.145206</electronic-resource-num></record></Cite></EndNote>], 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% [ADDIN EN.CITE <EndNote><Cite><Author>Ballenger-Browning</Author><Year>2009</Year><RecNum>95</RecNum><DisplayText>(Ballenger-Browning and Elder 2009)</DisplayText><record><rec-number>95</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1457032386">95</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Ballenger-Browning, K. K.</author><author>Elder, J. P.</author></authors></contributors><auth-address>Graduate School of Public Health, San Diego State University, San Diego, CA 92123, USA. kara.browning@med.navy.mil</auth-address><titles><title>Multi-modal Aedes aegypti mosquito reduction interventions and dengue fever prevention</title><secondary-title>Trop Med Int

Health</secondary-title></titles><periodical><full-title>Trop Med Int Health</full-title></periodical><pages>1542-51</pages><volume>14</volume><number>12</number><keywords><keyword>*Aedes</keyword><keyword>Animals</keyword><keyword>Clinical Trials as Topic</keyword><keyword>Dengue/epidemiology/*prevention & control/transmission</keyword><keyword>Disease Reservoirs</keyword><keyword>Entomology/*methods</keyword><keyword>Environmental Monitoring/*methods</keyword><keyword>Epidemiological Monitoring</keyword><keyword>Humans</keyword><keyword>Mosquito Control/*methods</keyword></keywords><dates><year>2009</year><pub-dates><date>Dec</date></pub-dates></dates><isbn>1365-3156 (Electronic)
1360-2276 (Linking)</isbn><accession-num>19788717</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/19788717</url></related-urls></urls><electronic-resource-num>10.1111/j.1365-3156.2009.02396.x</electronic-resource-num></record></Cite></EndNote>] and 50% as reported by FKMCD[47] but are increasingly ineffective due to the buildup of resistance mechanisms to the chemicals in use [ADDIN EN.CITE ADDIN EN.CITE.DATA]. 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 [ADDIN EN.CITE <EndNote><Cite><Author>Pierce</Author><Year>2005</Year><RecNum>209</RecNum><DisplayText>(Pierce et al. 2005)</DisplayText><record><rec-number>209</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463108827">209</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierce, R. H.</author><author>Henry, M. S.</author><author>Blum, T. C.</author><author>Mueller, E. M.</author></authors></contributors><auth-address>Mote Marine Laboratory, 1600 Ken Thompson Parkway, Sarasota, Florida 34236, USA. rich@mote.org</auth-address><titles><title>Aerial and tidal transport of mosquito control pesticides into the Florida Keys National Marine Sanctuary</title><secondary-title>Rev Biol Trop</secondary-title></titles><periodical><full-title>Rev Biol Trop</full-title></periodical><pages>117-25</pages><volume>53 Suppl 1</volume><keywords><keyword>*Air Movements</keyword><keyword>Animals</keyword><keyword>Dichlorvos/analysis/toxicity</keyword><keyword>Environmental Monitoring</keyword><keyword>Gas Chromatography-Mass Spectrometry</keyword><keyword>Insecticides/*analysis/toxicity</keyword><keyword>Lethal Dose 50</keyword><keyword>Naled/*analysis/toxicity</keyword><keyword>Permethrin/*analysis/toxicity</keyword><keyword>Seawater/*chemistry</keyword><keyword>*Water Movements</keyword></keywords><dates><year>2005</year><pub-dates><date>May</date></pub-

[47] [HYPERLINK "http://keysmosquito.org/wp-content/uploads/2015/05/2015-06-23-Reg-Mtg-Minutes.pdf"] [Accessed March 4, 2016]

dates></dates><isbn>0034-7744 (Print)
0034-7744 (Linking)</isbn><accession-num>17465151</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/17465151</url></related-urls></urls></record></Cite></EndNote>] and as they are non-discriminatory may harm beneficial insect species as well. Recent research however indicates that this risk may have been overstated [ADDIN EN.CITE ADDIN EN.CITE.DATA]. 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.

~~13.6.1~~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 [ADDIN EN.CITE <EndNote><Cite><Author>Juliano</Author><Year>2005</Year><RecNum>227</RecNum><DisplayText>(Juliano and Lounibos 2005)</DisplayText><record><rec-number>227</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463109971">227</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Juliano, S. A.</author><author>Lounibos, L. P.</author></authors></contributors><auth-address>Department of Biological Sciences, Behavior, Ecology, Evolution and Systematics Section, Illinois State University, Normal, IL 61790-4120, USA.</auth-address><titles><title>Ecology of invasive mosquitoes: effects on resident species and on human health</title><secondary-title>Ecol Lett</secondary-title></titles><periodical><full-title>Ecol Lett</full-title></periodical><pages>558-74</pages><volume>8</volume><number>5</number><dates><year>2005</year><pub-dates><date>May</date></pub-dates></dates><isbn>1461-0248 (Electronic)
1461-023X (Linking)</isbn><accession-num>17637849</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/17637849</url></related-urls></urls><custom2>1920178</custom2><electronic-resource-num>10.1111/j.1461-0248.2005.00755</electronic-resource-num></record></Cite></EndNote>]. Therefore, ~~larval~~ conditions for larval growth and development may have a significant impact on overall container-breeding insect 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. The effect of the 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 would involve a higher number of OX513A males released to the local *Ae. aegypti* male population at the trial site, which would 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 would be adapted to during the course of the trial to maintain over 50% of the female matings with OX513A.

13.6.213.5.2 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. There is some evidence that the presence of predators affects oviposition by *Ae. aegypti* (Albeny-Simoes *et al.*, 2014), where the mosquitoes 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.

Commented [KJ26]: This seems counterintuitive. Mosquitoes are attracted to vessels where they sense predators and lay their eggs?

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Potential routes of exposure involve different ecological guilds[48] of organisms. These guilds are summarized in [REF _Ref450334934 \h].

Table [SEQ Table * ARABIC]. 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

[48] 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.

urban or domestic mosquito closely associated with human habitations. Non-target organisms in these areas are not usually threatened or, 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 because it 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* presence in the food chain 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).

~~13.6.3.113.5.2.1~~ **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~~crepuscular. Furthermore, a study conducted on bats found that mosquitoes were not always available as food~~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, and variable field metabolic rates [ADDIN EN.CITE <EndNote><Cite><Author>Gonsalves</Author><Year>2013</Year><RecNum>234</RecNum><DisplayTe
xt>(Gonsalves et al. 2013)</DisplayText><record><rec-number>234</rec-number><foreign-keys><key
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type><contributors><authors><author>Gonsalves, L.</author><author>Bicknell,
B.</author><author>Law, B.</author><author>Webb, C.</author><author>Monamy,
V.</author></authors></contributors><auth-address>School of Arts & Sciences, Australian
Catholic University, North Sydney, New South Wales, Australia.</auth-address><titles><title>Mosquito
consumption by insectivorous bats: does size matter?</title><secondary-title>PLoS One</secondary-
title></titles><periodical><full-title>PLoS ONE</full-
title></periodical><pages>e77183</pages><volume>8</volume><number>10</number><keywords><k
eyword>Animals</keyword><keyword>*Body Size</keyword><keyword>Chiroptera/*anatomy &
histology/*physiology</keyword><keyword>*Culicidae/genetics</keyword><keyword>DNA/analysis</k
eyword><keyword>Diet/*veterinary</keyword><keyword>*Eating</keyword><keyword>Echolocation<
/keyword><keyword>*Mosquito

Control</keyword></keywords><dates><year>2013</year></dates><isbn>1932-6203 (Electronic)
1932-6203 (Linking)</isbn><accession-num>24130851</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24130851</url></related-urls></urls><custom2>3795000</custom2><electronic-resource-num>10.1371/journal.pone.0077183</electronic-resource-num></record></Cite></EndNote>]~~(Gonçalves et al., 2013)~~. The American Mosquito Control Association (AMCA) also reviews the role of bats for mosquito control on its~~their~~ website,[49] indicating that although bats do eat mosquitoes, ~~the consumption of~~ mosquitoes ~~by bats~~ comprised ~~of~~ less than 1% of the~~ir~~ 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 by [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Agosta</Author><Year>2002</Year><RecNum>267</RecNum><DisplayText>Agosta (2002)</DisplayText><record><rec-number>267</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1466707446">267</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Agosta, S.J.</author></authors></contributors><titles><title>Habitat use, diet and roost selection by the Big Brown Bat (Eptesicus fuscus) in North America: a case for conserving an abundant species</title><secondary-title>Mammal Review</secondary-title></titles><periodical><full-title>Mammal Review</full-title></periodical><pages>179-198</pages><volume>32</volume><number>3</number><dates><year>2002</year></dates><urls></urls></record></Cite></EndNote>]~~in Agosta 2002~~).- This is also confirmed by a study from [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Feldhamer</Author><Year>2009</Year><RecNum>279</RecNum><DisplayText>Feldhamer and Carter (2009)</DisplayText><record><rec-number>279</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1466736779">279</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Feldhamer, G.A.</author><author>Carter, T.C.</author></authors></contributors><titles><title>Prey consumed by eight species of insectovorous bats from Southern Illinois</title><secondary-title>American Midland Naturalist</secondary-title></titles><periodical><full-title>American Midland Naturalist</full-title></periodical><pages>43-51</pages><volume>162</volume><number>1</number><dates><year>2009</year></dates><urls></urls></record></Cite></EndNote>]~~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~~ likelihood that the mosquito~~y~~ ~~to~~ would form only a small part of the bat diet, it is unlikely that *Ae. aegypti* OX513A would~~ill~~ significantly impact ~~on~~ bats ~~in a significant way~~.

[49] [HYPERLINK "http://www.mosquito.org/faq"] (Accessed June 20, 2016).

~~13.6.2.2~~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[50] 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.[51] Therefore, due to the temporal separation in activity periods and that the mosquito is likely to form only a small part of the bird diet, it is unlikely that *Ae. aegypti* OX513A would~~ll~~ significantly impact ~~on~~ insectivorous~~e~~ birds ~~in a significant way~~.

~~13.6.2.3~~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 numbers for effective mosquito control.[52] Amphibians ~~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 is 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 a container breeding species ~~more~~ associated with human habitats, and salamanders are associated with seasonal pools and wetlands. Blum *et al.*, 1997 found ~~that~~ through a~~the~~ diet analysis of anurans (newts) that mosquitoes made up only 0.16% of the anuran diet's content.

Commented [KJ27]: Salamanders are not reptiles. I would change the sentence to "... amphibians such as frogs and salamanders.." This section is titled amphibians so not clear why reptiles mentioned at all

~~13.6.2.4~~13.5.2.4 Predatory invertebrates

Invertebrate predators form another group that is known to prey~~date~~ on mosquito larvae, in particular the predator mosquito species *Toxorhynchites*, 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,~~ *Toxorhynchites rutillus* is present in Florida, 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

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[50] [HYPERLINK "http://www.purplemartin.org"] [Accessed June 21, 2016].

[51] [HYPERLINK "http://www.mosquito.org/faq#purple%20martins"] [Accessed June 21, 2016].

[52] [HYPERLINK "http://www.michigan.gov/emergingdiseases/0,4579,7-186-25805_25824-75797--,00.html"] [Accessed June 21, 2016].

ED_006741_00009696-00120

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.

~~13.6.2.5~~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.

~~13.6.2.5.1~~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 [ADDIN EN.CITE <EndNote><Cite><Author>Nordin</Author><Year>2013</Year><RecNum>18</RecNum><DisplayText>(Nordin et al. 2013)</DisplayText><record><rec-number>18</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">18</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>Nordin, Oreenaiza</author><author>Donald, Wesley</author><author>Ming, Wong Hong</author><author>Ney, Teoh Guat</author><author>Mohamed, Khairul Asuad</author><author>Halim, Nor Azlina Abdul</author><author>Winskill, Peter</author><author>Hadi, Azahari Abdul</author><author>Muhammad, Zulkamal Safi'in</author><author>Lacroix, Renaud</author><author>Scaife, Sarah</author><author>McKemey, Andrew Robert</author><author>Beech, Camilla</author><author>Shahnaz, Murad</author><author>Alphey, Luke</author><author>Nimmo, Derric David</author><author>Nazni, Wasi Ahmed</author><author>Lee, Han Lim</author></authors></contributors><titles><title>Oral Ingestion of Transgenic RIDL Ae. aegypti Larvae Has No Negative Effect on Two Predator Toxorhynchites Species</title><secondary-title>PLoS ONE</secondary-title></titles><periodical><full-title>PLoS ONE</full-title></periodical><pages>e58805</pages><volume>8</volume><number>3</number><reprint-edition>Not in File</reprint-edition><dates><year>2013</year><pub-dates><date>2013</date></pub-dates></dates><isbn>1932-6203</isbn><label>18</label><urls><related-urls><url>http://dx.plos.org/10.1371/journal.pone.0058805</url></related-urls></urls><electronic-resource-num>10.1371/journal.pone.0058805</electronic-resource-num><access-date>3/28/2015</access-date></record></Cite></EndNote>]. 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~~The reason for the occurrence of these results ~~occurred~~ is unknown but there were no significant differences in any other measured parameters.

Commented [KJ28]: So the controls were not raised on tet? I think that could be the confounding fact. Most antibiotics do have some level of toxicity even in mammalian species and perhaps more so in mosquito species without the conditional lethality trait.

There was no evidence that the development, fecundity, or longevity of the two *Toxorhynchites* species were adversely affected 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 [ADDIN EN.CITE
<EndNote><Cite><Author>Nordin</Author><Year>2013</Year><RecNum>18</RecNum><DisplayText>(Nordin et al. 2013)</DisplayText><record><rec-number>18</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">18</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>Nordin, Oreenaiza</author><author>Donald, Wesley</author><author>Ming, Wong Hong</author><author>Ney, Teoh Guat</author><author>Mohamed, Khairul Asuad</author><author>Halim, Nor Azlina Abdul
/author><author>Winskill, Peter</author><author>Hadi, Azahari Abdul</author><author>Muhammad, Zulkamal Safi'in</author><author>Lacroix, Renaud</author><author>Scaife, Sarah</author><author>McKemey, Andrew Robert</author><author>Beech, Camilla</author><author>Shahnaz, Murad</author><author>Alphey, Luke</author><author>Nimmo, Derric David</author><author>Nazni, Wasi Ahmed</author><author>Lee, Han Lim</author></authors></contributors><titles><title>Oral Ingestion of Transgenic RIDL Ae. aegypti Larvae Has No Negative Effect on Two Predator Toxorhynchites Species</title><secondary-title>PLoS ONE</secondary-title></titles><periodical><full-title>PLoS ONE</full-title></periodical><pages>e58805</pages><volume>8</volume><number>3</number><reprint-edition>Not in File</reprint-edition><dates><year>2013</year><pub-dates><date>2013</date></pub-dates></dates><isbn>1932-6203</isbn><label>18</label><urls><related-urls><url>http://dx.plos.org/10.1371/journal.pone.0058805</url></related-urls></urls><electronic-resource-num>10.1371/journal.pone.0058805</electronic-resource-num><access-

date>3/28/2015</access-date></record></Cite></EndNote>]. This study is published by Nordin et al., 2013 in an open access journal[53].

13.6.2.5.213.5.2.5.2 **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 [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>OECD</Author><Year>1984</Year><RecNum>23</RecNum><DisplayText>OECD (1984)</DisplayText><record><rec-number>23</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">23</key></foreign-keys><ref-type name="Book">6</ref-type><contributors><authors><author>OECD</author></authors></contributors><titles><title>Test No. 204: Fish, Prolonged Toxicity Test: 14-Day Study</title></titles><reprint-edition>Not in File</reprint-edition><dates><year>1984</year><pub-dates><date>1984</date></pub-dates></dates><pub-location>Paris</pub-location><publisher>Organisation for Economic Co-operation and Development</publisher><isbn>9789264069985</isbn><label>24</label><urls><related-urls><url>http://www.oecd-ilibrary.org/content/book/9789264069985-en</url></related-urls></urls><access-date>4/27/2015</access-date></record></Cite></EndNote>] 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 under laboratory conditions. During the study, the fish were fed with OX513A mosquitoes or the non-GE control mosquitoes in the fish diet, daily, at the rate of 700 g mosquitoes/kg diet, following a range findings= study. The natural ratio for this fish species is approximately 50% (500 g 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 foundin [REF _Ref454268856 \h] immediately below; the study is appended (*Appendix H*).

Table [SEQ Table * ARABIC]. Summary of *P. reticulate* mortality, length, and weight after 14-day oral exposure to *Aedes aegypti*.

Endpoint	14-day mortality (%)	14-day length (mm)	14-day weight (mg)

[53] Nordin, O., Donald, W., Ming, W.H., Ney, T.G., Mohamed, K.A., Halim, N.A., Winskill, P., Hadi, A.A., Muhammad, Z.S., Laurok, 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.

