UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

SUBJECT: Human Health and Environmental Risk Assessment for the New Product OX5034 Containing the Tetracycline-Repressible Transactivator Protein Variant (tTAV-OX5034; New Active Ingredient) Protein, a DsRed2 Protein Variant (DsRed2-OX5034; New Inert Ingredient), and the Genetic Material (Vector pOX5034) Necessary for Their Production in OX5034 *Aedes aegypti*; Data and Information Were Provided in Support of a FIFRA Section 5 Application.

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FROM: Wiebke T. Striegel, Ph.D., Senior Scientist
Product Characterization and Human Health Reviewer
Emerging Technologies Branch
Biopesticides and Pollution Prevention Division (7511P)

Digitally signed by WIEBKE STRIEGEL
Date: 2020.04.30 12:44:49 -04'00'

AND

Amanda A. Pierce, Ph.D., Biologist
Environmental Fate and Effects Reviewer
Emerging Technologies Branch
Biopesticides and Pollution Prevention Division (7511P)

AMANDA PIERCE

Digitally signed by AMANDA PIERCE
Date: 2020.04.30 12:56:13 -04'00'

TO: Eric W. Bohnenblust, Ph.D., Risk Manager
Emerging Technologies Branch
Biopesticides and Pollution Prevention Division (7511P)

THROUGH: John L. Kough, Ph.D., Senior Scientist
Product Characterization and Human Health Reviewer
Risk Assessment Branch
Biopesticides and Pollution Prevention Division (7511P)

JOHN KOUGH Digitally signed by JOHN KOUGH Date: 2020.04.30 13:13:02 -04'00'

AND

Chris A. Wozniak, Ph.D., Biotechnology Special Assistant
Environmental Fate and Effects Reviewer
Biopesticides and Pollution Prevention Division (7511P)

AND

Mike Mendelsohn, Branch Chief
Emerging Technologies Branch
Biopesticides and Pollution Prevention Division (7511P)

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I. BACKGROUND AND EXECUTIVE SUMMARY

A. Background

Oxitec Ltd., (Oxitec or the applicant) requests an Experimental Use Permit (EUP) under FIFRA section 5 for a new end-use product OX5034 containing a variant of the new active ingredient tetracycline-repressible transactivator (tTAV-OX5034) protein, a variant of the new inert ingredient DsRed2 protein (DsRed2-OX5034), and the genetic material (vector pOX5034) necessary for their production in OX5034 *Aedes aegypti* (yellow fever mosquito). Oxitec requests this EUP to evaluate whether the product is efficacious in suppressing naturally occurring *Ae. aegypti* populations under field conditions.

OX5034 is described as a species-specific female-lethal trait that results in emergence of all-male progeny in the absence of tetracycline in the larval diet. The pesticidal effect of OX5034 is species-specific as it only affects the reproductive success of *Ae. aegypti* through mating between OX5034 *Ae. aegypti* males and *Ae. aegypti* females that are already present in the release area. OX5034 homozygous males alone will be released into the environment. Only female offspring from OX5034 matings are killed, while OX5034 hemizygous males survive to pass on the OX5034 female-lethal trait further. Unlike female mosquitoes, male mosquitoes do not bite humans. With continued field releases of OX5034 homozygous males, the *Ae. aegypti* population in the treatment area is thought to progressively decline due to the reduced number of females emerging each consecutive generation. In addition, OX5034 also expresses DsRed2-OX5034, a variant of the DsRed fluorescent protein form *Discosoma* spp., that allows for the visual identification of OX5034 hemizygous larvae collected from the field. A different transgenic mosquito developed by Oxitec, OX513A, is not covered under the current EUP application.[1]

With this application, Oxitec provided the experimental design for mosquito releases under the EUP. EPA evaluated this in its document "Review of Section G for an Experimental Use Permit 93167-EUP-E to Test OX5034 *Ae. aegypti* Mosquitoes Decision #549240; Submission #1047971," which is available in the www.regulations.gov docket established for this action (EPA-HQ-OPP-2019-0274). Oxitec requested a 24-month permit for a cumulative area of 6,600 acres, which will be divided into multiple treatment and control areas within Monroe, Co., Florida and Harris, Co., Texas.

Under the EUP, Oxitec is planning to test the efficacy of the product by deploying OX5034 mosquito eggs and adult males in the treatment areas. For egg releases, a known quantity of OX5034 eggs will be released in mosquito rearing boxes. Importantly, as described in Unit II.A.2, only male OX5034 mosquitoes will emerge from these eggs, no female OX5034 mosquitoes will be released. Mosquito rearing boxes will be physically isolated from the public whenever possible, or otherwise located discretely and out of public view. In the case of adult OX5034 male releases, known quantities of adult males will be released from containers either from a vehicle or on foot.

Monitoring and mosquito sampling will be done weekly in the treatment and control areas to monitor the adult mosquito population and to collect eggs. The egg collections will allow for evaluation of larvae

[1] The OX513A EUP application is no longer active at EPA (EPA File Symbol 93167-EUP-R; docket ID EPA-HQ-OPP-2017-0756). Unlike the OX5034 mosquitoes, neither OX513A males nor females are intended to survive without being reared on tetracyclines, although a small percentage of the offspring do.

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resulting from male OX5034 mosquito matings, which will provide monitoring to confirm no female OX5034 mosquitoes. Additional monitoring will also occur once releases have ceased to ensure that the OX5034 traits disappear from the male mosquitoes in the EUP locations as is expected.