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	>700	>700
LOER [g GE mosquitoes/kg diet]	>700	>700	>700
NOER [g GE mosquitoes/kg diet]	>700	>700	>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.

~~13.6.3~~13.5.3 ***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).

~~13.6.4~~13.5.4 ***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.

~~13.6.5~~13.5.5 *Ae. aegypti* as a resource for decomposers.

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A few organisms are known decomposers of *Ae. aegypti*; fungi such as *Metarhizium anisopliae*, a well-known entomopathogenic fungus[54] 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*, *Beauveria 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.

~~13.6.6~~13.5.6 *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 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;

[54] 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.

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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.

13.5.7 *Ae. aegypti* and threatened and endangered species

As described in Section [REF _Ref453244060 \r \h], the Stock Island Snail is the only species located in the physical vicinity of the proposed trial site. We determined that that the proposed investigational use of OX513A mosquitoes would not adversely affect the Stock Island Tree Snail because the Stock Island Tree Snail's habitat (hammock and beach berm) does not overlap with the domestic or peri-domestic environment of *Ae. aegypti*. Additionally, the proposed investigational trial does not intend to remove or modify the snail's habitat (hammock and beach berm). Therefore, FDA made a "no effect" determination under the ESA, 16 U.S.C. § 1531 *et seq.* The proposed investigational trial, as described in Section [REF _Ref453245461 \r \h], would not jeopardize the continued existence of the endangered Stock Island Tree Snail and would not result in the destruction or adverse modification of its critical habitat.

Commented [KJ29]: I think it may be a good idea to at least indicate that there is no indication that mosquitoes do not interact with this species. There is no reason to believe that any interaction would be in no material way different from interaction with the existing *Ae.aegypti* populations.

An overview of the wildlife refuges located in Monroe County is provided in Section [REF _Ref453940150 \r \h]. Because all of these refuges are located a considerable distance from the proposed trial site ~~of the study~~, it is highly unlikely that the proposed trial would have any effects on their environment. Thus, we conclude that the proposed trial would not jeopardize the continued existence of any other endangered species in wildlife refuges located in Monroe County or result in the destruction or adverse modification of other endangered species' critical habitat due to their being located a considerable distance from the proposed trial site.

13.5.8 Introgression of traits from OX513A to local wild-type *Ae. aegypti* at release site

The short duration of the release coupled with the lethal nature of the integrated trait limits the possibility of introgression of new traits into the local wild-type *Ae. aegypti* population. Further, results of insecticide testing indicate the absence of traits related to pyrethroid and organophosphate resistance including kdr mutations in OX513A mosquitoes. Thus, these traits cannot be introgressed into local mosquito populations. Lastly, *Ae. aegypti* strains continue to move around the globe by piggy backing on human modes of transportation such as cars, trucks, and buses on highways as well as ships and airplanes [ADDIN EN.CITE ADDIN EN.CITE.DATA] ~~(see Griffitts THD (1933). Air traffic in relation to public health. American Journal of Tropical Medicine 13:283-290 ; Guagliardo SA et al. (2014) Patterns of geographic expansion of Aedes aegypti in the Peruvian Amazon. PLoS NTD. 8(8): e3033 doi: 10.1371/journal.pntd.0003033; Miller MJ et al. (2015). Geographic expansion of the invasive mosquito Aedes albopictus across Panama—implications for control of dengue and chikungunya viruses. PLoS NTD 9(1): e0003333; Tatem AJ et al. (2006). Global traffic and disease vector dispersal. PNAS. 103(16):6242-6247.)~~ As a result~~This means that~~ introduction of new, non-native strains is ~~are~~ a constant possibility~~threat~~, especially to in an ~~high tourist~~ areas with a high number of visitors such as the Florida ~~k~~Keys. Thus, new traits could also introgress from these non-native strains that are introduced by

humans and unlike OX513A, go undetected due to the lack of readily observable phenotypic markers (e.g. DsRed2) and surveillance.

13.5.9 Question conclusions

FDA has determined that it is highly unlikely that the presence of OX513A mosquitoes and their progeny and suppression of the local population of *Ae. aegypti* would have any significant effects on the populations of predators, parasitoids, and decomposers at the proposed trial site. No adverse effect on the pollination of local plants is expected as well. Should the proposed field trial proceed, FDA has determined that the proposed trial would not jeopardize the continued existence of Stock Island Tree snails at the proposed trial site and would not result in the destruction or adverse modification of their habitat. Therefore, FDA makes a "no effect" determination under the ESA with regard to the Stock Island Tree Snail. Further, FDA does not expect any adverse effects on other endangered species in wildlife refuges located in Monroe County or destruction and modification of their habitats due to their considerable distance from the proposed trial site.

13.713.6 What is the likelihood that the rDNA expression products in OX513A mosquitoes would have adverse effects on humans or other animals? Analysis of the potential toxicity and allergenicity of the introduced proteins

13.7.113.6.1 Bioinformatics studies of the novel proteins expressed in OX513A

Because wild-type *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 survival of progeny from the OX513A mating (due to lack of complete penetrance of lethality trait) or a small amount number of OX513A females being released (due to lack of 100% sorting efficiency), two questions were examinedpursued:

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 wild-type Ae. aegypti 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 to aid with conduct of risk assessments for the safety of foods from produced with genetically engineered sources organisms and hence an oral route of exposure.

Moreover, Ssubcutaneous (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

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~~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.~~ [ADDIN EN.CITE
<EndNote><Cite><Author>Canonica</Author><Year>2014</Year><RecNum>66</RecNum><DisplayText>(Canonica et al. 2014)</DisplayText><record><rec-number>66</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1451409357">66</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Canonica, G. W.</author><author>Cox, L.</author><author>Pawankar, R.</author><author>Baena-Cagnani, C. E.</author><author>Blaiss, M.</author><author>Bonini, S.</author><author>Bousquet, J.</author><author>Calderon, M.</author><author>Compalati, E.</author><author>Durham, S. R.</author><author>van Wijk, R. G.</author><author>Larenas-Linnemann, D.</author><author>Nelson, H.</author><author>Passalacqua, G.</author><author>Pfaar, O.</author><author>Rosario, N.</author><author>Ryan, D.</author><author>Rosenwasser, L.</author><author>Schmid-Grendelmeier, P.</author><author>Senna, G.</author><author>Valovirta, E.</author><author>Van Bever, H.</author><author>Vichyanond, P.</author><author>Wahn, U.</author><author>Yusuf, O.</author></authors></contributors><auth-address>Respiratory and Allergy Clinic, DIMI-Department of Internal Medicine, University of Genoa, IRCCS Aou San Martino, Largo Rosanna Benzi 10, Genoa 1-16132, Italy. canonica@unige.it.</auth-address><titles><title>Sublingual immunotherapy: World Allergy Organization position paper 2013 update</title><secondary-title>World Allergy Organ J</secondary-title></titles><periodical><full-title>World Allergy Organ J</full-title></periodical><pages>6</pages><volume>7</volume><number>1</number><dates><year>2014</year></dates><isbn>1939-4551 (Electronic)
1939-4551 (Linking)</isbn><accession-num>24679069</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24679069</url></related-urls></urls><custom2>3983904</custom2><electronic-resource-num>10.1186/1939-4551-7-6</electronic-resource-num></record></Cite></EndNote>]~~(Canonica et al., 2014).~~ The argument that that both oral and subcutaneous exposure to known allergens would likely illicit the same immunological response in individuals allergic to these substances is based on the expert opinion of Dr. Ian Kimber, professor of toxicology at the University of Manchester, whose expert opinion is provided in Appendix I. Dr. Kimber states that the activity of allergens is independent of the route of exposure, pointing out that chicken ovalbumin, a known allergen, also has the ability to cause respiratory allergies in workers at poultry plants [ADDIN EN.CITE
<EndNote><Cite><Author>James</Author><Year>2007</Year><RecNum>76</RecNum><DisplayText>(James and Crespo 2007)</DisplayText><record><rec-number>76</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1451507618">76</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>James, J. M.</author><author>Crespo, J. F.</author></authors></contributors><auth-address>Colorado Allergy and Asthma Centers, 1136 East Stuart Street, Suite 3200, Fort Collins, CO 80524, USA. jm.james@coloradoallergy.com</auth-address><titles><title>Allergic reactions to foods by inhalation</title><secondary-title>Curr Allergy

Commented [KJ30]: I think you mean "elicit"

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Asthma Rep</secondary-title></titles><periodical><full-title>Curr Allergy Asthma Rep</full-title></periodical><pages>167-74</pages><volume>7</volume><number>3</number><keywords><keyword>*Allergens/administration & dosage/immunology</keyword><keyword>Animals</keyword><keyword>Cattle</keyword><keyword>Eggs</keyword><keyword>*Food Hypersensitivity</keyword><keyword>Humans</keyword><keyword>Inhalation</keyword><keyword>Milk</keyword><keyword>Seeds</keyword></keywords><dates><year>2007</year><pub-dates><date>Jun</date></pub-dates></dates><isbn>1529-7322 (Print)
1529-7322 (Linking)</isbn><accession-num>17448326</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/17448326</url></related-urls></urls></record></Cite></EndNote>]. In addition, there are other studies [ADDIN EN.CITE ADDIN EN.CITE.DATA] that evaluate the efficacy of sublingual (i.e., oral) and subcutaneous routes in allergen immunotherapy.[55] These studies show that for certain allergens sublingual exposure could be as effective as subcutaneous exposure with regard to the immunological response. Recent FDA approvals of GRASTEK [ADDIN EN.CITE <EndNote><Cite><Author>FDA</Author><Year>2014</Year><RecNum>69</RecNum><DisplayText>(FDA 2014a)</DisplayText><record><rec-number>69</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1451431341">69</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>FDA</author></authors></contributors><titles><title>April 11, 2014 Approval Letter - GRASTEK. http://www.fda.gov/BiologicsBloodVaccines/Allergenics/ucm393185.htm</title></titles><dates><year>2014</year></dates><urls></urls></record></Cite></EndNote>], ORALAIR [ADDIN EN.CITE <EndNote><Cite><Author>FDA</Author><Year>2014</Year><RecNum>70</RecNum><DisplayText>(FDA 2014b)</DisplayText><record><rec-number>70</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1451431496">70</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>FDA</author></authors></contributors><titles><title>FDA approves first sublingual allergen extract for the treatment of certain grass pollen allergies. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm391458.htm</title></titles><dates><year>2014</year></dates><urls></urls></record></Cite></EndNote>], and RAGWITEK [ADDIN EN.CITE <EndNote><Cite><Author>FDA</Author><Year>2014</Year><RecNum>71</RecNum><DisplayText>(FDA 2014c)</DisplayText><record><rec-number>71</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1451431559">71</key></foreign-keys><ref-

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[55] Immunotherapy involves the administration of gradually increasing amounts of allergen over a period of time to desensitize the subject. In sublingual immunotherapy, administration of allergens through oral, gingival, or sublingual mucosa can decrease the allergic response and desensitize the subject.

type name="Electronic Article">43</ref-type><contributors><authors><author>FDA</author></authors></contributors><titles><title>FDA approves Ragwitek for short ragweed pollen allergies. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm393820.htm </title></titles><dates><year>2014</year></dates><urls></urls></record></Cite></EndNote>] for treatment of allergic rhinitis using immunotherapy further support this statement. Thus, the weight of evidence suggests that the ability of allergens to illicit an immunological response in some instances may be independent of the route of exposure. ~~Therefore Three studies (Elifan, 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~~, we consider that the use of the Codex guidelines may be a suitable approach to evaluate the potential allergenicity and toxicity of tTAV and DsRed2 proteins. ~~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 Oxtec'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).~~

Commented [KJ31]: I agree that bioinformatics is a useful tool here but I don't know if I agree with Ian Kimber about all routes being the same for sensitizing and individual. Maybe elicitation is route independent in a sensitized individual but I find it hard to believe sensitization is too. My personal opinion: Ignore as you please.

~~13.7.1.1~~13.6.1.1 tTAV potential toxicity and ~~potential~~ allergenicity assessment

The ~~C~~conditional lethal element, known as the tTA system, which was developed by [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Gossen</Author><Year>1992</Year><RecNum>12</RecNum><DisplayText>Gossen and Bujard (1992)</DisplayText><record><rec-number>12</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">12</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Gossen, Manfred</author><author>Bujard, Hermann</author></authors></contributors><titles><title>Tight control of gene expression in mammalian cells by tetracycline-responsive promoters</title><secondary-title>PNAS</secondary-title></titles><periodical><full-title>PNAS</full-title></periodical><pages>5547-5551</pages><volume>89</volume><number>12</number><reprint-edition>Not in File</reprint-edition><dates><year>1992</year><pub-dates><date>1992</date></pub-dates></dates><label>12</label><urls><related-urls><url>http://www.pnas.org/content/89/12/5547.short</url></related-urls></urls><access-date>3/28/2015</access-date></record></Cite></EndNote>], and subsequent variants of that system, haves 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" [ADDIN EN.CITE ADDIN EN.CITE.DATA] and/or interference with ubiquitin-dependent proteolysis. It is the interference of high levels of tTA 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 tTA protein is turned off.

Although some potential symptoms of toxicity have been reported in transgenic mice expressing high levels of tTA or its variants [ADDIN EN.CITE <EndNote><Cite><Author>Whitsett</Author><Year>2006</Year><RecNum>187</RecNum><DisplayText>(Whitsett and Perl 2006)</DisplayText><record><rec-number>187</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463107223">187</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Whitsett, J. A.</author><author>Perl, A. K.</author></authors></contributors><titles><title>Conditional control of gene expression in the respiratory epithelium: A cautionary note</title><secondary-title>Am J Respir Cell Mol Biol</secondary-title></titles><periodical><full-title>Am J Respir Cell Mol Biol</full-title></periodical><pages>519-20</pages><volume>34</volume><number>5</number><keywords><keyword>Animals</keyword><keyword>Gene Expression Regulation/*drug effects</keyword><keyword>Integrases/genetics/metabolism/toxicity</keyword><keyword>Mice</keyword><keyword>Respiratory Mucosa/*drug effects/*metabolism</keyword><keyword>Tetracycline/pharmacology</keyword></keywords><dates><year>2006</year><pub-dates><date>May</date></pub-dates></dates><isbn>1044-1549 (Print)
1044-1549 (Linking)</isbn><accession-num>16618785</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/16618785</url></related-urls></urls><electronic-resource-num>10.1165/rcmb.F310</electronic-resource-num></record></Cite></EndNote>], other papers have reported observinged no apparent toxicity [ADDIN EN.CITE ADDIN EN.CITE.DATA].

~~Any evidence of an effect on cognitive behavior in mice is irrelevant to OX513A mosquitoes and their phenotype related to performance in the field as evidenced by results from suppression trials in other areas outside of the U.S.~~

The potential toxicity and allergenicity of the tTAV and DsRed2 proteins ~~was~~ were 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 the literature 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 and DsRed2 search strategies (FASTA3; BLASTP algorithm) using Allergenonline version 13 and NCBI Entrez protein databases.

The predicted amino acid sequence of tTAV is given in [REF _Ref453239562 \h] below.

<tTAV

MGSRLDKSKVINSALELLNEVGIEGLTTRKLAQKLGVEQPTLYWHVKNKRALLDALAIEM
LDRHHTHFCPLEGESWQDFLRNNAKSFRCALLSHRDGAKVHLGTRPTEKQYETLENQLAF
LCQQGFSLENALYALSAVGHFTLGCVLEDQEHQVAKEERETPTTDSMPPLLRQAIELFDH
QGAEPAFLFGLELIICGLEKQLKCESGSGPAYSRARTKNNYGSTIEGLLDLPDDDAPEEA
GLAAPRLSFLPAGHTRRLSTAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDS
PGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDEYGG

Figure [SEQ Figure * ARABIC]. Amino acid sequence of the tTAV protein.

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 September, 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 (~~[REF _Ref453239744 \h]~~). In OX513A, there are three additional amino acids (MAR) at the N-terminus, which are from a cloning linker sequence.~~

~~13.7.1.2~~13.6.1.2 **DsRed2 potential toxicity and allergenicity assessment**

DsRed2 is a marker protein which is expressed constitutively in the developmental life stages of the OX513A mosquito. DsRed is a naturally occurring fluorescent protein which was originally found in various *Discosoma spp*. DsRed2 was developed *in vitro* from native DsRed to enhance the fluorescence and improve the solubility of the protein, which in turn increases the sensitivity of detection (Shagin, 2004; Bevis, 2000; Matz, 1999; Lukyanov, 2000; CLONTECHniques, 2001) of cells expressing this enhanced DsRed2 protein. The DsRed2 DNA sequence used in #OX513A was obtained from Clontech Laboratories ([REF _Ref454528850 \h]). The N-terminus of DsRed2 protein expressed by OX513A mosquitoes has three additional amino acids (MAR) from a cloning linker sequence.