B. Executive Summary of Human Health and Environmental Risk Assessment

In assessing the risk to human health and the environment from the limited releases of OX5034 mosquitoes in Monroe, Co., Florida and Harris, Co., Texas over two (2) years, several key factors played a significant role.

- Only male OX5034 mosquitoes will be released into the environment. Because male mosquitoes do not feed on humans (they do not bite), they do not pose a human health risk.
- Female mosquitoes feed on human blood, but only once they become adults.
- Oxitec's OX5034 female mosquitoes do not survive to become adults without tetracycline. Tetracycline acts as an antidote to the OX5034 female mosquito-lethal trait.
- EPA evaluated penetrance of the OX5034 female-lethal trait.
  - Penetrance for the OX5034 mosquitoes refers to the proportion of female insects that die before reaching adulthood, i.e. does it consistently work. EPA found that it does.
- EPA evaluated human health risk of OX5034 mosquitoes.
  - A determination of the toxicity and allergenicity of the two substances in Oxitec's OX5034 mosquitoes that 1) kill female mosquitoes, tTAV-OX5034, and 2) allow trained personnel to identify OX5034 via fluorescence, DsRed2-OX5034, has not been made.
  - However, because no OX5034 female mosquitoes are being released or are expected to emerge in the environment, exposure is negligible and therefore, so is the potential risk from tTAV-OX5034 and DsRed2-OX5034 (Risk = Exposure x Hazard).
- EPA evaluated introgression risk.
  - Introgression for the OX5034 mosquitoes refers to the movement of background traits from the non-GE portion of the OX5034 mosquito genome to local mosquitoes, i.e. will releases of OX5034 mosquitoes increase the ability of wild mosquitoes in the release area to vector/transmit disease, result in larger populations numbers, or result in more robust mosquitoes. EPA found this impact is unlikely. As part of this analysis, EPA collaborated with the United States Centers for Disease Control and Prevention (CDC) in reviewing laboratory data, a meta-analysis, and rationale submitted by the applicant comparing the vectorial capacity of OX5034 mosquitoes to that of wild mosquitoes.
- EPA evaluated the risk of OX5034 mosquitoes to non-target organisms (bats, amphibians, etc.).
  - No direct adverse effects due to consumption of OX5034 males by non-target organisms is expected based on acute oral toxicity studies and bioinformatics analyses.
  - *Ae. aegypti* mosquitoes (of which OX5034 mosquitoes are) are not a sole or critical food source for non-target organisms, so no indirect adverse effects are expected should there be a decrease in the local mosquito population.

Based on the above factors and analyses discussed in EPA's science assessment (Unit II), EPA determined that there will be no unreasonable adverse effects to humans or the environment as a result of

the experimental permit to release Oxitec's OX5034 male mosquito. Below are EPA's risk conclusions for the human health and environmental risk assessment, which can also be found in Unit III, "Human Health & Environmental Risk Conclusions:"

EPA has reviewed the OX5034 manufacturing process detailing the production and quality assurance processes used in the development and manufacture of OX5034 mosquitoes, associated standard operating procedures, and other pertinent information characterizing OX5034 mosquitoes on a genetic and phenotypic level. EPA determined this information to be adequate to support a finding of no unreasonable adverse effects to man and the environment during the proposed EUP.

EPA has determined that there will be no unreasonable adverse effects for humans as a result of the experimental permit to release *Ae. aegypti* OX5034 male mosquitoes provided such releases do not take place within 500 m of commercial citrus growing areas or wastewater treatment sites due to considerations regarding the impact of environmental sources of tetracyclines on female OX5034 mosquito survival. A compilation of release recapture studies around the world found that most *Ae. aegypti* are recovered within 20 m to 50 m of the release point, with a small percentage found 170 m but generally not more than 200 m from the release point. Therefore, a restriction of 500 m from potential sources (200 m for released OX5034 males + 200 m for mated *Ae. aegypti* females + 100 m of additional buffer) provides a conservative buffer zone. The human health assessment considered data provided on the mammalian toxicity and allergenicity of the tTAV-OX5034 (active ingredient) and DsRed2-OX5034 (inert ingredient) proteins and the potential routes through which humans may be exposed to these substances as a result of OX5034 application. While no determination has been made on the potential of either protein to pose mammalian hazard, the human health risk was found to be negligible, as exposure to female mosquitoes carrying these traits was determined to be negligible given that the penetrance of the tTAV-OX5034 lethal trait was shown to be 100% in female mosquitoes and the restrictions on access to potential tetracycline sources.

EPA has determined that there will be no unreasonable adverse effects for humans or the environment due to introgression of OX5034 background strain genetics into the local *Ae. aegypti* population. EPA evaluated OX5034 mosquitoes for key traits that could increase the ability of mosquitoes to transmit disease, result in larger populations numbers, or result in more robust mosquitoes. Based on a combination of laboratory data, meta-analyses, and a review of the scientific literature, EPA finds it is unlikely that the local mosquito population would pose any increased risk to humans or the environment as a result of releases of OX5034 mosquitoes and introgression of OX5034 background strain genetics.

EPA has also determined that no unreasonable adverse effects are anticipated for non-target organisms as a result of the experimental permit to release *Ae. aegypti* OX5034 male mosquitoes. No direct adverse effects due to consumption of OX5034 males by non-target organisms is expected based on acute oral toxicity studies and bioinformatics analyses. There are also no indirect adverse effects anticipated from reduction in *Ae. aegypti* as a food source should the release of OX5034 mosquitoes successfully reduce the local *Ae. aegypti* population. In the case of *Ae. aegypti*, their status as invasive species and their oviposition choice behavior makes it less likely that they serve an integral role in newly invaded ecosystems. Additionally, *Ae. aegypti* are regularly subjected to other control methods such as insecticide

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treatment and source reduction and it is therefore unlikely any predator species or plant is dependent on *Ae. aegypti* presence.