Commented [KJ32]: These 3 added amino acids do not seem to be reflected in the provided sequence information unless MASS is supposed to be MARS-

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

Figure [SEQ Figure * ARABIC]. Amino acid sequence of the DsRed2 protein

The DsRed2 marker protein has been evaluated in a New Protein Consultation by the FDA Center for Food Safety and Applied Nutrition (CFSAN) ~~in the USA~~ for human safety, and they raised no objections to its use in corn plants [ADDIN EN.CITE <EndNote><Cite><Author>FDA</Author><Year>2010</Year><RecNum>73</RecNum><DisplayText>(Pioneer Hi-Bred International 2006; FDA 2010)</DisplayText><record><rec-number>73</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1451496120">73</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>FDA</author></authors></contributors><titles><title>NPC 000004: Agency Response Letter. http://www.fda.gov/Food/FoodScienceResearch/GEPlants/Submissions/ucm222920.htm</title></titles><dates><year>2010</year></dates><urls></urls></record></Cite><Cite><Author>Pioneer Hi-Bred International</Author><Year>2006</Year><RecNum>79</RecNum><record><rec-number>79</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1451584513">79</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>Pioneer Hi-Bred International, Inc.</author></authors></contributors><titles><title>Early food safety evaluation for a Red Fluorescent Protein: DsRed2. http://www.fda.gov/downloads/Food/Biotechnology/Submissions/UCM219002.pdf</title></titles><dates><year>2006</year></dates><urls></urls></record></Cite></EndNote>] ~~(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 [ADDIN EN.CITE <EndNote><Cite><Author>Pioneer Hi-Bred International</Author><Year>2006</Year><RecNum>79</RecNum><DisplayText>(Pioneer Hi-Bred International 2006)</DisplayText><record><rec-number>79</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1451584513">79</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>Pioneer Hi-Bred International, Inc.</author></authors></contributors><titles><title>Early food safety evaluation for a Red Fluorescent Protein: DsRed2. http://www.fda.gov/downloads/Food/Biotechnology/Submissions/UCM219002.pdf</title></titles><dates><year>2006</year></dates><urls></urls></record></Cite></EndNote>]. Additional information on the lack of toxicity of DsRed2 is presented in a reviewed by [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Millwood</Author><Year>2010</Year><RecNum>160</RecNum><DisplayTex

ED_006741_00009696-00133

t>Millwood et al. (2010)</DisplayText><record><rec-number>160</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106826">160</key></foreign-keys><ref-type name="Book Section">5</ref-type><contributors><authors><author>Millwood, Reginald J.</author><author>Moon, Hong S.</author><author>Neal Stewart, C.</author></authors><secondary-authors><author>Geddes, Chris D.</author></secondary-authors></contributors><titles><title>Fluorescent Proteins in Transgenic Plants</title><secondary-title>Reviews in Fluorescence 2008</secondary-title></titles><pages>387-403</pages><volume>2008</volume><dates><year>2010</year><pub-dates><date>2010</date></pub-dates></dates><pub-location>New York, NY</pub-location><publisher>Springer New York</publisher><isbn>978-1-4419-0828-5 978-1-4419-1260-2</isbn><urls><related-urls><url>http://link.springer.com/10.1007/978-1-4419-1260-2_16</url></related-urls></urls><remote-database-provider>CrossRef</remote-database-provider><access-date>2015/03/28/04:14:08</access-date></record></Cite></EndNote>] and [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Stewart</Author><Year>2006</Year><RecNum>179</RecNum><DisplayText>Stewart (2006)</DisplayText><record><rec-number>179</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106827">179</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Stewart, C. Neal</author></authors></contributors><titles><title>Go with the glow: fluorescent proteins to light transgenic organisms</title><secondary-title>Trends in Biotechnology</secondary-title><short-title>Go with the glow</short-title></titles><periodical><full-title>Trends in Biotechnology</full-title></periodical><pages>155-162</pages><volume>24</volume><number>4</number><dates><year>2006</year><pub-dates><date>2006/04//</date></pub-dates></dates><isbn>01677799</isbn><urls><related-urls><url>http://linkinghub.elsevier.com/retrieve/pii/S0167779906000308</url><url>http://ac.els-cdn.com/S0167779906000308/1-s2.0-S0167779906000308-main.pdf?_tid=6507413a-18b3-11e6-91e3-00000aab0f6c&acdnat=1463107125_e9ac98915605a6468edc33298d723e9b</url></related-urls></urls><electronic-resource-num>10.1016/j.tibtech.2006.02.002</electronic-resource-num><remote-database-provider>CrossRef</remote-database-provider><language>en</language><access-date>2015/03/28/04:21:52</access-date></record></Cite></EndNote>], including oral studies in rats [ADDIN EN.CITE <EndNote><Cite><Author>Richards</Author><Year>2003</Year><RecNum>171</RecNum><DisplayText>(Richards et al. 2003)</DisplayText><record><rec-number>171</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106827">171</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Richards, Harold A.</author><author>Han, Chung-Ting</author><author>Hopkins, Robin G.</author><author>Failla, Mark L.</author><author>Ward, William W.</author><author>Stewart, C. Neal</author></authors></contributors><titles><title>Safety assessment of recombinant green fluorescent protein orally administered to weaned rats</title><secondary-title>The Journal of nutrition</secondary-title></titles><periodical><full-title>The Journal of nutrition</full-title></periodical><pages>1909-

1912</pages><volume>133</volume><number>6</number><dates><year>2003</year><pub-dates><date>2003</date></pub-dates></dates><urls><related-urls><url>http://jn.nutrition.org/content/133/6/1909.short</url><url>http://jn.nutrition.org/content/133/6/1909.full.pdf</url></related-urls></urls><remote-database-provider>Google Scholar</remote-database-provider><access-date>2015/03/28/04:19:21</access-date></record></Cite></EndNote>]. It has been further evaluated in an EA by the United States Department of Agriculture, Animal Plant Health Inspection Service (APHIS),[56] which concluded that the corn transformation event that contained the DsRed2 gene was unlikely to become a plant pest risk. APHIS conducted Aan additional EA on a GE pink bollworm expressing fluorescent genes similar to DsRed2 ~~has also been conducted~~ (USDA, 2001)[57] ~~and~~ that concluded in a Finding of No Significant Impact ~~(FONSI)~~ on the environment. Furthermore, DsRed2 and members of ~~its~~ the related Green Fluorescent Protein family, ha~~ve~~s 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.~~

~~13.7.1.3~~13.6.1.3 Bioinformatics assessment results

The ~~P~~potential allergenicity assessment examined the presence of known allergenic sequences in the tTAV and DsRed2 proteins. Oxitec performed several bioinformatic analyses as per Codex Alimentarius guidelines (2003) to determine potential IgE binding epitopes as well as the potential for cross-reaction with other known allergens. The use of Codex guidelines is appropriate as they provide a robust risk assessment paradigm for both food and non-food exposure to the two proteins as there is no single predictive criterion for the potential allergenicity of newly expressed proteins. A search of allergenic sequences in Version 13 of the Food Allergy Research and Resource Program (FARRP) Allergenonline.org using the complete sequences of tTAV and DsRed2 did not yield any cross matches that had greater than 35% amino acid identity. The same results were returned for an 80 amino acid sliding window sequence homology search of the same A~~a~~llergenonline database. A third search for any known allergens in the Allergenonline database for any match of any eight contiguous amino acid segments was also negative. There were also no matches with more than 50% identity over the full sequence length of both proteins. BLASTP searches of NCBI Entrez using DsRed2 protein sequence and the keyword allergen returned 6 proteins with E scores <10 and all had either low identity and/or short regions of alignment making the matches highly unlikely to cause cross reactivity in humans. When a similar BLASTP search was conducted using full length tTAV protein sequence only one match with a sequence length of 20 amino acids and 55% identity was returned. These short regions of identity suggest that the overall structure of the query is unlikely to match the known allergenic epitopes of the proteins in the database.

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[56] [HYPERLINK "http://www.aphis.usda.gov/brs/aphisdocs/08_33801p_dpra.pdf" \h][Accessed June 21, 2016~~March 19, 2013~~].

[57] [HYPERLINK "http://www.gpo.gov/fdsys/pkg/FR-2006-04-19/html/E6-5878.htm" \h][Accessed June 21, 2016~~March 14 2013~~]

Although Codex Guidelines are primarily intended to evaluate food safety concerns from GE organisms (mucosal route of administration) the risk assessment paradigms are applicable to other routes of exposure such as bites and mosquito saliva. Severe anaphylactic reactions to mosquito bites that could be life threatening are rare and the multiple levels of protection described above make it extremely unlikely that humans would be exposed to these two proteins via a mosquito bite and that this would result in a serious anaphylactic reaction. Additionally, an independent food safety assessment for DsRed2 indicates that it should be rapidly digested by gastric enzymes if orally ingested.[58] Taken together these data suggest that there are unlikely to be epitopes that are known to cause allergenic reactions in humans. ~~the sequence alignment of thefull length tTAV and DsRed2 protein sequences with protein sequences in the allergenonline 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 (as per Codex , 2003). 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[59] database, the only public, peer-reviewed allergen database available for safety evaluation. There were no matches with more than 50% identity over the full sequence length for either protein.~~

- ~~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 conservative guideline that, but will likelyprobably 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[60] 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.~~

Commented [KJ33]: Sorry I didn't check the whole document above but is there any data to show that these proteins are even found in mosquito salivary glands? The lack of exposure in female salivary glands would add weight to the safety argument.

[58] [58] Independent food safety assessment for DsRed2 protein- [HYPERLINK "http://www.fda.gov/downloads/Food/Biotechnology/Submissions/UCM219002.pdf"]

[59] [HYPERLINK "http://www.allergenonline.org/" \h]~~(Accessed June 21, 2016).~~

[60] [HYPERLINK "http://www.allergenonline.org/" \h]~~(Aaccessed 22 June 21, 2016an 22, 2013).~~

~~The study was initially conducted in 2013 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 A*.~~

~~The study concluded that results of the bioinformatics analysis analyses of tTAV and DsRed2 protein amino acid sequences indicated that there was no more risk of allergy allergenicity or toxicity that was no 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 in the allergenonline and NCBI databases. 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 (see Section~~ [REF _Ref453677594 \r \h]~~).~~ These results indicated that ~~there was~~ no convincing evidence was found to suggest tTAV or DsRed2 proteins expressed in OX513A mosquitoes represent risks of allergy to humans 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 [REF _Ref453570514 \r \h] and [REF _Ref453331867 \r \h]), 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, b~~ Because risk is a function of both exposure and hazard, Oxitec provided a ~~n additional~~ study on whether the introduced proteins can be detected in OX513A female mosquito saliva.

Commented [KJ34]: Oops sorry! Ignore the above comment.

~~13.7.2~~13.6.2 Analysis of expression of the introduced proteins in female mosquito saliva

Saliva from *Aedes* species mosquitoes contains secreted proteins that~~which~~ play a role in sugar and blood feeding [ADDIN EN.CITE ADDIN EN.CITE.DATA]. These have been characterized by proteomic studies of saliva itself [ADDIN EN.CITE
<EndNote><Cite><Author>Chisenhall</Author><Year>2014</Year><RecNum>114</RecNum><DisplayText>(Chisenhall et al. 2014)</DisplayText><record><rec-number>114</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463104195">114</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Chisenhall, D. M.</author><author>Christofferson, R. C.</author><author>McCracken, M. K.</author><author>Johnson, A. M.</author><author>Londono-Renteria, B.</author><author>Mores, C. N.</author></authors></contributors><auth-address>Department of Pathobiological Sciences, Vector-borne Disease Laboratories, Louisiana State

University, School of Veterinary Medicine, Baton Rouge, LA, USA. cmores@lsu.edu.</auth-address><titles><title>Infection with dengue-2 virus alters proteins in naturally expectorated saliva of Aedes aegypti mosquitoes</title><secondary-title>Parasit Vectors</secondary-title></titles><periodical><full-title>Parasit Vectors</full-title></periodical><pages>252</pages><volume>7</volume><keywords><keyword>Aedes/*physiology/*virology</keyword><keyword>Animals</keyword><keyword>Dengue Virus/classification/*physiology</keyword><keyword>Gene Expression Regulation</keyword><keyword>Insect Proteins/chemistry/genetics/*metabolism</keyword><keyword>Saliva/*chemistry</keyword></keywords><dates><year>2014</year></dates><isbn>1756-3305 (Electronic)
1756-3305 (Linking)</isbn><accession-num>24886023</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24886023</url></related-urls></urls><custom2>4057903</custom2><electronic-resource-num>10.1186/1756-3305-7-252</electronic-resource-num></record></Cite></EndNote>], as well as by studies of the sialome (the set of messages and proteins expressed in salivary 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, [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Capurro</Author><Year>2000</Year><RecNum>133</RecNum><DisplayText>Capurro et al. (2000)</DisplayText><record><rec-number>133</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106826">133</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Capurro, M. de Lara</author><author>Coleman, J.</author><author>Beerntsen, B. T.</author><author>Myles, K. M.</author><author>Olson, K. E.</author><author>Rocha, E.</author><author>Krettli, A. U.</author><author>James, A. A.</author></authors></contributors><titles><title>Virus-expressed, recombinant single-chain antibody blocks sporozoite infection of salivary glands in Plasmodium gallinaceum-infected Aedes aegypti</title><secondary-title>The American Journal of Tropical Medicine and Hygiene</secondary-title><alt-title>Am J Trop Med Hyg</alt-title></titles><periodical><full-title>The American Journal of Tropical Medicine and Hygiene</full-title></periodical><alt-periodical><full-title>Am J Trop Med Hyg</full-title></alt-periodical><pages>427-433</pages><volume>62</volume><number>4</number><dates><year>2000</year><pub-dates><date>2000/04/01/</date></pub-dates></dates><isbn>0002-9637</isbn><urls><related-urls><url>http://www.ajtmh.org/content/62/4/427</url><url>http://www.ncbi.nlm.nih.gov/pubmed/11220756</url><url>http://www.ajtmh.org/content/62/4/427.long</url><url>http://www.ajtmh.org/content/62/4/427.full.pdf</url></related-urls><pdf-urls><url>http://www.ajtmh.org/content/62/4/427.full.pdf</url></pdf-urls></urls><remote-database-provider>www.ajtmh.org</remote-database-provider><language>en</language><access-date>2015/12/23/15:28:13</access-date></record></Cite></EndNote>] confirm that a mosquito secretory signal sequence, fused to the upstream region of the coding sequence is required in order to secrete engineered short chained variable fragment (scFV) antibodies into ~~the~~ saliva for, ~~a mosquito secretory signal sequence, fused to the upstream region of the coding sequence is required for~~

functional expression in mosquitoes. This signal sequence is cleaved during the process of protein secretion into saliva in mosquitoes (e.g., [ADDIN EN.CITE ADDIN EN.CITE.DATA]). Neither tTAV nor DsRed2 contains ~~no~~ such a signal sequence for secretion nor does ~~it~~ they have any sequences with homology to such signal sequences; therefore, tTAV and DsRed2 ~~is~~ proteins are 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 [REF _Ref453570581 \r \h] 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.~~ [ADDIN EN.CITE ADDIN EN.CITE.DATA] ~~In the study, Dr. Calvo *et al.* analyzed saliva samples containing the pooled saliva of ten OX513A female mosquitoes. A sample containing the pooled saliva of ten wild-type *Ae. aegypti* female mosquitoes served as a negative control; while samples from whole pupae extracts from the OX513A and OX4358B[64] lines of GE mosquitoes served as positive controls. The investigators did not detect any tTAV protein in saliva samples from OX513A and wild-type females, whereas positive controls showed a band of the~~

~~[64] The OX4358B line of *Ae. albopictus* mosquitoes was developed by Oxitec and also expresses tTAV protein.~~ [ADDIN EN.CITE <EndNote><Cite><Author>Labbe</Author><Year>2012</Year><RecNum>80</RecNum><DisplayText>Labbe GM, Scaife S, Morgan SA, Curtis ZH, Alphey L. 2012. Female-specific flightless (fsRIDL) phenotype for control of Aedes albopictus. <style face="italic">PLoS Negl Trop Dis</style> <style face="bold">6</style>: e1724.</DisplayText><record><rec-number>80</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1451591030">80</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Labbe, G. M.</author><author>Scaife, S.</author><author>Morgan, S. A.</author><author>Curtis, Z. H.</author><author>Alphey, L.</author></authors></contributors><auth-address>Oxitec Limited, Oxford, United Kingdom.</auth-address><titles><title>Female-specific flightless (fsRIDL) phenotype for control of Aedes albopictus</title><secondary-title>PLoS Negl Trop Dis</secondary-title></titles><periodical><full-title>PLoS Negl Trop Dis</full-title></periodical><pages>e1724</pages><volume>6</volume><number>7</number><keywords><keyword>Actins/genetics</keyword><keyword>Aedes/genetics/*physiology</keyword><keyword>Animals</keyword><keyword>*Disease Vectors</keyword><keyword>Female</keyword><keyword>*Flight, Animal</keyword><keyword>Male</keyword><keyword>Mosquito Control/*methods</keyword><keyword>Phenotype</keyword></keywords><dates><year>2012</year></dates><isbn>1935-2735 (Electronic)
1935-2727 (Linking)</isbn><accession-num>22802980</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/22802980</url></related-urls></urls><custom2>3393675</custom2><electronic-resource-num>10.1371/journal.pntd.0001724</electronic-resource-num></record></Cite></EndNote>].