II. SCIENCE ASSESSMENT

A. Product Characterization

The manufacturing process and the characteristics of the resulting pesticide product provide the foundation for assessing its risk for human health and the environment. This section describes the OX5034 *Ae. aegypti* strain, how the strain was developed, the purpose of the genetic elements integrated into the mosquito genome, and the mechanisms underlying the female-specific lethality. The applicant submitted data that supported a finding of no unreasonable adverse effects to humans and the environment during the proposed EUP. A summary of these data is provided in Table 1 and within the risk assessment below. Full review of the information is contained within the Data Evaluation Records (DERs). Some of the references cited in this assessment were included in the studies provided by the applicant within the MRIDs cited. Other references were included from the open literature that pertained to specific topics discussed below.

Table 1. Classification of the data submitted for the product characterization and manufacturing process of OX5034 *Ae. aegypti*.

Study type/ Title	OPPTS Guideline No.	Results Summary and Classification	MRID No.
Manufacturing process	880.1200	The information contained in this MRID describes the production and quality assurance processes used in the development and manufacture of the OX5034 *Ae. aegypti* mosquitoes. The plasmid map detailing the genetic elements, the procedures for microinjection, and the identification of transformants are appropriately detailed as part of the production of the end-use product, the OX5034 male mosquitoes. A thorough screening process with selective criteria and the demonstration that no females are produced (in the absence of tetracyclines) regardless of zygosity is critical and described in detail. The procedures and quality assurance protocols described in this MRID are acceptable for these purposes. **Classification: Acceptable.**	50889424
Supplemental information to OX5034 description of starting materials, production and formulation process	880.1200	Oxitec has adequately clarified the production of eggs and adult OX5034 male mosquitoes, as well as construction of rearing boxes for mosquito release at their UK, Florida and Texas facilities. Key to this analysis is the indication that there will be no egg production or blood feeding of adult female mosquitoes or use of tetracycline-class antibiotics taking place at the Florida or Texas facilities. Eggs will be shipped from the UK facility for deployment in the US Further, the description of the dedicated containment suite at the UK facility, as used for production of OX5034 eggs, and the restriction of personnel who have visited other production suites from entering this dedicated insectary address production aspects of interest to the Agency. **Classification: Acceptable.**	51130901

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Standard Operating Procedures for Production of *Aedes aegypti* OX5034	880.1200	The information contained in this MRID details the protocols for production and quality assurance of male mosquitoes for release to the environment. Descriptions of egg, larval, pupal and adult mosquito production included are scientifically sound and the attention to quality assurance / quality control, certification of technical support staff, and record keeping are scientifically sound and based upon accepted methodologies. A description of sexing protocols and enumeration of larvae, pupae and adults are discussed. The study is acceptable for use in production of OX5034. **Classification: Acceptable.**	50889427
OX5034 *Aedes aegypti*: Product Identity and Composition, Discussion of the Formation of Impurities, Preliminary Analysis, Certified Limits, and Enforcement Analytical Method	880.1100 880.1400 830.1700 830.1750 830.1800	The information contained in this MRID characterizes the genetic construct used to create OX5034 *Ae. aegypti*, the active and inert ingredients, the female-specific lethality of the active ingredient, as well as other relevant characteristics of the end-use product. For example, information was provided on the enforcement analytical methods and a discussion of the likelihood for resistance development. The MRID was overall acceptable to characterize the OX5034 *Ae. aegypti* end-use product. **Classification: Acceptable.**	50889401 51115102
Quantitative Western Blot Analysis of Expressed Proteins tTAV-OX5034 and DsRed2-OX5034 in OX5034 *Aedes aegypti* male adults, pupae and larvae	N/A	The data presented in this study allowed for a conservative estimation of the DsRed2-OX5034 protein abundance in homozygous OX5034 males at all life stages. The data showed that a tTAV protein variant is present in older homozygous OX5034 males but were not adequate to determine total tTAV-OX5034 protein abundance. The supplemental classification is a result of several methodological deficiencies identified in the immunoblot assays. **Classification: Supplemental.**	50889419 51094401
Amended Quantitative Western Blot Analysis of Expressed Proteins tTAV-OX5034 and DsRed2-OX5034 in OX5034 *Ae. aegypti* male Adults, Pupae and Larvae	N/A	Review of the original study that quantified the tTAV-OX5034 and DsRed2-OX5034 proteins in OX5034 determined the tTAV-OX5034 protein abundance in adult male OX5034 was potentially underestimated (508894-19). The recalculation of the "combined" tTAV-OX5034 protein amount in adult OX5034 males, presented in MRID 511151-01, was adequate for use in this EUP, as this value likely overestimates tTAV-OX5034 abundance in these individuals. **Classification: Acceptable.**	51115101
Evaluation of Insecticide Resistance in OX5034	N/A	The data and information presented in the four MRIDs were acceptable to address the insecticide susceptibility of the OX5034 *Ae. aegypti* strain. OX5034 was susceptible to the pesticide active ingredients temephos (larvicide), permethrin, deltamethrin, and malathion (three adulticides), and the strain does not carry pyrethroid resistance-associated *kdr* mutations. It is expected that these four insecticides are effective for controlling OX5034 *Ae. aegypti* in the field. While the strain showed some resistance to Propoxur, this chemical is not approved for uses on mosquitoes in the US and thus any resistance	50698717 50698718 50889418 50973405

		associated with Propoxur will not affect current mosquito control practices. **Classification: Acceptable.**	
Arbovirus testing	N/A	For the EUP the colony will be maintained at Oxitec's insectary in Milton Park, UK and OX5034 male eggs will be shipped to rearing facilities in the US for deployment to release sites. The Agency concludes that because neither *Ae. aegypti* nor the arboviruses for which it is a vector are present in the UK, the risk of arbovirus infection of the source colony is low. Therefore, arbovirus testing for the EUP is not required. The study was classified as supplemental because the rationale by the company for not requiring testing was provided based on the presence of arboviruses at the release sites, i.e., FL and TX. This rationale was found to be inadequate to determine the likelihood of arboviral infection of the source colony reared in the UK. It should be noted that because testing is not required for the EUP, the test kits cited in Table 1 were not reviewed in detail and a determination regarding the adequacy of the proposed testing protocol outline has not been made at this time. **Classification: Supplemental.**	51094403
The self-limiting phenotype, penetrance, longevity and egg clutch size of *Aedes aegypti*, OX5034	N/A	Hemizygous and homozygous OX5034 mosquitoes were evaluated for longevity and compared against LWT mosquitoes. Similar longevity between hemizygous OX5034 males and LWT males but reduced longevity in homozygous OX5034 males was found. For egg clutch analysis, crosses were performed between homozygous OX5034 and LWT mosquitoes. Egg clutches are smaller from an OX5034 mating compared to LWT, indicating a potential fitness cost of the OX5034 rDNA. **Classification: Acceptable.**	50889417

1. Transformation system and genetic elements

The OX5034 *Ae. aegypti* line was developed in 2013 by transformation of a "Latin American *Ae. aegypti* wild-type" strain (LWT) with the vector pOX5034. The strain was subsequently backcrossed several times to obtain the OX5034 homozygous *Ae. aegypti* for which the EUP is sought. The background of the LWT strain is comprised of genetics from ten separate *Ae. aegypti* colonies. These colonies were established from mosquitoes that were collected in the Mexican State of Chiapas in 2006 (Wise de Valdez et al. 2011).

The OX5034 expression cassette contains several genetic elements in addition to the sequences coding for the active ingredient tetracycline-repressible transactivator protein variant (tTAV-OX5034) and the inert ingredient DsRed2-OX5034. Briefly, basal expression of *tTAV-OX5034* is driven by the DmHsp70 minimal promoter and 5' UTR from *Drosophila melanogaster* located downstream of a tetracycline-responsive operator ($tetO_7$). This basal expression of the protein is required for the initiation of the positive feedback loop that is characteristic of the Tet-OFF system (Unit II.A.2). Dimeric tTAV (cleaved variant of tTAV-OX5034) binds to the $tetO_7$ operator and enhances expression of *tTAV-OX5034* in the absence of tetracyclines. tTAV-OX5034 is a chimeric fusion protein that consists of three basic functional units: at the N-terminus, a modified version of the *Ae. aegypti doublesex* gene (*Aeadsx*) (Salvemini et al.

2011), followed by ubiquitin (UBQ; *D. melanogaster*), and tTAV. tTAV itself is a fusion of the tetracycline-binding domain protein (tetR) and the viral tegument protein 16 (VP16; transcriptional activator) from the herpes simplex virus-1 (HSV-1) (Gossen and Bujard 1992).

DsRed2-OX5034 is virtually identical to DsRed2 (Yanushevich et al. 2002), but in addition contains a bipartite nuclear localization sequence (NLS) and linker sequences at its N-terminus and C-terminus. The expression of the *DsRed2-OX5034* gene is driven by the IE1 promoter and Hr5 enhancer, both of which are derived from *Autographa californica* nucleopolyhedrovirus (AcNPV). DsRed2-OX5034 is the fluorescent marker that allows for visual identification of *Ae. aegypti* individuals carrying the OX5034 genetic cassette in larvae collected from the field. Expression from these regulatory elements is expected to be constitutive, although overexpression of other variants of the tetracycline-repressible transactivator protein (tTA) in insects has also been observed to positively affect the expression of fluorescence markers located on the same genetic construct. For example, in the transgenic *Ae. aegypti* strain LA513, which expresses different variants of the *tTAV-OX5034* and *DsRed2-OX5034* genes, tTAV presence is reported to positively affect *DsRed2* expression from a *D. melanogaster* Actin 5C promoter (Alphey 2015). Similarly, positive correlation between *tTA* expression and expression of the fluorescent marker ZsGreen was observed in a transgenic strain of *D. melanogaster* (Knudsen et al. 2020).

Characterization of the genomic DNA isolated from OX5034 homozygous *Ae. aegypti*, using restriction enzyme digestion and Southern blot analysis, indicate that the genetic cassette from plasmid pOX5034 inserted as a single, intact copy into the mosquito genome (MRID 50889401). The genomic locus of insertion was identified by sequencing the genomic region flanking the insertion site at the 5' end and comparison of that sequence with the *Ae. aegypti* AaegL3 genome assembly (2014) deposited in VectorBase (Giraldo-Calderon et al. 2015). Based on the data provided, it is unlikely that the inserted OX5034 gene cassette disrupts any open reading frames within the genome of OX5034 *Ae. aegypti*. To obtain a homozygous strain, the originally transformed strain was backcrossed several times. In doing so, any pOX5034 vector backbone that may have inserted into the genome at a location other than the expression cassette, would be segregated out. PCR analysis of the OX5034 genome using backbone-specific primers demonstrated the absence of backbone DNA in the OX5034 genome. The observation that the DsRed2-OX5034 fluorescence phenotype segregates in a Mendelian pattern of inheritance indicates the stability and inheritance of the inserted DNA across several generations.