~~expected size that corresponds to the relative molecular weight of the tTAV protein. These data suggest that tTAV in the OX513A female saliva is below the limit of detection (LOD) but the study did not attempt to determine that LOD. 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.~~

~~13.7.2.1~~13.6.2.1 Study on detection of tTAV and DsRed2 in the saliva of OX513A females

~~Because the previous preliminary study did not establish the LOD for the tTAV antibody and did not assay for potential presence of DsRed2,~~ Oxitec conducted a study to determine whether tTAV and DsRed2 proteins would be detectable in the saliva of OX513A female mosquitoes and to determine the limit of detection (LOD) for each of the proteins. ~~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, respectively) were 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~~ antibodies was carried out, using an Enhanced Chemiluminescence (ECL) approach. Sample integrity was confirmed using an antibody ~~detecting~~ to Aegyptin a secreted salivary protein found in mosquitoes~~, Aegyptin, as in the previous study conducted at NIH~~. Aegyptin detection was also used ~~as a basis~~ to determine that equivalent amounts of salivary proteins were loaded in ~~control and sample lanes between the test saliva samples of OX513A and the WT control~~all saliva samples tested.

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

The ~~introduced~~ engineered proteins, tTAV and DsRed2 were not detected in OX513A female *Aedes aegypti* saliva at and above these LODs in the 5 µl of saliva analysed. In this study 5 µl of OX513A saliva ~~equates~~ corresponded to the ~~quantity~~ volume 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*~~homozygous OX513A ~~*Ae. aegypti*~~ adult females ~~homozygous for the #OX513 rDNA construct~~).The study report is provided in *Appendix K*.

~~13.7.3~~13.6.3 ~~Conclusion on the toxicity and allergenicity potential of the introduced proteins~~Question conclusions

Based on the Western immuno-blot assays performed by Oxitec, we conclude that the levels of tTAV and DsRed2 proteins in saliva of OX513A *Ae. aegypti* females homozygous for the #OX513 rDNA

construct are below the limit of detection for that assay. Therefore, we consider that it is highly unlikely that humans or other animals would be exposed to these proteins even if they were to be bitten by OX513A female mosquitoes. A stepwise, weight-of-evidence approach evaluating the toxic and allergenic potential of tTAV and DsRed2 proteins based on Codex guidelines and a scientific literature search did not identify any evidence suggesting the allergenicity or toxicity of tTAV and DsRed2 proteins. Bioinformatics analysis of amino acid sequences of tTAV and DsRed2 proteins did not identify any similarities with known toxins or allergens. Therefore, FDA concludes that tTAV and DsRed2 proteins lack any toxic or allergenic potential and do not pose any significant risks to humans or non-target animals. [REF _Ref453764606 \r \h] [REF _Ref454525518 \r \h][][ADDIN EN.CITE <EndNote><Cite><Author>Pioneer Hi-Bred International</Author><Year>2006</Year><RecNum>79</RecNum><DisplayText>(Pioneer Hi-Bred International 2006)</DisplayText><record><rec-number>79</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1451584513">79</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>Pioneer Hi-Bred International, Inc.</author></authors></contributors><titles><title>Early food safety evaluation for a Red Fluorescent Protein: DsRed2. http://www.fda.gov/downloads/Food/Biotechnology/Submissions/UCM219002.pdf</title></titles><dates><year>2006</year></dates><urls></urls></record></Cite></EndNote>]Taking the combined probability of all three steps into consideration, it is highly unlikely that tTAV and DsRed2, if present in the saliva of female GE OX513A mosquitoes, would cause an allergic reaction in healthy human hosts due to blood feeding. In summary, saliva from OX513A mosquitoes poses no greater risk to human health due to allergic response than that from wild-type mosquitoes.

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 [REF _Ref453924807 \r \h] 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.**~~

~~Based on the Western immuno blot assays performed by Oxitec, we conclude that the levels of tTAV and DsRed2 proteins in saliva of OX513A *Ae. aegypti* females homozygous for the #OX513 rDNA construct are below the limit of detection for that assay. Therefore, we consider that it is highly unlikely that humans or other animals would be exposed to these proteins even if they were to be bitten by OX513A female mosquitoes. A stepwise, weight-of-evidence approach evaluating the toxic and allergenic potential of tTAV and DsRed2 proteins based on Codex guidelines and a scientific literature search did not identify any evidence suggesting the allergenicity or toxicity of tTAV and DsRed2 proteins. Bioinformatics analysis of amino acid sequences of tTAV and DsRed2 proteins did not identify any similarities with known toxins or allergens. Therefore, based on our evaluation of submitted data and information, we conclude that tTAV and DsRed2 proteins lack any toxic or allergenic potential and do not pose any significant risks to humans.~~

14 Measures used to minimize potential impacts

Commented [EEA35]: We plan to change the order of sections 14 and 15 after reviews are completed.

14.1 Physical containment

Physical containment measures would beare 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[62], Benedict *et al.*, 2003). These include both primary and secondary level containments and are summarized below and in [REF _Ref450336935 \h].

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 9 CFR 71.54.[63]

- *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 to prevent arthropod survival and escape (e.g., heated to over the lethal

[62] 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 [HYPERLINK "http://www.astmh.org/subgroups/acme#arthropod"] [Accessed June 21, 2016]http://www.astmh.org/AM/Template.cfm?Section=ACME&Template=/CM/ContentDisplay.cfm&ContentID=1450.

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[63] [HYPERLINK "http://www.cdc.gov/od/eaipp/inspection/docs/Import_Permit_Checklist_ACL-2.pdf"] [Accessed June 17Mar 31, 2016]

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°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.

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- *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 Design*. 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.

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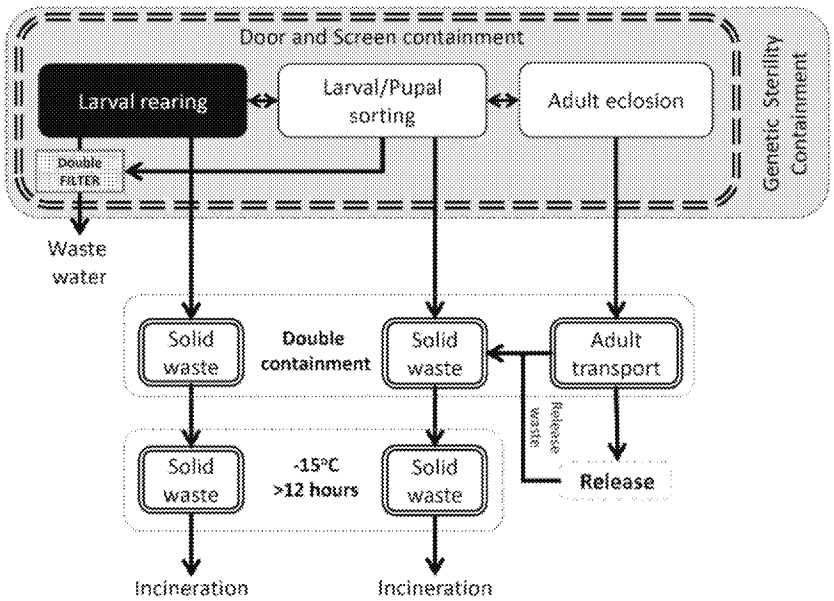

Figure [SEQ Figure * ARABIC]. Summary schematic of containment measures for egg production facility in the U.S.

14.2 Biological containment

Any potential escapees from the HRU would~~ill~~ be homozygous for the OX513A insertion and as the integrated rDNA ~~insertion~~ lethality trait is >95% penetrant in the laboratory, it is anticipated that >95% would~~ill~~ 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. However, even if 5% of the progeny survive, they will not live any longer than wild-type *Ae. aegypti* -because they are functionally no more fit than the wild-type. Some evidence of ~~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 [ADDIN EN.CITE <EndNote><Cite><Author>Lacroix</Author><Year>2012</Year><RecNum>43</RecNum><DisplayText>(Lacroix et al. 2012)</DisplayText><record><rec-number>43</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">43</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>Lacroix, R.</author><author>McKemey, A.R.</author><author>Raduan, N.</author><author>Kwee Wee, L.</author><author>Nordin, O.</author></authors></contributors><titles><title>Open Field Release of Genetically Engineered Sterile Male Aedes aegypti in Malasia</title><secondary-title>PLoS

ONE</secondary-title></titles><periodical><full-title>PLoS ONE</full-title></periodical><pages>e42771</pages><volume>7</volume><number>8</number><reprint-edition>Not in File</reprint-edition><keywords><keyword>Aedes</keyword><keyword>Aedes aegypti</keyword></keywords><dates><year>2012</year><pub-dates><date>2012</date></pub-dates></dates><label>44</label><urls></urls></record></Cite></EndNote>] and the Cayman Islands [ADDIN EN.CITE ADDIN EN.CITE.DATA] to assess the longevity of released OX513A 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 mosquito 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 mosquito strain ~~mosquitoes~~ 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. In the event such mutations were to occur, resulting in a loss of function of the tTAV lethality trait, these mosquitoes would be functionally no different than existing wild-type *Ae. aegypti*. Additionally, a loss of tTAV function in the field (i.e., in released adults) as opposed to in the rearing process ~~within the HRU (i.e. in the breeding stock)~~ will not affect future batches of OX513A adults produced and released as live mosquitoes from the field are not returned to the production facility and cannot influence the genetics of the production stock.

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 [REF _Ref453317568 \r \h]. 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~~ using quality control processes that ensure accuracy of the sorting does not exceed a maximum of 0.2% ~~an accuracy averaging over 99.9%~~ [ADDIN EN.CITE ADDIN EN.CITE.DATA] ~~(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 the lethality trait expression is assessed by comparing the mortality of the OX513A mosquito (scored by fluorescence and confirmed by PCR) and wild-type progeny, as described in the ~~proposed investigational field trial section protocol~~ (Section [REF _Ref453245461 \r \h]). If these results indicate that there is no statistically significant difference in mortality, then the lethality 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 lethality 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 such as larvicides or adulticides can ~~continued to~~ be applied ~~such as the use of larvicides or adulticides~~.

14.3 Geographical/geophysical containment

Ae. aegypti ~~can~~ are present ~~survive~~ in the environment in Florida, where it is regarded as an invasive species by some ([ADDIN EN.CITE <EndNote><Cite><Author>Juliano</Author><Year>2005</Year><RecNum>227</RecNum><DisplayText>(Juliano and Lounibos 2005)</DisplayText><record><rec-number>227</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463109971">227</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Juliano, S. A.</author><author>Lounibos, L. P.</author></authors></contributors><auth-address>Department of Biological Sciences, Behavior, Ecology, Evolution and Systematics Section, Illinois State University, Normal, IL 61790-4120, USA.</auth-address><titles><title>Ecology of invasive mosquitoes: effects on resident species and on human health</title><secondary-title>Ecol Lett</secondary-title></titles><periodical><full-title>Ecol Lett</full-title></periodical><pages>558-74</pages><volume>8</volume><number>5</number><dates><year>2005</year><pub-dates><date>May</date></pub-dates></dates><isbn>1461-0248 (Electronic)
1461-023X (Linking)</isbn><accession-num>17637849</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/17637849</url></related-urls></urls><custom2>1920178</custom2><electronic-resource-num>10.1111/j.1461-0248.2005.00755</electronic-resource-num></record></Cite></EndNote>] and CDC[64]), but for the purposes of this EA *Ae. aegypti* will be referred to as a non-native species. It is the intention of the proposed field trial ~~investigational use~~ for ~~the~~ OX513A males to mate with wild-type females ~~of the wild population of *Ae. aegypti*~~ at the proposed release site. The proposed field trial would include the following ~~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.

[64] [HYPERLINK "http://www.cdc.gov/dengue/entomologyecology/"] [Accessed ~~30 June 17~~~~Mar 30~~, 201~~55~~]

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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°C (~50°F -86°F) [ADDIN EN.CITE <EndNote><Cite><Author>Tun-Lin</Author><Year>2000</Year><RecNum>34</RecNum><DisplayText>(Tun-Lin et al. 2000)</DisplayText><record><rec-number>34</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">34</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Tun-Lin, W.</author><author>Burkot, T.R.</author><author>Kay, B.H.</author></authors></contributors><titles><title>Effects of temperature and larval diet on development rates and survival of the dengue vector Aedes aegypti in north Queensland, Australia</title><secondary-title>Medical and Veterinary Entomology</secondary-title></titles><periodical><full-title>Medical and Veterinary Entomology</full-title></periodical><pages>31-37</pages><volume>14</volume><number>1</number><reprint-edition>Not in File</reprint-edition><keywords><keyword>Aedes</keyword><keyword>Aedes aegypti</keyword><keyword>Australia</keyword><keyword>Dengue</keyword><keyword>dengue vector</keyword><keyword>development time</keyword><keyword>environmental effects</keyword><keyword>larval diet</keyword><keyword>Queensland</keyword><keyword>survival rate</keyword><keyword>temperature</keyword><keyword>thermal constant</keyword><keyword>wing-length</keyword></keywords><dates><year>2000</year><pub-dates><date>2000</date></pub-dates></dates><isbn>1365-2915</isbn><label>35</label><urls><related-urls><url>http://onlinelibrary.wiley.com/doi/10.1046/j.1365-2915.2000.00207.x/abstract</url><url>jsessionid=2B2B294E14FFE326306EE14AB7739DB6.f02t03</url></related-urls></urls><electronic-resource-num>10.1046/j.1365-2915.2000.00207.x</electronic-resource-num><access-date>5/14/2015</access-date></record></Cite></EndNote>]. 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 [ADDIN EN.CITE <EndNote><Cite><Author>Clements</Author><Year>2000</Year><RecNum>272</RecNum><DisplayText>(Clements 2000)</DisplayText><record><rec-number>272</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1466709433">272</key></foreign-keys><ref-type name="Book">6</ref-type><contributors><authors><author>Clements, A.N.</author></authors></contributors><titles><title>The biology of mosquitoes: development, nutrition, and reproduction</title></titles><dates><year>2000</year></dates><pub-location>Oxford</pub-location><publisher>CABI Publishing</publisher><urls></urls></record></Cite></EndNote>]. High temperatures alone (>40°C)[104°F] are unlikely to limit the species but low temperatures are limiting with the threshold being the 10-15°C (~50-59 °F) isotherm. At temperatures lower than 15^{0}C (59°F), *Ae. aegypti* becomes torpid, unable to fly, or moves its limbs only slowly. Lower temperatures can slow development time to

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such a degree that the species is prevented from establishing itself because, 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. Low temperatures affect the ability of eggs to hatch with significant decrease in hatching seen at -5- -7°C and no hatching seen when temperatures fell to -10°C. The duration of exposure to cold temperatures had less effect as temperatures decreased. No Aedes aegypti eggs survived and hatched when exposed to -15°C for even an hour (Thomas SM. Et al. (2012) Low-temperature threshold for egg survival of a post-diapause and non-diapause European aedine strain, Aedes albopictus (Diptera: Culicidae) Parasites and Vectors. 5:100). Thus, even brief exposure to -10°C or lower temperatures can have significant impacts on egg banks and mosquito populations in future seasons.

Temperature sensitivity of the OX513A strain line has been investigated and is reported in Section [REF _Ref453319789 \r \h].