Information on the genetic stability of the OX5034 traits was provided, which supports the conclusion that integration of the genetic cassette is stable. The OX5034 expression cassette was inserted into the *Ae. aegypti* genome using a piggyBac vector derived transposon system (MRID 50889424). The specific system used in the creation of OX5034 is comprised of two components that were co-transformed into *Ae. aegypti* embryos via microinjection: a vector carrying the genetic cassette (pOX5034) and mRNA encoding for the transposase enzyme for excision of the cassette from the vector and subsequent integration into the mosquito genome. Briefly, the genetic cassette that codes for *tTAV-OX5034* and *DsRed2-OX5034* and its regulatory elements was cloned between two inverted terminal repeat sequences (ITRs), which were originally derived from a transposon of the cabbage looper moth *Trichoplusia ni* (Tamura et al. 2000, Handler 2002, Kuwayama et al. 2006, Labbe et al. 2010). The transposase recognizes the ITRs and integrates the intervening DNA into the genome, preferentially at TTAA sites, although non-canonical integration has also been observed (Sethuraman et al. 2007). The piggyBac transposon system generally integrates intact genetic cassettes. Re-excision from the genome is not expected to occur as the transposase is only transiently expressed in transformed cells and the integrated

cassette does not itself encode for a transposase enzyme. Other transgenic *Ae. aegypti* created using piggyBac-derived transposon systems have been reported to be genetically stable, possibly due to a low proportion of transposon-specific piRNAs in this mosquito species (Sethuraman et al. 2007). Relatedly, remobilization of the OX5034 expression cassette in OX5034 *Ae. aegypti* has not been observed in over 27 generation equivalents.

2. Pesticidal activity of the active ingredient tTAV-OX5034

OX5034 female lethality is attributed to the overexpression of the tTAV-OX5034 protein in immature females, a process that is thought to interfere with the transcriptional machinery of the insect and consequently normal cellular function. As a result, homo- and hemizygous females carrying the OX5034 genetic cassette survive only until the early larval stages (L2/L3). The tTAV-associated effect is commonly referred to as transcriptional squelching (Gill and Ptashne 1988). One study in *D. melanogaster* suggested that the overexpression of tTAV leads to the stochastic differential expression of genes in the transgenic organism in a manner that is specific to the site of transgene integration (Bryk et al. 2017). The latter may therefore also contribute to the lethal effect.

Overexpression of *tTAV-OX5034* is achieved through a gene circuit that is based on the "Tet-OFF" system that was first described in *E. coli* (Gossen and Bujard 1992). Here, basal *tTAV-OX5034* expression is enhanced through a positive feedback loop that can be suppressed through the addition of tetracyclines to the larval diet. Briefly, basal expression of *tTAV-OX5034* is driven by the DmHsp70 minimal promoter, which is required to initiate the positive feedback loop. Alternate splicing patterns in developing males and females lead to preferential expression of the full-length *tTAV-OX5034* mRNA in females. Once translated, tTAV-OX5034 is cleaved to release tTAV, which dimerizes and, in the absence of tetracyclines, binds to $tetO_7$, enhancing the expression of *tTAV-OX5034* (Fu et al. 2007) (Figure 1). Because the tTAV homodimer preferentially binds to tetracycline over $tetO_7$, the addition of tetracycline to the system quenches *tTAV-OX5034* expression. Thus, in order to be able to sustain an OX5034 homozygous *Ae. aegypti* colony in the laboratory, tetracyclines must be added to the rearing medium, as no intervention would lead to the death of OX5034 females.

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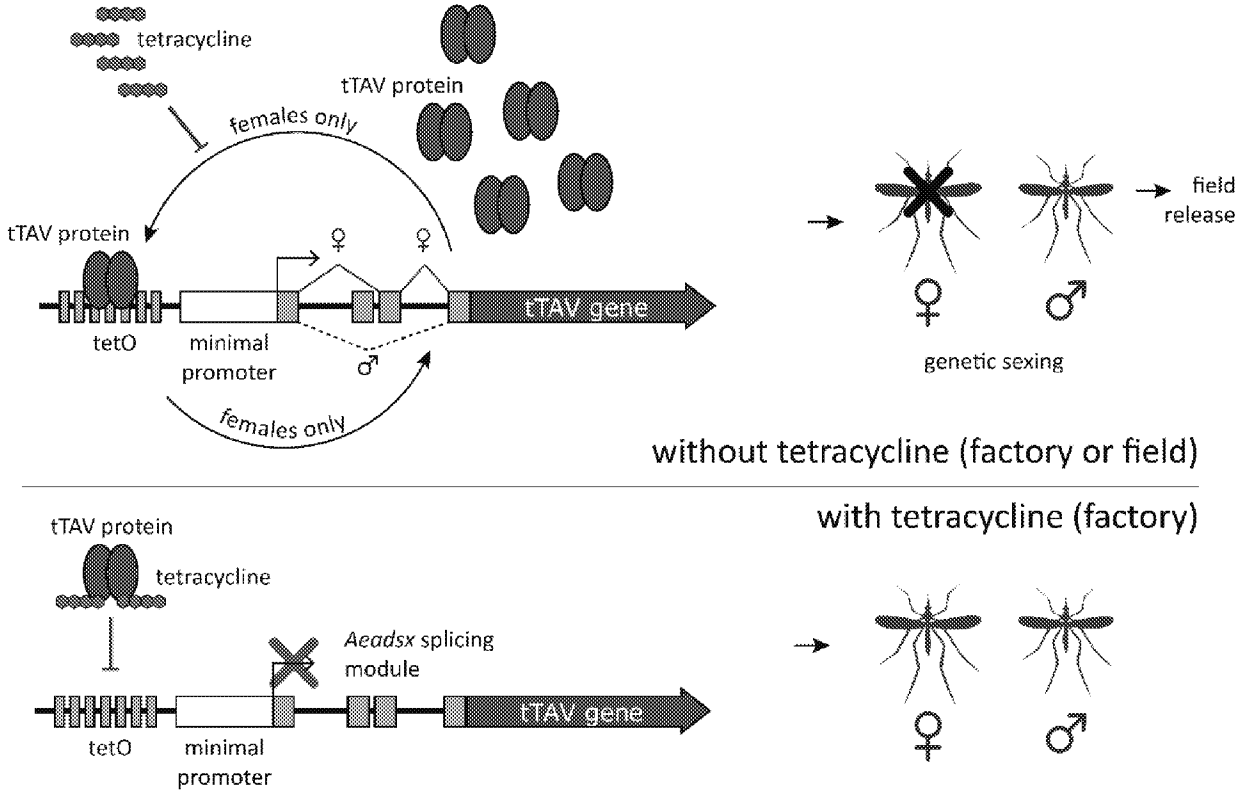

Figure 1. Schematic representation of the OX5034 female-lethal trait mediated through sex-specific *tTAV-OX5034* expression. Top: In the absence of tetracyclines, basal expression of *tTAV-OX5034* in female OX5034 *Ae. aegypti* results in the production of the tTAV-OX5034 protein. The *Aeadsx* splicing module located at the 5'-end of the *tTAV-OX5034* gene is alternately spliced in males and females, leading to the preferential expression of the full-length *tTAV-OX5034* mRNA isoform in females. Once the protein is translated it is cleaved by endogenous deubiquitinases at the UBQ-tTAV junction, releasing tTAV. tTAV then dimerizes (purple ovals). The positive feedback loop is closed when the VP16-domains of the tTAV protein bind to the $tetO_7$ operator, which enhances the expression of the *tTAV-OX5034* gene. Through this mechanism of overexpression, cellular functions are affected resulting in cell death in the developing larvae. Bottom: In the absence of tetracyclines, basal expression of *tTAV-OX5034* still occurs. Because tTAV preferentially binds to tetracycline than to $tetO_7$, expression is not enhanced.

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3. Conditional female-specific expression of *tTAV-OX5034*

The genetic construct integrated into the *Ae. aegypti* genome was designed such that the active ingredient is primarily expressed in female mosquitoes. Briefly, female-specific lethality of tTAV-OX5304 is achieved by inclusion of a splicing module upstream of the genetic sequence coding for *tTAV*. The splicing module is derived from the *Ae. aegypti Aeadsx* (*doublesex*) gene, which is differentially spliced in males and females as part of the sexual differentiation pathway (Salvemini et al. 2011). The genetic sequence of exon 5b of *Aeadsx* in OX5034 was minimally altered from exon 5b present in the native gene. OX5034 females produce two mRNA isoforms of *tTAV-OX5034*, F1 and F2, and males primarily produce a single M isoform (Figure 2). Both the F1 and M isoforms contain a premature stop codon and are unlikely to be translated. The F2 isoform produced in OX5034 females is translated into tTAV-OX5034 and thus, the pesticidal mode of action is female-specific.