14.3.2 Water storage and rainfall

Dessicated *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 Water storage vessels for personal use, such as washing and drinking, can serve as attractive oviposition sites for female mosquitoes if the vesselscontainers 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 water from all containers that they might have on their land (source reduction). 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[65] 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 [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Ramasamy</Author><Year>2011</Year><RecNum>168</RecNum><DisplayText>Ramasamy et al. (2011)</DisplayText><record><rec-number>168</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463106826">168</key></foreign-keys><ref-type name="Journal Article">17</ref-

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

type><contributors><authors><author>Ramasamy, Ranjan</author><author>Surendran, Sinnathamby N.</author><author>Jude, Pavilupillai J.</author><author>Dharshini, Sangaralingam</author><author>Vinobaba, Muthuladchumy</author></authors><secondary-authors><author>Barrera, Roberto</author></secondary-authors></contributors><titles><title>Larval Development of Aedes aegypti and Aedes albopictus in Peri-Urban Brackish Water and Its Implications for Transmission of Arboviral Diseases</title><secondary-title>PLoS Neglected Tropical Diseases</secondary-title></titles><periodical><full-title>PLoS Neglected Tropical Diseases</full-title></periodical><pages>e1369</pages><volume>5</volume><number>11</number><dates><year>2011</year><pub-dates><date>2011/11/22/</date></pub-dates></dates><isbn>1935-2735</isbn><urls><related-urls><url>http://dx.plos.org/10.1371/journal.pntd.0001369</url><url>http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3222631/pdf/pntd.0001369.pdf</url></related-urls></urls><electronic-resource-num>10.1371/journal.pntd.0001369</electronic-resource-num><remote-database-provider>CrossRef</remote-database-provider><language>en</language><access-date>2015/03/28/04:18:33</access-date></record></Cite></EndNote>] showed that they ~~have been found~~were able to survive to a limited extent in brackish waters with lower saline levels (3 g/L), as described~~reviewed~~ in Section [REF _Ref453320591 \r \h]. 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 that are targeted~~examined~~ for *Aedes* control using conventional means such as insecticides. FKMCD recommends that standing water be~~s~~ removed from boats.[66]

~~14.3.4 Tetracycline in the environmentInsufficient tTetracycline in the environment~~

14.3.4

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Tetracyclines in the environment can come from human ~~or and~~ animal drugs, or non-drug sources~~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 o~~Oral tetracyclines used at that time included tetracycline, (oxytetracycline, and chlortetracycline,) ~~in~~Many uses of these drugs have been discontinued for use in humans. The ~~of which are no longer available or are used in veterinary medicine only. More modern~~ forms of tetracycline most commonly used in human medicine today include, for example, doxycycline and minocycline ~~which are much more commonly used and have similar indications~~. Both doxycycline and minocycline are prescription drugs. Currently, tetracycline is most frequently used for upper respiratory and skin and soft tissue infection in humans. Tetracycline~~s~~ is used ~~for veterinary~~ therapeuticaily in animals. Oxytetracycline and chlortetracycline are used both therapeutically and for production (growth)

[66] [HYPERLINK "http://keysmosquito.org/mosquito-protection/"] [Accessed June 17, 2016].~~http://keysmosquito.org/programs-domestic-field-ent-services-offshore-trucks-aerial/ http://keysmosquito.org/programs-domestic-field-ent-services-offshore-trucks-aerial/~~

purposes use are used widely for the prevention of infection. They have also been used in the USA for growth promotion purposes in food-producing animals, although FDA has issued a guidance documentspolicy with recommendations for ending discouraging production use for such purposes in feed or drinking water by January 1, 2017 (see Guidance for Industry 209152[67] and Guidance for Industry 213[68]). According to an Animal Health Institute survey in 2007 (AHI, 2009) Based on 2014 tetracycline sales data, 6,600,849 kgs of active ingredient were sold that year for use over 30 million pounds of tetracyclines are used in animalsthe livestock industry in the U.S.[69] According to the U.S. Pharmacopeia Safety Data Sheet, Ttetracycline has known environmental toxicity to fish with LC50[70]a lethal concentration of 186.9-258.9 mg/L.[71] The sensitivity of the OX513A strain line has been evaluated in Section [REF _Ref453319679 \r \h] and will not be repeated here, but in summary, minimum concentrations of 1 µg/mL are required to fully rescue the phenotype from the lethality trait.

Aquaculture facilities, agricultural farms, hospitals, or municipal sewage facilities are the only sources that theoretically could introduce into the environment sufficiently high levels of tetracycline to allow survival of OX513A progeny in the environment. Our survey of the area showed that there are no farms, including aquaculture facilities or citrus groves, agricultural farms, or hospitals/medical centers inat the proposed trial site.[72] The closest hospital is located on another island, separated from the trial site by more than 250 m of saline water and dense vegetation that prevents the spontaneous dispersion of the OX513A *Ae. aegypti* mosquitoes. The proposed field trial site has a waste water treatment plant (WWTP) that serves the residents of Key Haven. The WWTP is located at the southern end of Key Haventhe island at the junction of the Buffer and Untreated Comparator Areas ([REF _Ref450310557 \h]). This site is approximately 400 m away from the Treatment Area, which is considerably farther than an average spontaneous flight distance of *Ae. aegypti* mosquitoes. IfEven if released OX513A mosquitoes somehow werewould be able to reach the WWTP, it is highly unlikely that their progeny would find any considerablesuitable concentration of tetracycline in its water to enable rescue of the lethality trait because the WWTP provides services to residential customers only.

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[67] 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 [HYPERLINK "http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM216936.pdf" \t "_self"] (Accessed June 21, 2016).

[68] [HYPERLINK "http://www.fda.gov/downloads/animalveterinary/guidancecomplianceenforcement/guidanceforindustry/ucm299624.pdf"] (Accessed June 23, 2106).

[69] FDA 2014 Summary Report on Antimicrobials Sold or Distributed for Use in Food Producing Animals. [HYPERLINK "http://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM476258.pdf"] (Accessed June 21, 2016).

[70] LC50 is the lethal dose at which 50% of the test subjects die.

[71] [HYPERLINK "http://static.usp.org/pdf/EN/referenceStandards/msds/1651009.pdf"] (Accessed June 16, 2016).

[72] [HYPERLINK "https://www.google.com/maps/@24.5821038,-81.7370013,16z"] (Accessed June 16, 2016)

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Further, tetracycline and its derivatives are sensitive to ultra-violet light and degrade quickly when exposed to sunlight [ADDIN EN.CITE <EndNote><Cite><Author>Bautitz</Author><Year>2007</Year><RecNum>9</RecNum><DisplayText>(Bautitz and Nogueira 2007)</DisplayText><record><rec-number>9</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">9</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Bautitz, Ivonete Rossi</author><author>Nogueira, Raquel F.P.</author></authors></contributors><titles><title>Degradation of tetracycline by photo-Fenton process - Solar irradiation and matrix effects</title><secondary-title>J Photochem. Photobiol A: Chemistry</secondary-title></titles><pages>33-39</pages><volume>187</volume><number>1</number><reprint-edition>Not in File</reprint-edition><dates><year>2007</year><pub-dates><date>2007</date></pub-dates></dates><isbn>10106030</isbn><label>9</label><urls><related-urls><url>http://linkinghub.elsevier.com/retrieve/pii/S1010603006005053</url></related-urls></urls><electronic-resource-num>10.1016/j.jphotochem.2006.09.009</electronic-resource-num><access-date>3/28/2015</access-date></record></Cite></EndNote>]. They are strongly adsorbed by soil and clays, which significantly decrease their mobility and bioavailability [ADDIN EN.CITE ADDIN EN.CITE.DATA]. [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Curtis</Author><Year>2015</Year><RecNum>86</RecNum><DisplayText>Curtis et al. (2015)</DisplayText><record><rec-number>86</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1454680517">86</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Curtis, Z.</author><author>Matzen, K.</author><author>Oviedo, M. N.</author><author>Nimmo, D.</author><author>Gray, P.</author><author>Winskill, P.</author><author>Locatelli, M. A. F.</author><author>Jardim, W. F.</author><author>Warner, S.</author><author>Alphey, L.</author><author>Beech, C.</author></authors></contributors><titles><title>Assessment of the Impact of Potential Tetracycline Exposure on the Phenotype of Aedes aegypti OX513A: Implications for Field Use</title><secondary-title>Plos Neglected Tropical Diseases</secondary-title></titles><periodical><full-title>PLoS Neglected Tropical Diseases</full-title></periodical><volume>9</volume><number>8</number><dates><year>2015</year><pub-dates><date>Aug</date></pub-dates></dates><isbn>1935-2735</isbn><accession-num>WOS:000360708200046</accession-num><urls><related-urls><url><Go to ISI>://WOS:000360708200046</url></related-urls></urls><custom7>e0003999</custom7><electronic-resource-num>10.1371/journal.pntd.0003999</electronic-resource-num></record></Cite></EndNote>] analyzed environmental concentrations of tetracycline and its derivatives in samples from Campinas and Itu, Sao Paolo, Brazil. The samples were collected from three different creeks impacted by sewage or poultry production, one private fish production lake, rain and tap water, and multiple discarded containers that~~which~~ contained larvae at the time of sampling. The analysis showed that the levels of tetracycline, and its analogs- oxytetracycline and chlortetracycline were below the limit of quantification for each of the samples. In general, *Ae. aegypti* prefer~~entially use~~ man-made containers such as gutters, water

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containers, and tires that hold rainwater or clean still water for their breeding sites [ADDIN EN.CITE <EndNote><Cite><Author>Hribar</Author><Year>2001</Year><RecNum>47</RecNum><DisplayText>(Tun-Lin et al. 1995; Hribar et al. 2001)</DisplayText><record><rec-number>47</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432063934">47</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Hribar, L.J.</author><author>Smith, J.M.</author><author>Vlach, J.J.</author><author>Verna, T.N.</author></authors></contributors><titles><title>Survey of Containter-Feeding Mosquitoes from the Florida Keys, Monroe County, Florida.</title><secondary-title>J Am Mosquito Contr Association</secondary-title></titles><periodical><full-title>J Am Mosquito Contr Association</full-title></periodical><pages>245-248</pages><volume>17</volume><number>4</number><dates><year>2001</year></dates><urls></urls></record></Cite><Cite><Author>Tun-Lin</Author><Year>1995</Year><RecNum>35</RecNum><record><rec-number>35</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">35</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Tun-Lin, W.</author><author>Kay, B.H.</author><author>Barnes, A.</author></authors></contributors><titles><title>Understanding productivity, a key to Aedes aegypti surveillance</title><secondary-title>Am J Trop Med Hyg</secondary-title></titles><periodical><full-title>Am J Trop Med Hyg</full-title></periodical><pages>595-601</pages><volume>53</volume><number>6</number><reprint-edition>Not in File</reprint-edition><keywords><keyword>Aedes</keyword><keyword>Aedes aegypti</keyword></keywords><dates><year>1995</year><pub-dates><date>1995</date></pub-dates></dates><isbn>0002-9637</isbn><label>36</label><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/8561260</url><url>http://europepmc.org/abstract/med/8561260</url></related-urls></urls><access-date>5/14/2015</access-date></record></Cite></EndNote>]. It is highly unlikely that these sites would contain any ~~levels of~~ tetracycline at all. ~~Although~~ [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Hribar</Author><Year>2004</Year><RecNum>15</RecNum><DisplayText>Hribar et al. (2004)</DisplayText><record><rec-number>15</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">15</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Hribar, Lawrence J.</author><author>Vlach, Joshua J.</author><author>DeMay, David J.</author><author>James, Shannon S.</author><author>Fahey, Jennifer S.</author><author>Fussell, Edsel M.</author></authors></contributors><titles><title>Mosquito larvae (Culicidae) and other Diptera associated with containers, storm drains, and sewage treatment plants in the Florida Keys, Monroe County, Florida</title><secondary-title>Florida Entomologist</secondary-title></titles><pages>199-203</pages><volume>87</volume><number>2</number><reprint-edition>Not in File</reprint-edition><dates><year>2004</year><pub-dates><date>2004</date></pub-dates></dates><label>15</label><urls><related-urls><url>http://www.bioone.org/doi/abs/10.1653/0015-

4040(2004)087%5B0199:MLCAOD%5D2.0.CO%3B2</url></related-urls></urls><access-date>3/28/2015</access-date></record></Cite></EndNote>] site that sewage treatment plants, septic tanks, and cesspits may serve as larval development sites for *Ae. aegypti* and a few other mosquito species, these data should be interpreted with a caution as they do not differentiate between *Ae. aegypti* larvae found in active chambers containing sewage effluent and deactivated chambers containing rainwater. It is highly unlikely that *Ae. aegypti* would use sewage waters at the Key Haven WWTP as their breeding site as with EDA confirmed by the FKMCD surveillance records (Appendix I). The FKMCD records indicate that no *Ae. aegypti* larvae were found at the Key Haven WWTP during the 2004-2015 period. There have been some reports of *Ae. aegypti* larvae being found in the surface clear water layer of septic tanks [ADDIN EN.CITE ADDIN EN.CITE.DATA](Hribar, 2011, Burke, 2010), but this is unusualnot common and usually occurs where the lid is cracked or broken, providing access to the female access asto a novel oviposition site. Key West and surrounding areas in Monroe County have eliminated 99.9% of septic tanks[73][74] and uses mains sewerageea public sewage system as the major means of waste disposal.

Additionally, any potential sources of tetracycline in and around residences in the TA due to the presence of pet or human food from animal-derived sources with potential tetracycline residues would also have a low probability of affecting OX513A survival (see Section [REF _Ref453329832 \r \h]).

Thus, we conclude that it is highly unlikely that OX513A mosquitoes or their progeny would be exposed to any exogenous tetracycline and its derivatives in the environment that would allow them to establish or persist at the proposed trial site.

From a review of the accessible environments (Section [REF _Ref411522794 \r \h * MERGEFORMAT] 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 [REF _Ref412017276 \r \h * MERGEFORMAT].

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[74] Monroe County Engineering Division: Keys Wastewater Plan Nov 2007 [HYPERLINK "http://www.monroecounty-fl.gov/DocumentCenter/Home/View/478"] (Accessed June 9, 2016)

15 What are the likely consequences to, or effects on the environment of the U.S. associated with the proposed investigational use of OX513A ~~mosquitoes~~ mosquitoes?~~Consequences of potential escape, establishment, and spread~~

Potential impacts associated with the proposed released of OX513A mosquitoes would depend on the general fitness of the released OX513A males, their role in ecosystem, their interaction with other species in the ecosystem, and potential for dispersal~~spread,~~ and establishment~~, or persistence~~. Therefore, potential adverse effects associated with the release of OX513A mosquitoes may be divided into two broad categories: Gconsequences for the environment and Gconsequences for human/animal health, which are discussed below.

~~14.4~~15.1 Consequences for the environment

Potential impacts onto the environment ~~identified in the current EA~~ are summarized in [REF _Ref453328110 \h] and include interbreeding with related mosquito species, effects of tetracycline on ecological services, effects on flora, effects on predators, effects on decomposers, effects on endangered or threatened species, development of resistance to insecticides ~~in mosquito population~~, and persistence or establishment of OX513A mosquitoes at the trial site.

It is highly unlikely that OX513A males would interbreed with other, related mosquito species present at the proposed trial site. Studies show that *Ae. aegypti* matings with closely related mosquito species do not produce viable offspring [ADDIN EN.CITE ADDIN EN.CITE.DATA]. This question is~~was~~ discussed in greater detail in Section [REF _Ref453329698 \r \h]. Further, in the highly unlikely event that OX513A male mosquitoes do mate with other closely related mosquito species, it is highly unlikely that the rDNA construct would spread in the population of these mosquitoes due to the lethality phenotype ~~trait~~ conferred by this rDNA construct. Therefore, the likelihood of OX513A mosquitoes ~~males~~ breeding with other mosquito species~~the adverse event~~ would be extremely low as would be the case for survival of any potential progeny produced as a result of such matings.