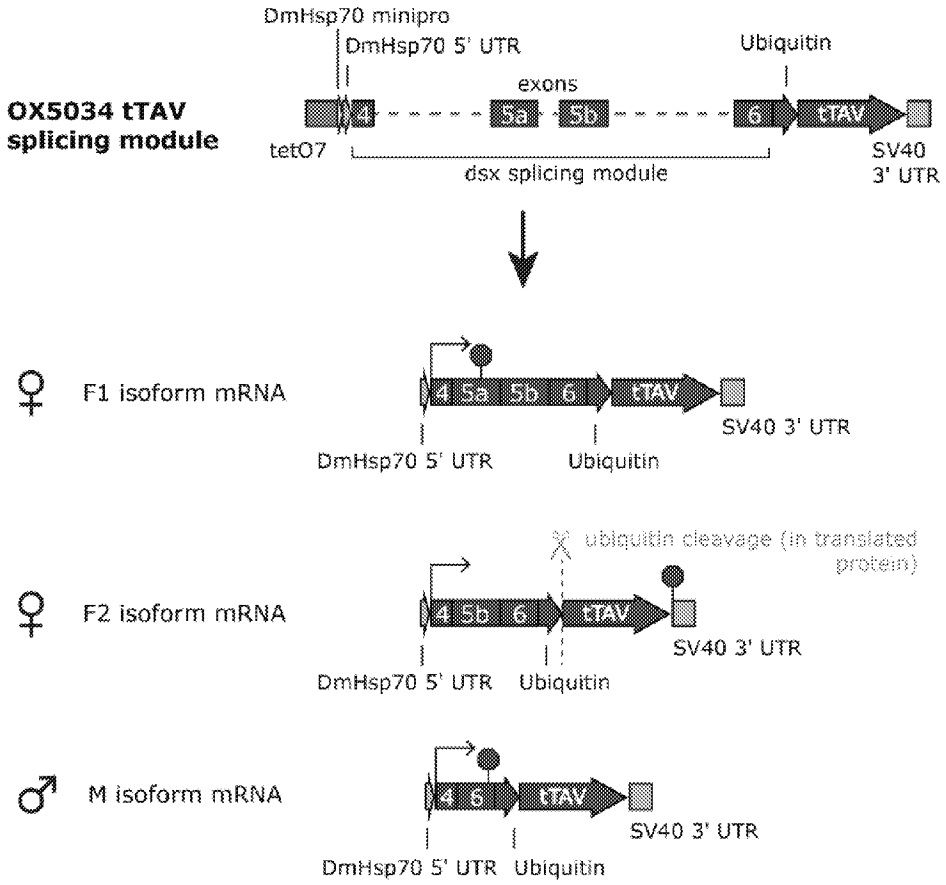

Figure 2. Schematic representation of the splicing module in *tTAV-OX5034*. The *Aeadsx* splice module consists of exons 4, 5a, 5b, and 6, together with the fragments of *Aeadsx* introns 4, 5, and 6. The arrow indicates the position of the start codon and the red octagon indicates that of a stop codon.

Oxitec provided data that demonstrate that the minimally altered *Aeadsx* splice module in *tTAV-OX5034* results in the differential expression of the mRNA in male and female pupae and that the F2 isoform that codes for the active ingredient is primarily expressed in female mosquitoes. To allow for female development to the pupal life stage all individuals were reared in the presence of tetracyclines. Basal expression of *tTAV-OX5034* is expected to occur under these conditions (Unit II.A.1). Using *tTAV-OX5034*-specific primers, female OX5034 pupae were shown to express both female mRNA isoforms, F1

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and F2, with F2 being the most abundant. OX5034 females do not produce the male isoform M, or this isoform was present below the assay's limit of detection. Similar results were obtained in the LWT background when analyzing the isoforms of the native *Aeadsx* transcript.

Male OX5034 pupae were shown to express the M isoform as well as a small amount of the F2 isoform. The study remarks that this may be the result of ineffective splicing that occurs at low levels (Unit II.A.4; MRID 50889419). While mRNA abundance was not specifically evaluated in adult males, the F2 isoform is likely also transcribed in these individuals as the tTAV-OX5034 protein is detected in adult males at 1- and 6-days post eclosion (Unit II.A.4.b). If the F2 isoform of the native *Aeadsx* gene is expressed in male LWT pupae, abundance is below the detection limit of this assay.

4. Protein abundance

The abundance of the tTAV-OX5034 and DsRed2-OX5034 proteins was determined in OX5034 male mosquitoes at several life stages. OX5034 males were reared in the absence of tetracyclines, which is also how they will be reared in preparation for environmental releases (Unit II.A.5). The protein content in homozygous adult males is the most relevant for determining the amount of pesticide applied during the experimental program as these individuals will be released into the environment; while eggs will also be deployed, they are contained in the mosquito rearing boxes from which adult homozygous males will emerge.

As presented in Unit II.A.3, OX5034 male pupae, like OX5034 females, produce the F2 mRNA isoform of *tTAV-OX5034* and thus it is conceivable that adult males also produce the tTAV-OX5034 protein. Once translated, tTAV-OX5034 is thought to be cleaved at the UBQ-tTAV junction to release the tTAV protein which, once dimerized, will initiate the positive feedback loop characteristic of the Tet-OFF gene circuit (Figure 1) (Gossen and Bujard 1992, Fu et al. 2007). Consequently, three variations of the tTAV-OX5034 protein may be present within mosquitoes: tTAV-OX5034 (full-length protein), monomeric tTAV (cleaved), and dimerized tTAV (the transcriptionally active form of the protein). In addition to tTAV-OX5034, homozygous males were expected to produce DsRed2-OX5034 protein, as *DsRed2-OX5034* is under the control of a constitutive promoter. The presence of DsRed2-OX5034 is furthermore evident in hemizygous *Ae. aegypti* male (and female) larvae where the protein can be visualized through its fluorescent properties (Units II.A.1 and II.A.7).

a. DsRed2-OX5034

DsRed2-OX5034 protein abundance was determined in the lysate of OX5034 homo- and hemizygous males at the larval, pupal, and two adult life stages (Table 2). Mosquitoes were reared in the absence of tetracyclines. DsRed2-OX5034 protein abundance was quantified using immunoblot analysis with polyclonal primary antibody for DsRed2 (TaKaRa; Clontech) in mosquito lysate of a volume equivalent to that of a single individual. Total protein abundance in the mosquito lysates was not determined.

The abundance of endogenously produced DsRed2-OX5034 was determined by simultaneous probing of known quantities of a recombinant DsRed2 protein standard, followed by densitometric analysis of the protein bands and normalization of these bands to a housekeeping protein.

The highest protein abundance was detected in adult males at 6-days post eclosion. This relative increase in DsRed2-OX5034 compared to immature life stages may be the result of protein accumulation and/ or an increase in *DsRed2-OX5034* expression in later life stages (Unit II.A.1). It is relevant to note that while the DsRed2-OX5034 protein was below the limit of detection of this immunoblot assay in hemizygous larvae and pupae, fluorescence analysis of larvae demonstrates that DsRed2-OX5034 is present in larvae carrying the OX5034 construct (Unit II.A.7). As a general remark, the protein values in Table 2 may represent the sum of DsRed2-OX5034 and one or more endogenous protein(s) and thus may overestimate the DsRed2-OX5034 abundance. This is because the antibody used for the detection of DsRed2-OX5034 also recognized one or more endogenous proteins of similar molecular weight in the non-transgenic LWT strain.