It is highly unlikely that the use of tetracycline in the production of OX513A mosquitoes would have any adverse effects on the environment. The levels of tetracycline in the HRU waste water would be small (grams/week). Moreover, these low levels are~~this is~~ expected to be rapidly broken down in the environment as tetracycline is sensitive to light (as described in Section 14.3.4~~14.3.4~~). ~~Tetracycline is rapidly degraded by ultra-violet radiation.~~ [ADDIN EN.CITE <EndNote><Cite><Author>Bautitz</Author><Year>2007</Year><RecNum>9</RecNum><DisplayText>(Bautitz and Nogueira 2007)</DisplayText><record><rec-number>9</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">9</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Bautitz, Ivonete Rossi</author><author>Nogueira, Raquel F.P.</author></authors></contributors><titles><title>Degradation of tetracycline by photo-Fenton process - Solar irradiation and matrix effects</title><secondary-title>J Photochem. Photobiol A:

Chemistry</secondary-title></titles><pages>33-39</pages><volume>187</volume><number>1</number><reprint-edition>Not in File</reprint-edition><dates><year>2007</year><pub-dates><date>2007</date></pub-dates></dates><isbn>10106030</isbn><label>9</label><urls><related-urls><url>http://linkinghub.elsevier.com/retrieve/pii/S1010603006005053</url></related-urls></urls><electronic-resource-num>10.1016/j.jphotochem.2006.09.009</electronic-resource-num><access-date>3/28/2015</access-date></record></Cite></EndNote>]~~, in the presence of iron or other metal catalysts (Reyes, Fernadez et al., 2006), with total deactivation obtained in 70 minutes.~~ The use of tetracycline and its fate in the environment was reviewed by [ADDIN EN.CITE <EndNote><Cite AuthorYear="1"><Author>Sarmah</Author><Year>2006</Year><RecNum>199</RecNum><DisplayText>Sarmah et al. (2006)</DisplayText><record><rec-number>199</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1463108152">199</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Sarmah, A. K.</author><author>Meyer, M. T.</author><author>Boxall, A. B.</author></authors></contributors><auth-address>Landcare Research New Zealand Limited, Private Bag 3127, Hamilton, New Zealand. sarmahA@LandcareResearch.co.nz</auth-address><titles><title>A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment</title><secondary-title>Chemosphere</secondary-title></titles><periodical><full-title>Chemosphere</full-title></periodical><pages>725-59</pages><volume>65</volume><number>5</number><keywords><keyword>Animals</keyword><keyword>*Anti-Bacterial Agents/pharmacology/toxicity</keyword><keyword>Bacterial Infections/drug therapy/*veterinary</keyword><keyword>Drug Resistance, Microbial</keyword><keyword>Environmental Monitoring</keyword><keyword>*Environmental Pollutants/metabolism/toxicity</keyword><keyword>*Global Health</keyword><keyword>Humans</keyword><keyword>Manure/microbiology</keyword><keyword>Risk Assessment</keyword><keyword>Soil Microbiology</keyword><keyword>*Veterinary Drugs/pharmacology/toxicity</keyword></keywords><dates><year>2006</year><pub-dates><date>Oct</date></pub-dates></dates><isbn>0045-6535 (Print)
0045-6535 (Linking)</isbn><accession-num>16677683</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/16677683</url></related-urls></urls><electronic-resource-num>10.1016/j.chemosphere.2006.03.026</electronic-resource-num></record></Cite></EndNote>]~~, 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). The fate of tetracycline and its derivatives in the environment is was discussed in Sections~~ [REF _Ref453329832 \r \h] ~~and 14.3.4 of the EA. Therefore, the likelihood of adverse effects associated with the use tetracycline for production of OX513A mosquitoes would be is expected to be extremely low.~~

~~It is highly unlikely that the release of OX513A male mosquitoes will have any adverse effects on the populations of predators, decomposers, threatened or endangered species or flora at the proposed investigational trial site. As discussed in Section~~ [REF _Ref453329905 \r \h] ~~of the EA, due to the unique~~

habitat occupied by *Ae. aegypti*, they are subject only to opportunistic predators that prey on *Ae. aegypti* larvae and adults, if and when they encounter them. The anthropophilic behavior of *Ae. aegypti* mosquitoes limits the probability of encounter with potential predatory species and, therefore, we identified no species that would rely on these mosquitoes in its~~their~~ diet. Further, with the exception of generalist parasitoids infecting a number of mosquito species, we did not identify any specific parasitoid species associated with *Ae. aegypti* (Section [REF _Ref453330141 \r \h]). No adverse effects on decomposers were identified as well. 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 that could be involved in the breakdown of *Ae. aegypti*, including but not limited to organisms from classes of *Oligochaeta, Diplopoda, Isopoda, Nematodes, Collembola,* and *Acari* as well as species of Protozoa, Fungi, and Bacteria were identified but none of them are specifically involved in decomposition of *Ae. aegypti* (Section [REF _Ref453330160 \r \h]). It is highly unlikely that *Ae. aegypti* mosquitoes play any significant role in pollination because, being non-native species, *Ae. aegypti* mosquitoes have not been present in the Florida ecosystem sufficiently long to develop such a function (Section [REF _Ref453330174 \r \h]). With respect to ~~the~~ threatened and endangered species (Section [REF _Ref453244060 \r \h]), we established that the proposed trial is not likely to adversely affect the Stock Island Tree Snail whose habitat is in the vicinity of the proposed ~~investigational~~ trial site. Further, it is highly unlikely that the proposed trial would have any significant effects on wildlife refuges located in Monroe County due to a considerable distance from the proposed trial site. Therefore, the likelihood of the adverse effects on the populations of predators, parasitoids, decomposers, and threatened and endangered species or flora is expected to be extremely low.

Commented [KJ36]: Rather than say this in terms of co-evolution, I would just say that their pollination function is so slight that their presence or absence would not have any effect on this ecological function.

Commented [KJ37]: Rather than relying on the distance argument, I would also add the fact that males only are released and unlikely to present any hazard or risk to ESA species.

It is highly unlikely that ~~the~~ released OX513A mosquitoes would introduce insecticide resistance to the local *Ae. aegypti* mosquito population. Insecticide resistance studies have shown that ~~the~~ OX513A mosquitoes are susceptible to insecticides used for mosquito control (Section [REF _Ref453330256 \r \h]). ~~Additionally, as they are insecticide-susceptible OX513A mosquitoes they could introgress these susceptibility alleles into the local wild-type *Ae. aegypti* population and increase the susceptibility of the local wild-type population to existing insecticides.~~ Therefore, the likelihood of adverse effects associated with introduction of insecticide resistance into the local population of *Ae. aegypti* is expected to be extremely low.

As discussed in Section [REF _Ref453330318 \r \h] ~~of the EA~~, it is highly unlikely that the OX513A mosquitoes would be able to establish ~~or persist~~ at the proposed ~~investigational~~ trial site. The OX513A line of *Ae. aegypti* mosquitoes carries a repressible dominant lethality trait that prevents progeny inheriting the #OX513A rDNA construct from surviving to functional adulthood in the absence ~~causes lethality in mosquitoes and early pupal stage progeny unless reared in the presence~~ of tetracycline. Data and information provided in Section [REF _Ref453330473 \r \h] ~~of the EA~~ and peer-reviewed scientific journals [ADDIN EN.CITE ADDIN EN.CITE.DATA] indicate that more than 95% of OX513A progeny die before reaching viable~~the~~ adulthood if reared without tetracycline. Our evaluation did not identify any sources at the proposed ~~investigational~~ trial site that potentially could have sufficiently high levels of

tetracycline to allow survival of OX513A progeny in the environment (Sections [REF _Ref453331467 \r \h] and [REF _Ref453330513 \r \h]). Although the introduced lethality trait does not appear to have a significant effect on the mating competitiveness of OX513A males, it does appear to have a significant impact on longevity by reducing their fitness (Section [REF _Ref453331554 \r \h]). Dispersal of OX513A mosquitoes also appears to be adversely affected as measured by mean distance traveled, but not by maximum distance traveled, indicating, that, in general, the population of OX513A mosquitoes is not expected to exhibit geographical dispersion significantly different from wild-type *Ae. aegypti*. The location of the proposed field trial~~study~~ site would also limit dispersion because of its relative isolation and existing natural geophysical barriers. Further, given that this trial would be carried out concurrently with the existing FKMCD integrated ~~pest management~~vector control program ~~mosquito control measures~~ currently in place, it is unlikely that OX513A mosquitoes would disperse beyond the trial site~~area~~ (Sections [REF _Ref453331678 \r \h] and [REF _Ref453331695 \r \h]). Therefore, the likelihood of adverse effects associated with establishment ~~or persistence~~ of OX513A at the proposed trial site is extremely low. ~~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~~ [REF _Ref411866173 \r \h * MERGEFORMAT] ~~*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 tetracyclines (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).~~

The ~~One element of the~~ proposed ~~investigational use is that the~~ trial would~~ill~~ include ~~be~~ continuously monitor~~ing~~ed of the mosquito population by different (egg and adult) trapping and molecular methods. ~~,~~ This ~~which~~ would~~ill~~ ~~also~~ allow monitoring of the continued expression ~~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~~.

~~14.5~~15.2 Consequences for human and ~~other~~ non-target animal health

Potential impacts onto human or ~~other~~ non-target animal health ~~identified in the EA~~ are summarized in [REF _Ref453328110 \h] and include potential toxic ~~or allergenic~~ effects in humans or ~~other~~ non-target animals or allergenic effects in humans, transfer of the rDNA construct to humans or ~~other~~ non-target animals, increase in transmission of dengue or other diseases transmitted by mosquitoes, increase in population of other mosquitoes that may contribute to the increase of diseases, development of antimicrobial resistance, inadvertent release of OX513A females at the trial site, and a failure of the introduced traits in OX513A mosquitoes.

The risk to human health due to allergenicity of novel proteins can be assessed in a stepwise manner with the final pathway to harm resulting from the multiplication of the probability of occurrence of each step.

The first step is the presence in the local environment of adult GE female mosquitoes that are capable of flying, locating human hosts, and taking a blood meal from these human hosts. The investigational field trial proposes to release primarily male GE mosquitoes. Because male mosquitoes do not bite, they are not a hazard in terms of allergenicity from salivary proteins injected during blood feeding. As described in Section [REF _Ref453764606 \r \h], the trial protocol calls for use of a sex sorting method based on the size difference between male and female pupae with quality control processes that ensure accuracy of the sorting does not exceed a maximum of 0.2%. Thus, the overall probability of an OX513A female mosquito being released during the investigational trial is very low (0.2% at most) and the probability of this released female locating a human host and taking a blood meal is also low based on the total human population in the trial area. As described in Section [REF _Ref454525518 \r \h], the actual number of GE mosquitoes released during the trial depends on the initial level of infestation in the trial area, duration of the trial, overflooding ratio, and adaptive management-related adjustments in numbers. Under high initial infestation levels, the total number of OX513A females that would be released is estimated at <29,000 over 104 weeks or 0.6 female mosquitoes per person per week at the highest initial infestation levels and no adaptive management.

The second step is for the recombinant proteins to be expressed in the salivary glands of the OX513A female mosquitoes and be secreted into the mosquito's saliva so that these proteins could be injected into the human host during blood feeding. As described above (Section [REF _Ref453331762 \r \h]), results from western immunoblot assays performed by Oxitec indicate the LOD to be 0.8 ng and 2.5-5 ng for tTAV and DsRed2 respectively when four times the amount of salivary protein injected in a bite was used per sample (i.e., at approximately 0.2 and 0.625-1.25 ng for the amount of protein in a single bite). Expert opinion provided by Dr. Jose Ribeiro (NIAID) states that saliva volume is not a relevant estimator of hazard during biting as saliva volume is dependent on active flow of water through the cells in response to serotonin during blood feeding. Total protein content in the salivary gland before and after blood feeding is a better estimator of hazard. In general, an adult *Aedes* female mosquito has ~3 µg of total protein in the salivary gland of which ~1.5 µg is injected and ~0.75 µg is re-ingested into its gut during blood feeding, resulting in a net ~0.75- 1 µg of salivary protein remaining in the bitten host. Additionally, *Aedes* saliva contains about 100 polypeptides with a wide variation in relative abundance. The most highly expressed salivary protein, Aegyptin, is no more than 30% of total salivary protein or ~300 ng, with the least expressed proteins being less than 1 ng. Known allergenic proteins in mosquito saliva are expressed in the dozens or hundreds of ng range and the least expressed proteins in mosquito saliva are expressed at the single ng level. Because both tTAV and DsRed2 proteins were undetectable by this assay, the data supports the hypothesis that, if they are expressed and secreted in saliva at all, these proteins are likely expressed below or close to the 1 ng range per *Aedes* female bite. tTAV and DsRed2 are, therefore, very unlikely to cause an allergic response in a healthy human host that is bitten by an OX513A female because the protein level would be close to or below the level at which Dr. Ribeiro indicates mosquito saliva proteins that have been identified as human allergens are present.

Commented [LE38]: Ashley: Should we attach as an appendix?

The third step is the presence of known allergenic sequences in the tTAV and DsRed2 proteins. As discussed in Section [REF _Ref453570581 \r \h]~~13.6.1.3~~. Oxitec performed several bioinformatics analyses as per Codex Alimentarius guidelines (2003) to determine potential IgE binding epitopes as well as the potential for cross-reaction with other known allergens. Taken together these data suggest that there are unlikely to be epitopes that are known to cause allergenic reactions in humans.

~~It is highly unlikely that the release of OX513A male mosquitoes would result in toxic or allergenic effects in humans or other animalsRs 0~~[REF _Ref454289524 \r \h]~~0Based on the sensitive Western blot assays demonstrating that the concentrations of tTAV and DsRed2 proteins in saliva of OX513A females is below the level of detection, it is highly unlikely that humans or other animals would be exposed to these proteins even if they are bitten by an OX513A female mosquito (Section~~ [REF _Ref453331762 \r \h]~~). Therefore, the immunological response to the bites from OX513A female mosquitoes is not expected to be any different from the immunological response to the bites from wild-type Ae. aegypti mosquitoes. Bioinformatics analysis and opinions of allergenicity experts suggest that tTAV and DsRed2 proteins lack any toxic or allergenic potential and do not pose any significant risks to human or other non-target animal health (Sections~~ [REF _Ref453331856 \r \h]~~and~~ [REF _Ref453331867 \r \h]~~). Further, as described in Section~~ [REF _Ref453331917 \r \h]~~, none of the species~~animal species~~ fed a diet comprised of the OX513A larvae exhibited any adverse effects. Therefore, the likelihood of toxic or allergenic effects in humans or other non-target animals is expected to be extremely minor.~~

It is highly unlikely that the rDNA construct could be transferred to humans or ~~other~~ non-target animals. Our evaluation of the possibility for such transfer focused on two potential pathways (Section [REF _Ref453332100 \r \h]). First, we evaluated the possibility of ~~the~~ #OX513 rDNA construct transfer to humans or ~~other~~ animals via biting. We determined that it is highly unlikely that the #OX513A rDNA construct could be transferred to humans or ~~other~~ animals via biting because the rDNA construct is stably integrated in~~to~~ the mosquito genome and is not capable of re-mobilization even when treated with appropriate transposases due to altered ITR sequences (Section [REF _Ref453332132 \r \h]). Also, it is highly unlikely that naked, full length #OX513A DNA would be present in saliva. Additionally, mosquitoes have been feeding on humans and other animals for millennia but there is no ~~any~~ evidence of DNA transfer between mosquitoes and humans or ~~other~~ animals. ~~Second, w~~We also evaluated the possibility of ~~the~~ #OX513 rDNA construct transfer to microorganisms (e.g., bacteria in the intestine of OX513A mosquitoes, humans, or other animals; bacteria present in soil and involved in decomposition of organic matter) (Section [REF _Ref453332315 \r \h]). We determined that such transfer is highly unlikely due to a number of physical, biochemical, and genetic barriers that restrict horizontal gene transfer. Despite the fact that prokaryotes are exposed to an~~the~~ abundance of genetic material from eukaryotic organisms, the presence of eukaryotic genes in the genome of prokaryotes is extremely limited and suggests the existence of functional and selective barriers that limit the acquisition of eukaryotic genes by bacteria. Therefore, the likelihood of adverse effects associated with a potential transfer of the rDNA construct to humans or other non-target animals is extremely low.

It is highly unlikely that the release of OX513A mosquitoes would result in an~~the~~ increase in transmission of dengue or other diseases transmitted by mosquitoes. ~~Released~~ OX513A male mosquitoes do not bite

~~humans or other animals~~ and, consequently, do not transmit diseases. A small number of females ~~that~~ may be co-released with OX513A male mosquitoes or be present at the site of the proposed release as a result of incomplete penetrance of the introduced lethality trait. Hhowever, there is no evidence to suggest that OX513A females are fitter than wild-type *Aedes aegypti* [ADDIN EN.CITE <EndNote><Cite><Author>Lee</Author><Year>2009</Year><RecNum>244</RecNum><DisplayText>(Lee et al. 2009b)</DisplayText><record><rec-number>244</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1465585323">244</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lee, H.L.</author><author>Joko, H.</author><author>Nazni, W.A.</author><author>Vasan, S.</author></authors></contributors><titles><title>Comparative life parameters of transgenic and wild strain of Aedes aegypti in the laboratory </title><secondary-title>Dengue Bulletin</secondary-title></titles><periodical><full-title>Dengue Bulletin</full-title></periodical><pages>103-114</pages><volume>33</volume><dates><year>2009</year></dates><urls></urls></record></Cite></EndNote>]. There is also no evidence that OX513A females have increased vector competence than wild-type *Ae. aegypti*. In fact, evidence suggests OX513A females have a decreased vector competence because any inadvertently released OX513A females will die in 2-3 days time as the lack of tetracycline in the environment will turn on the lethality trait resulting in a lifespan too short to vector viral disease. This is because the short lifespan of the OX513A females is too brief for arboviruses such as dengue and Zika to cross the mosquito midgut barrier, reach the salivary glands, and multiply sufficiently (this period is defined as the external incubation period, EIP) to be transmitted to a human host at a subsequent blood feeding. Disease transmission by OX513A females requires that they can locate a human host that is infected with a sufficient titer of virus and blood feed adequately, that the EIP is sufficiently long to allow virus multiplication and secretion into saliva, and that the female lives long enough to blood feed again after the EIP is complete, thereby transmitting the virus to a human host. EIP for dengue is estimated at 10-14 days. "Long-lived vectors contribute most to pathogen transmission and small decreases in vector life expectancy can cause large reductions in transmission rates." [ADDIN EN.CITE <EndNote><Cite><Author>Cook</Author><Year>2007</Year><RecNum>245</RecNum><DisplayText>(Cook et al. 2007)</DisplayText><record><rec-number>245</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1465924312">245</key></foreign-keys><ref-type name="Book Section">5</ref-type><contributors><authors><author>Cook, P.E.</author><author>McMeniman, C.J.</author><author>O'Neill, S.L.</author></authors><secondary-authors><author>Aksoy, S.</author></secondary-authors></contributors><titles><title>Modifying Insect Population age structure to control vector-borne disease.</title><secondary-title>Transgenesis and the management of vector-borne disease.</secondary-title></titles><dates><year>2007</year></dates><publisher>Landes Bioscience</publisher><urls></urls></record></Cite></EndNote>]. All of these factors combined suggest that, if anything, OX513A females would have a lower overall vectorial capacity as compared to wild-type *Ae. aegypti*. ~~have relatively short time, which limits their ability to interact with humans and participate in transmission of diseases.~~ Further, OX513A mosquitoes are produced under disease-free conditions that~~which~~ further limit the possibility of transmitting any diseases (Section [REF

_Ref453332380 \r \h]. Therefore, the likelihood of adverse effects associated with an increase in transmission of dengue or other diseases transmitted by mosquitoes is extremely low.

It is highly unlikely that the release of OX513A mosquitoes would lead to an~~the~~ increase in the population of other mosquito species that might~~tay~~ contribute to an~~the~~ increase ~~of~~in diseases transmission at the proposed trial site. A suppression field trial using OX513A in Panama resulted in an 82% suppression of *Ae. aegypti* over an 84-day period without an increase in *Ae. albopictus* at the same site [ADDIN EN.CITE
<EndNote><Cite><Author>Gorman</Author><Year>2016</Year><RecNum>249</RecNum><DisplayText>(Gorman et al. 2016)</DisplayText><record><rec-number>249</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1466182262">249</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Gorman, K.</author><author>Young, J.</author><author>Pineda, L.</author><author>Marquez, R.</author><author>Sosa, N.</author><author>Bernal, D.</author><author>Torres, R.</author><author>Soto, Y.</author><author>Lacroix, R.</author><author>Naish, N.</author><author>Kaiser, P.</author><author>Tepedino, K.</author><author>Philips, G.</author><author>Kosmann, C.</author><author>Caceres, L.</author></authors></contributors><auth-address>Oxitec Limited, Abingdon, Oxfordshire, UK.
Gorgas Memorial Institute for Human Health, Ciudad de Panama, Panama.</auth-address><titles><title>Short-term suppression of Aedes aegypti using genetic control does not facilitate Aedes albopictus</title><secondary-title>Pest Manag Sci</secondary-title></titles><periodical><full-title>Pest Manag Sci</full-title></periodical><pages>618-28</pages><volume>72</volume><number>3</number><keywords><keyword>Ox513a</keyword><keyword>Panama</keyword><keyword>chikungunya</keyword><keyword>dengue</keyword><keyword>mosquito</keyword><keyword>transgenic</keyword></keywords><dates><year>2016</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1526-4998 (Electronic)
1526-498X (Linking)</isbn><accession-num>26374668</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/26374668</url></related-urls></urls><electronic-resource-num>10.1002/ps.4151</electronic-resource-num></record></Cite></EndNote>]. This suggests that a short term field trial as proposed for Key Haven, FL should not have an effect on local *Ae. albopictus* populations via niche expansion. Additionally, *Ae. aegypti* is found more frequently in areas that are coastal and at low altitude, while *Ae. albopictus* is more likely to be present in locations that are inland and at higher altitude [ADDIN EN.CITE ADDIN EN.CITE.DATA]. Because the proposed trial site is in a coastal, low altitude location, it is a more likely habitat for *Ae. aegypti* than *Ae. albopictus*, further lessening the likelihood of *Ae. aegypti* replacement with *Ae. albopictus*. ~~Use of ovitraps and BG-Sentinel traps will allow Oxitec to monitor for potential presence of *Ae. albopictus*. Moreover, replacement~~ ~~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.~~ Additionally, the wild-type *Ae. aegypti* population would be ~~is~~ expected to recover to pre-trial numbers after the cessation of OX513A mosquito releases. Therefore, the likelihood of adverse effects associated

with increase in population of other mosquitoes that may contribute to the increase of diseases at the proposed trial site is extremely low. However, use of ovitraps and BG Sentinel traps will allow Oxitec to monitor for potential presence of *Ae. albopictus* at the trial site and adjust use of insecticides to address any such unlikely increase in *Ae. albopictus*.

It is highly unlikely that the production and release of OX513A mosquitoes would lead to development of antimicrobial resistance in part because . It is highly unlikely that resistant bacteria, even if present in the larval or pupal stages, would be highly unlikely to be present in adult OX513A mosquitoes because their gut bacteria are lost during mosquito metamorphosis from larvae to adults. It is also would be highly unlikely that any antimicrobial resistance would arise in bacteria in the rearing water and that this trait would be transferred to other bacteria that could cause food or water-borne diseases due to thea short duration of the mosquito development cycle and well as the trial in general. Sewage water from the OX513A production facility is treated at a local waste water treatment facility in accordance with existing local and state laws, further precluding the exposure of humans and other animals to mosquito larvae-contaminated water. The process controls that would be implemented at the HRU (e.g., use of personal protective equipment) would eliminate the potential for transfer of antibiotic resistance to personnel involved in production of OX513A mosquitoes (Section [REF _Ref453332428 \r \h]). Therefore, the likelihood of the adverse effects associated with development of anti-microbial resistance is extremely low.

Inadvertent release of OX513A females is highly unlikely due to SOPs and quality control procedures that Oxitec would implemented by Oxitec (Section [REF _Ref453741905 \r \h]). In the a highly unlikely event that , if a person were bitten by an OX513A female inadvertently released at the trial site or by the female OX513A progeny that survived, the immunological response to these bites in humans and other animals would is not be expected to be any different from the immunological response to bites by wild-type *Ae. aegypti* mosquitoes as discussed above. In fact, we anticipate that it would be less dangerous in several respects: (1) released mosquitoes would be are disease-free as they are maintained in conditions and with procedures that prevent contamination with virus, and (2) dengue virus takes a long time to develop in a mosquito to the point when it can be transmitted (EIP- Section 15.2), shorter-lived females such as the OX513A females are less likely to pass on diseases. Male mosquitoes do not bite humans. Therefore, the likelihood of the adverse effects associated with the release of OX513A females at the trial site is expected to be extremely low.

It is highly unlikely that the failure of the introduced traits in OX513A male mosquitoes would lead to any adverse effects. The stability of the #OX513 rDNA construct was confirmed over multiple generations of OX513A mosquitoes (Section [REF _Ref453332462 \r \h]). In thea highly unlikely event that the introduced lethality trait is compromised, resulting in a loss of function of the tTAV lethality trait, these mosquitoes would be functionally no different than existing wild-type *Ae. aegypti*. Oxitec would will be monitoring the performance of OX513A mosquitoes during the investigational trial (Section [REF _Ref453245461 \r \h]11) and would be able to detect the failure of the traits and stop the trial. Therefore, the likelihood of the adverse effects associated with the failure of the introduced traits is expected to be extremely low.

15.3 Question conclusions

Data and information on the ~~The~~ consequences of release, survival, establishment, and spread of OX513A in the environment ~~have been extensively studied: data and information from these studies~~ indicate that the proposed investigational use of OX513A *Ae. aegypti* mosquitoes ~~Based on the information provided in this EA, the release of *Ae. aegypti* OX513A~~would ~~is~~ not be expected to cause any significant adverse impacts on the environment or human and ~~other~~ non-target a~~human~~nimal health beyond those caused by wild-type~~other~~ mosquitoes ~~(e.g., local reactions at the site of bites)~~.

15.4 Consequences of~~or~~ the No Action Alternative

As described earlier (Section [REF _Ref453245565 \r \h]), ~~there is only one likely scenario to consider as a result of~~ the no action alternative would be ~~s~~for Oxitec ~~would not~~ to not carry out the field trial in Key Haven, Florida~~L~~. As a result, Oxitec could continue development and commercialization of the product at locations outside of the United States with no intent to conduct a field trial~~market the product~~ in the United States, or they could select another location in the United States to conduct a~~the~~ field trials. With respect to the former, there would be no consequences or potential environmental impacts arising from that scenario – as there would be no trial in Key Haven, Florida~~L~~. With respect to the latter, Oxitec would prepare a new environmental assessment evaluating potential environmental impacts associated with that investigational release~~their new proposed trial at a new location~~.

15.5 Cumulative Impacts

As defined in CEQ regulations, cumulative impacts are "the impact on the environment which results from the incremental impact of the present action when added to other past, present, and reasonably foreseeable future actions..." 40 CFR 1508.7. There would be no "incremental impact" because this is the first proposed field trial using OX513A *Ae. aegypti* mosquitoes at Key Haven, FL. As a result, there would be no cumulative impacts on the environment of the United States. Moreover, consideration of any future field trials at this time would be purely speculative. ~~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.~~

~~15~~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.~~Our risk assessment approach relies on the environmental risk issues associated with the introduction or escape of GE animals into the environment, which are identified in the 2002 National Research Council (NRC) report entitled "Animal Biotechnology: Science Based Concerns" [ADDIN EN.CITE <EndNote><Cite><Author>NRC</Author><Year>2002</Year><RecNum>44</RecNum><DisplayText>(NRC 2002)</DisplayText><record><rec-number>44</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">44</key></foreign-keys><ref-type name="Book">6</ref-type><contributors><authors><author>NRC</author></authors></contributors><titles><title>Animal biotechnology: science-based concerns</title></titles><reprint-edition>Not in File</reprint-edition><dates><year>2002</year><pub-dates><date>2002</date></pub-dates></dates><pub-location>Washington, DC</pub-location><publisher>The National Academic Press</publisher><isbn>0-309-08439-3</isbn><label>138</label><urls></urls></record></Cite></EndNote>]. According to the NRC report, risk (R) is the joint probability of exposure (P(E)) and the conditional probability of harm (i.e., adverse effects) given that the exposure to a hazard has occurred (P(H|E)): Risk = P(E) x P(H|E) or Risk = Exposure x Adverse Effect. Therefore, there must be both exposure and an adverse effect to pose a risk. If one of the components is negligible then the risk would be negligible as well.

~~In its report on Animal Biotechnology Science Based Concerns (NRC 2002) the National Academies of Science~~Further, the report 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.

Because ~~F~~for a GE animal to prove a hazard it must spread and establish in the community in which it is released, the NRC report further ~~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; and
- Determination of risk by the multiplication of the resulting outcomes on harm and exposure.

In our assessment we The identifiedication of and evaluated potential hazards, likelihood of exposure and potential consequences (likelihood of adverse effectsharm being realized) associated with the proposed trial 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 [REF _Ref450424660 \h] below.

Table [SEQ Table * ARABIC]. Estimation of risk matrix.

LIKELIHOOD	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

Note: the risk assessment matrix and definitions are taken from the Australian OGTR Risk Analysis Framework[75].

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

[75] [HYPERLINK "http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/content/riskassessments-1" \h] [Accessed 24 Oct 24, 2013]

- 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 proposed 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 GE mosquito modified insect) was identified and characterised.

TA summary of the potential adverse effects associated withharms envisaged from the proposed investigational use of the OX513A *Ae. aegypti* mosquito are are summarised in [REF _Ref453328110 \h]. .These potential adverse effects have been classified as direct or indirect, immediate or delayed and have been grouped according to their likely area of impact: human health, or other non-target animal health and, or environmental health.

A direct harmadverse effect 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 harmadverse effect refers to a causal chain of events being established whereby the harm is reached though mechanisms not directly related to the OX513A mosquito insect itself, such as interaction with other organisms, transfer of the rDNA constructgenetic material, or changes in use or management at the release site.

Classifying the harm adverse effect as immediate or direct will facilitate the monitoring activities during the trial. An immediate effect refers to a potential harm adverse effect 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 adverse effects 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 [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 [REF _Ref453328110 \h] and brings together all the information previously presented in the EA regarding potential ~~harms,~~ hazards, likelihood of exposure and adverse effects ~~and consequences~~ along with the data endpoints that have been considered in the analysis.

Table [SEQ Table * ARABIC]. Risk assessment.

Risk category	Adverse effect/ consequence	Direct/ indirect	Likelihood of exposure	Likelihood of adverse effects	Estimation of risk	Comments
Human or animal health	Toxic ~~or allergenic~~ effects in humans or ~~other~~ non-target animals or allergenic effects in humans	Direct	Highly unlikely (HUL)	Extremely low (EL)	Negligible (HUL x EL)	Western blot assays ~~performed by Oxitec~~ demonstrated that the levels of tTAV and DsRed2 proteins in OX513A female saliva are below the LOD~~limit of detection~~. Further, bioinformatics analysis demonstrated the lack of toxic and allergenic potential for humans (Section [REF _Ref453739750 \r \h]). The expressed proteins have been shown to have no homology to known toxins following bioinformatics evaluations carried out according to international guidelines. Therefore, the immunological response to the bites from OX513A female mosquitoes is not expected to be any different from the immunological response to the bites from wild-type *Ae. aegypti* mosquitoes.
Human or animal health	Transfer of the rDNA construct to humans or ~~other~~ non-target animals	Direct	Highly unlikely (HUL)	Extremely low (EL)	Negligible (HUL x EL)	The rDNA construct is stably integrated in the mosquito genome (Section [REF _Ref453332132 \r \h]) and is incapable of being transferred through non-sexual means (Section [REF _Ref453332100 \r \h]). No adverse effects on predator species were identified when they were fed a diet comprised of OX513A larvae exclusively (Section [REF _Ref453331917 \r \h]).

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Risk category	Adverse effect/ consequence	Direct/ indirect	Likelihood of exposure	Likelihood of adverse effects	Estimation of risk	Comments
Human or animal health	Increase in transmission of dengue or other diseases transmitted by mosquitoes	Direct	Highly unlikely (HUL)	Extremely low (EL)	Negligible (HUL x EL)	Male mosquitoes do not bite or transmit disease. A small number of OX513A females that may be co-released (not to exceed 0.2%) or are present in the environment as a result of incomplete penetrance of the introduced lethality trait would have a relatively short lifespan~~time~~, which would limit their ability to interact with humans and ~~participate in~~ transmit~~ssion of~~ diseases. In the ~~a~~ highly unlikely event that, ~~if~~ an OX513A female feeds on an ~~the~~ infected person, the OX513A mosquito ~~it~~ would not be able to transmit the infection further because OX513A lifespan~~time~~ is considerably shorter than the EIP required for ~~infection~~viral development (Section [REF _Ref453740982 \r \h]).

Risk category	Adverse effect/ consequence	Direct/ indirect	Likelihood of exposure	Likelihood of adverse effects	Estimation of risk	Comments
Human or animal health	Increase in population of other mosquitoes that may contribute to the increase of diseases	Indirect	Unlikely (UL)	Extremely low (EL)	Negligible (UL x EL)	It is highly unlikely that the release of OX513A mosquitoes would lead to an increase in population of other mosquito species that might contribute to an increase of disease transmission at the proposed trial site. A suppression field trial using OX513A in Panama resulted in an 82% suppression of *Ae. aegypti* over an 84 day period without an increase in *Ae. albopictus* at the same site. This suggests that a short term field trial should not have an effect on local *Ae. albopictus* populations via niche expansion. *Ae. aegypti* is found more frequently in areas that are coastal and low altitude, while *Ae. albopictus* is more likely to be present in locations that are inland and at higher altitude. The proposed trial site is in a coastal, low altitude location. ~~Monitoring of the trial would allow for tracking for potential presence of *Ae. Albopictus* mosquitoes at the investigational site. 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.~~

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Risk category	Adverse effect/ consequence	Direct/ indirect	Likelihood of exposure	Likelihood of adverse effects	Estimation of risk	Comments
Human or animal health	Development of anti-microbial resistance	Indirect	Highly unlikely (HUL)	Extremely low (EL)	Negligible (HUL x EL)	It is highly unlikely that resistant bacteria, even if present in the larval or pupal stages, would be present in adult OX513A mosquitoes because their gut bacteria are lost during mosquito metamorphosis from larvae to adults. It is highly unlikely that any antimicrobial resistance would arise in bacteria in the rearing water and that this trait would be transferred to other bacteria that could cause food or water-borne diseases due to ~~a~~the short duration of the mosquito development cycle and the trial in general. ~~Sewage water from the OX513A production facility is treated at a local waste water treatment facility in accordance with existing local and state laws further precluding the exposure of humans and other animals to mosquito larvae-contaminated water.~~ The process controls implemented at the HRU (e.g., use of personal protective equipment) eliminate the potential for transfer of antibiotic resistance (if present) to personnel, if present.

Risk category	Adverse effect/ consequence	Direct/ indirect	Likelihood of exposure	Likelihood of adverse effects	Estimation of risk	Comments
Human or animal health	Release of OX513A females at the trial site	Direct	Highly unlikely (HUL)	Extremely low (EL)	Negligible (HUL x EL)	SOPs and quality control procedures would be ~~are~~ in place to ensure accuracy of the sorting does not exceed a maximum of 0.2% ~~preclude inadvertent release of~~ OX513A females. Male mosquitoes do not bite. If a person were bitten by an OX513A female inadvertently released at the trial site or by ~~the~~ female OX513A progeny that survived, the immunological response to these bites in humans and other animals is not expected to be any different from the immunological response to bites by wild-type *Ae. aegypti* mosquitoes. Inadvertently released female OX513A mosquitoes would not be likely ~~able~~ to transmit any diseases because (1) released mosquitoes would be ~~are disease-free as they are~~ maintained in conditions and with procedures that prevent contamination with virus, and (2) dengue virus takes a long time to develop in a mosquito to the point when it can be transmitted, so that shorter-lived females such as the OX513A females are less likely to pass on diseases. ~~Male mosquitoes do not bite humans.~~
Human or animal health	Failure of the introduced traits	Direct	Highly unlikely (HUL)	Extremely low (EL)	Negligible (HUL x EL)	Stability of the inserted rDNA construct was confirmed over multiple generations of OX513A mosquitoes. In the highly event that ~~unlikely scenario, if~~ the introduced traits is compromised, mosquitoes could~~s~~ be controlled using alternative techniques ~~such as fogging and use of larvicides~~. No such instability in the introduced traits has been observed to date over 100~~fifl~~ generations.

Risk category	Adverse effect/ consequence	Direct/ indirect	Likelihood of exposure	Likelihood of adverse effects	Estimation of risk	Comments
Environmental	Interbreeding with related mosquito species	Direct	Highly unlikely (HUL)	Extremely low (EL)	Negligible (HUL x EL)	Biological data from experiments conducted and literature shows that cross-species mating results in non-viable progeny (Section [REF _Ref453329698 \r \h]).

Risk category	Adverse effect/ consequence	Direct/ indirect	Likelihood of exposure	Likelihood of adverse effects	Estimation of risk	Comments
Environmental	Effect of tetracycline on the environment	Indirect	Highly Unlikely (UL)	Extremely low (EL)Marginal(MM)	Negligible (HUL x EL)	The levels of tetracycline in the waste water are expected to be relatively low and would be quickly degraded or adsorbed in the environment (Section [REF _Ref453329832 \r \h]).[ADDIN EN.CITE <EndNote><Cite><Author>Bautitz</Author><Year>2007</Year><RecNum>9</RecNum><DisplayText>(Bautitz and Nogueira 2007)</DisplayText><record><rec-number>9</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">9</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Bautitz, Ivonete Rossi</author><author>Nogueira, Raquel F.P.</author></authors></contributors><titles><title>Degradation of tetracycline by photo-Fenton process - Solar irradiation and matrix effects</title><secondary-title>J Photochem. Photobiol A: Chemistry</secondary-title></titles><pages>33-39</pages><volume>187</volume><number>1</number><reprint-edition>Not in File</reprint-edition><dates><year>2007</year><pub-dates><date>2007</date></pub-dates></dates><isbn>10106030</isbn><label>9</label><urls><related-urls><url>http://linkinghub.elsevier.com/retrieve/pii/S1010603006005053</url></related-urls></urls><electronic-resource-num>10.1016/j.jphotochem.2006.09.009</electronic-resource-num><access-date>3/28/2015</access-date></record></Cite></EndNote>]

Risk category	Adverse effect/ consequence	Direct/ indirect	Likelihood of exposure	Likelihood of adverse effects	Estimation of risk	Comments
Environmental	Effect on flora	Direct/ indirect	Highly unlikely (HUL)	Extremely low (EL)	Negligible (HUL x EL)	There are no reports that *Ae. aegypti* is a pollinator for any plant species~~Ae. aegypti is not a pollinator~~. It is highly unlikely that the rDNA construct could be transferred to other species that may be involved in pollination of ~~the~~ plants.
Environmental	Effect on predators	Direct	Highly unlikely (HUL)	Extremely low (EL)	Negligible (HUL x EL)	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 a 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 opportunist-ically eaten by bats or birds. Additionally, the FKMCD currently controls mosquitoes ~~are currently being controlled~~ by chemical, biological, or source reduction methods and, therefore, impacts on non-targets are not likely to differ ~~significantlysignificantly be greater than those of~~from ~~the~~ existing control mechanisms in the event the proposed trial ~~is a success and~~ reduces local *Aedes aegypti* population significantly.

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Risk category	Adverse effect/ consequence	Direct/ indirect	Likelihood of exposure	Likelihood of adverse effects	Estimation of risk	Comments
Environmental	Effect on decomposers	Direct	Unlikely (UL)	Extremely low (EL)	Negligible (UL x EL)	No decomposers that are specifically involved in decomposition of *Ae. aegypti* were identified (Section [REF _Ref453330160 \r \h]). No adverse effects have been identified in open releases conducted in Malaysia, Cayman Islands, Panama and Brazil.
Environmental	Development of resistance to insecticides in the local population of *Ae. aegypti*	Indirect	Highly unlikely (HUL)	Extremely low (EL)~~Minor (MN)~~	Negligible (HUL x EL)	OX513A are susceptible to insecticides used for mosquito control.
Environmental	Persisting or establishing at the trial site	Direct	Highly unlikely (HUL)	Extremely low (EL)~~Marginal (MR)~~	Negligible (HUL x EL)	It is highly unlikely that OX513A mosquitoes and their progeny would be able to ~~persist or~~ establish in the environment due to selective disadvantage conferred by the lethality trait and compromised fitness.

Risk category	Adverse effect/ consequence	Direct/ indirect	Likelihood of exposure	Likelihood of adverse effects	Estimation of risk	Comments
Environmental	Effect on endangered or threatened species	Direct	Highly unlikely (HUL)	Extremely low (EL)	Negligible (HUL x EL)	The mosquitoes are already controlled by chemical, biological or source reduction methods and therefore impacts on endangered or threatened species are not likely to be greater than those of the existing control mechanisms. With the exception of the Stock Island Tree Snail, there is no habitat overlap of OX513A mosquitoes with threatened or endangered species as *Ae. aegypti* is a n-urban or domestic mosquito closely associated with human habitats. The trial is not likely to adversely affect the Stock Island Tree Snail as it does not propose removal or modification of its habitat. National wildlife refuges are located considerable distance away from the proposed trial site and would not be affected by the proposed trial.

~~15.1~~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:

- What is the likelihood ~~offor~~ inadvertent release of OX513A mosquitoes outside of the proposed trial site~~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 would be~~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 would~~ill~~ 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 would~~ill~~ be trained in the procedures for the rearing of OX513A by Oxitec staff.

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[76] and a Hurricane Preparedness Policy being in place, where adult and larval insect life stages would~~ill~~ 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 would~~ill~~ not live longer than their short lifespan and the introduced lethality trait and the dependence on the presence of tetracycline for survival would~~ill~~ prevent establishment in the environment.

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[76] 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 130-156 mph; 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" [ADDIN EN.CITE <EndNote><Cite><Author>NOAA</Author><Year>2015</Year><RecNum>72</RecNum><DisplayText>NOAA. 2015. Saffir-Simpson Hurricane Wind Scale. http://www.nhc.noaa.gov/aboutsshws.php.</DisplayText><record><rec-number>72</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1451484603">72</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>NOAA</author></authors></contributors><titles><title>Saffir-Simpson Hurricane Wind Scale. http://www.nhc.noaa.gov/aboutsshws.php</title></titles><dates><year>2015</year></dates><urls></urls></record></Cite></EndNote>]).

- What is the likelihood ~~of spread that for~~ OX513A mosquitoes and their progeny would disperse from the proposed trial site ~~*Ae. aegypti* will survive and disperse once released into the environment~~?

There is a high degree of confidence that OX513A released males would~~ll~~ have limited dispersal, based on results from previous trials of OX513A in other countries and information from the published literature and the location and features of the proposed trial site. ~~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 Section~~ [REF _Ref411529408 \r \h * MERGEFORMAT] ~~). 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[22] 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.~~

• What is the likelihood for establishment of OX513A mosquitoes at the proposed trial site?

Sufficient information from previous field releases of OX513A, where the lifespan of the released insects was approximately 1-3 days [ADDIN EN.CITE <EndNote><Cite><Author>Lacroix</Author><Year>2012</Year><RecNum>43</RecNum><DisplayText>(Lacroix et al. 2012)</DisplayText><record><rec-number>43</rec-number><foreign-keys><key app="EN" db-id="sa90t0tfyvfaw7e0pdc5xssda55xesz0sss5" timestamp="1432047849">43</key></foreign-keys><ref-type name="Electronic Article">43</ref-type><contributors><authors><author>Lacroix, R.</author><author>McKemey, A.R.</author><author>Raduan, N.</author><author>Kwee Wee, L.</author><author>Nordin, O.</author></authors></contributors><titles><title>Open Field Release of Genetically Engineered Sterile Male Aedes aegypti in Malasia</title><secondary-title>PLoS ONE</secondary-title></titles><periodical><full-title>PLoS ONE</full-title></periodical><pages>e42771</pages><volume>7</volume><number>8</number><reprint-edition>Not in File</reprint-edition><keywords><keyword>Aedes</keyword><keyword>Aedes

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~~[22] Semi-field describes a study that has been done in containment, but under natural environmental conditions; i.e., a field house or field cage.~~

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aegypti</keyword></keywords><dates><year>2012</year><pub-dates><date>2012</date></pub-dates></dates><label>44</label><urls></urls></record></Cite></EndNote>] and the fact that more than 95% of progeny die before reaching adulthood as well as evidence from the scientific literature on potential sources of tetracycline provide a high degree of certainty that the OX513A would be~~is~~ unlikely to establish in the environment.

~~16~~17 Conclusions

~~Using weight-of-evidence risk-based approach,~~ Based on the foregoing, FDA ~~performed a rigorous environmental assessment and concludes found no evidence~~ that the investigational use of OX513A *Ae. aegypti* mosquitoes in Key Haven, Florida~~,~~ would not result in significant impacts on the environment of the United States. The agency's conclusions are summarized below: ~~Information relevant to the pertinent risk questions described throughout this document and summarized in Section~~ [REF _Ref411866517 \r \h * MERGEFORMAT]~~, have been presented in this draft EA with the following conclusions:~~

- What is the likelihood ~~offer~~ inadvertent release of OX513A mosquitoes outside of the proposed trial site?

  FDA concludes~~determines~~ that the likelihood ~~offer~~ inadvertent release of OX513A mosquitoes outside of the proposed trial site is ~~very~~ low due to the physical containment measures and standard operating procedures implemented for the rearing and transportation of OX513A. ~~Can OX513A *Ae. aegypti* escape the confined conditions in which it is reared?~~
  ~~The likelihood of escape from confined conditions is negligible.~~

- ~~Wha~~What is the likelihood ~~offer~~ establishment of OX513A mosquitoes at the proposed trial site?

~~t is the likelihood that OX513A *Ae. aegypti* will survive and disperse once released into the environment?~~
The OX513A line of *Ae. aegypti* mosquitoes carries a repressible dominant lethality trait that prevents progeny inheriting the ~~causes death of #~~OX513A rDNA construct from surviving to functional adulthood ~~mosquito progeny at the early pupal or larval stage unless reared~~ in the absence~~presence~~ of tetracycline. Although it appears that the introduced lethality trait did not affect mating competitiveness of OX513A males, data demonstrating hemizygous females reared without tetracycline have a median lifespan of two days relative to a wild-type median lifespan of 68 ~~we conclude that it had a significant impact on survival of OX513A mosquitoes dramatically reducing their lifespan. The lack of exogenous tetracycline and its derivatives in the environment would~~ further reduce the likelihood of survival of OX513A mosquitoes and their progeny. FDA therefore concludes that it is highly unlikely that OX513A mosquitoes and their progeny would be able to

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

* What is the likelihood ~~that OX513A *Ae. aegypti* can reproduce and establish in the environment into which they are released?~~of dispersal of~~spread for~~ OX513A mosquitoes and their progeny from the proposed trial site?

Based on our analysis of data available in the literature, we ~~see~~conclude~~sides~~ that dispersal of OX513A mosquitoes appears to be adversely affected as measured by *MDT*, but not by *maximum distance traveled*, indicating, that in general, the population of OX513A is not expected to exhibit dispersion greater than wild-type *Ae. aegypti*. The location of the proposed trial site and mosquito control measures implemented by FKMCD would considerably limit the dispersal~~ion~~ of OX513A mosquitoes as well. FDA therefore concludes that it is highly unlikely that OX513A mosquitoes and their progeny would be able to establish ~~or persist~~ at the proposed field trial site, or spread beyond its boundaries, should the trial proceed.

* What is the likelihood that the rDNA construct could be transferred to humans or other organisms?

Based on evaluation of data and information submitted by Oxitec, FDA determined that the #OX513 rDNA construct is stably integrated in the OX513A mosquito genome and completely refractory to remobilization, even when deliberately re-exposed to *piggyBac* transposase. Should the proposed field trial proceed, FDA considers that it is highly unlikely that the #OX513 rDNA construct could be transmitted to other closely related species by inter-breeding, as *Ae. aegypti* mating behavior is highly species-specific. Horizontal or non-sexual transfer of the rDNA construct to humans and other animals is also highly unlikely due to complexity of the process. Mosquitoes have been feeding on humans and other animals for millennia with no evidence of DNA transfer between humans and mosquitoes.

* What is the likelihood that release of OX513A mosquitoes would have an adverse effect on non-target species at the proposed trial site?

FDA has determined that it is highly unlikely that the presence of OX513A mosquitoes and their progeny and suppression of the local population of *Ae. aegypti* would have any significant effects on the populations of predators, parasitoids, and decomposers at the proposed trial site. No adverse effect on the pollination of local plants is expected as well. Should the proposed field trial proceed, FDA has determined that the proposed trial would not jeopardize the

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continued existence of Stock Island Tree snails found in the vicinity of ~~at~~ the proposed trial site and would not result in the destruction or adverse modification of their habitat. Therefore, FDA makes a "no effect" determination under the ESA with regard to the Stock Island Tree Snail. Further, FDA does not expect any adverse effects on other endangered species in wildlife refuges located in Monroe County or destruction and modification of their habitats due to their considerable distance from the proposed trial site

- What is the likelihood that the rDNA expression products in OX513A mosquitoes would have adverse effects on humans or other animals?

  Based on the Western immuno-blot assays performed by Oxitec, we conclude that the levels of tTAV and DsRed2 proteins in saliva of OX513A *Ae. aegypti* females homozygous for the #OX513 rDNA construct are below the limit of detection for that assay. Therefore, we consider that it is highly unlikely that humans or other animals would be exposed to these proteins even if they were to be bitten by OX513A female mosquitoes. A stepwise, weight-of-evidence approach evaluating the toxic and allergenic potential of tTAV and DsRed2 proteins based on Codex guidelines and a scientific literature search did not identify any evidence suggesting the allergenicity or toxicity of tTAV and DsRed2 proteins. Bioinformatics analysis of amino acid sequences of tTAV and DsRed2 proteins did not identify any similarities with known toxins or allergens. Therefore, FDA concludes that tTAV and DsRed2 proteins lack any toxic or allergenic potential and do not pose any significant risks to humans or non-target animals.

- What are likely consequences to, or effects on the environment of the United States associated with the investigational use of OX513A mosquitoes?

  The consequences of release, ~~survival,~~ establishment, and dispersal of OX513A in the environment have been extensively studied; data and information from these studies indicate that the proposed investigational use of OX513A *Ae. aegypti* mosquitoes is not expected to cause any significant adverse impacts on the environment or human and non-target animal health beyond those caused by wild-type mosquitoes.

In summary, data and information presented and evaluated in the EA indicates that the investigational use of OX513A *Ae. aegypti* mosquitoes, as described in this EA, would not result in significant effects on the quality of the human environment in the United States. ~~There is a high likelihood that OX513A *Ae. aegypti* can reproduce, as reproduction with local females is the intended effect of the proposed release. However, there is a low likelihood that they would~~~~ill 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 ~~would be~~ 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 ~~would~~ are likely to be negligible. The impact on the environment and non-target organisms ~~would~~ is likely to be less than the use of broad-spectrum insecticides for mosquito control.

~~17~~18 Listing of agencies and persons consulted~~Preparation of the EA~~

U.S. Environmental Protection Agency
Office of Pesticide Programs
Biopesticides and Pollution Prevention Division

Centers for Disease Control and Prevention
Division of Vector-Borne Diseases

National Institutes of Health
National Institute of Allergy and Infectious Diseases
Laboratory of Malaria and Vector Research

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