Table 2. Mean expression levels of DsRed2-OX5034 in various life stages of male OX5034 *Ae. aegypti*. ND = not detectable, below the limit of detection (LoD).

OX5034 life stage	Assay LoD	Zygosity	DsRed2-OX5034 ng/ mosquito (volume lysate)
L4 larvae	6.25 ng/ larva	Homozygous	7.7 ± 2.4 (ND - 16.7 ng range)
		Hemizygous	ND
Pupae	6.25 ng/ pupa	Homozygous	7.9 ± 0.4 (7.3 - 8.4 ng range)
		Hemizygous	ND
Adults 1-day post eclosion	7.5 ng/ adult	Homozygous	ND
Adults 6-day post eclosion			35.3 ± 6.5 (17.2 - 48.9 ng range)

b. tTAV-OX5034

tTAV-OX5034 protein abundance was determined in the lysate of homo- and hemizygous OX5034 males at the larval, pupal, and two adult life stages. Mosquitoes were reared in the absence of tetracyclines, which means that protein abundance in these individuals is expected to be representative of those levels found in OX5034 males in the environment. A VP16 primary antibody was used to detect tTAV-OX5034 protein in these samples. The VP16 domain is part of the tTAV portion of tTAV-OX5034 and therefore the primary antibody is expected to recognize all three variations of the tTAV-OX5034 protein (full-length, cleaved, and dimerized).

A single band likely representing the monomeric tTAV variant was detected in lysate of homozygous adult OX5034 males at 1 and 6-days post eclosion. No tTAV-associated bands were identified in any of the immature life stages. The lysate of the adult life stages was simultaneously probed with a second primary antibody that recognized the housekeeping protein Hsp70 to normalize the band intensity of the target protein (tTAV-OX5034). However, because the molecular weight of the Hsp70 protein is indistinguishable from both the full-length tTAV-OX5034 protein and the dimeric tTAV, it is possible that the Hsp70 protein band obfuscated the presence of these variants in the OX5034 lysate. As a result, the sum of tTAV-OX5034 associated proteins may have been underestimated. To account for that possibility, a conservative approach was taken in which it was assumed that the Hsp70-associated protein band in OX5034 homozygous adult males solely consists of tTAV-OX5034 and dimeric tTAV. That

value was then added to the value determined for the monomeric tTAV to give the result presented in Table 3. It should be noted that a different housekeeping protein (GAPDH) was used for probing of immature life stages. GAPDH has a molecular weight that is clearly distinguishable from all three tTAV-OX5034 protein variations.

Table 3. Mean expression levels of tTAV-OX5034 in various life stages of male OX5034 *Ae. aegypti*. ND = not detectable, below the limit of detection (LoD).

OX5034 life stage		**Zygosity**	**tTAV-OX5034 ng/ mosquito (volume lysate)**
L4 larvae	3.13 ng/ larva	Homozygous	ND
		Hemizygous	ND
Pupae	0.78 ng/ pupa	Homozygous	ND
		Hemizygous	ND
Adults 1-day post eclosion	0.39 ng/ adult	Homozygous	3.3 ± 0.3 ng (2.8 - 4.1 ng range)
Adults 6-day post eclosion			2.2 ± 0.2 ng (1.8 - 2.7 ng range)

c. Protein susceptibility to environmental proteases

tTAV-OX5034 and DsRed2-OX5034 are proteins and as such are susceptible to the biotic processes of degradation in the environment through microbial activity. To further characterize protein behavior and environmental fate to gain insight into how long they may persist in the environment, susceptibility of tTAV-OX5034 and DsRed2-OX5034 to two environmental proteases was assessed. Two appropriate microbial proteases were chosen for this assessment: the serine proteases proteinase K and subtilisin A. Proteinase K was originally identified in the fungus *Tritirachium album* and subtilisins are produced by several *Bacillus* species (Jacobs et al. 1985, Couto et al. 1993, Rao et al. 1998, Hu and Leger 2004). Bioinformatic programs were used to predict the presence of proteolytic cleavage sites in the tTAV-OX5034 and DsRed2-OX5034 protein sequences. Secondly, an *in vitro* time-course assay was conducted on recombinant protein, using HN-tagged tTAV (as expressed in OX513A, which is Oxitec's 1st Generation mosquito; EPA File Symbol 93167-EUP-R) and DsRed2 (Clontech) as proxies for the two OX5034-expressed proteins.

In silico analyses were computed using the Expert Protein Analysis System (ExPASy; Swiss Institute of Bioinformatics) PeptideCutter tool (Gasteiger et al. 2003, Gasteiger et al. 2005). The protein sequences of the full-length tTAV-OX5034 protein, the monomeric form tTAV, and DsRed2-OX5034 demonstrate that these proteins contain several predicted cleavage sites for proteinase K, indicating that they would be susceptible to its proteolytic activity. PeptideCutter does not include the option to predict the presence of subtilisin A cleavage sites and no such information was provided for tTAV-OX5034 and DsRed2-OX5034. The *in vitro* assays confirmed the *in silico* predicted susceptibility of tTAV (monomeric) to proteinase K, which was shown to be degraded at 37 °C within minutes. The remaining *in vitro* studies that investigated the susceptibility of tTAV-OX5034 and DsRed2-OX5034 to environmental proteases were inconclusive, for various reasons, including potential autolysis of proteinase K and subtilisin A. In addition, the lack of protein quantification hindered the determination of kinetics of these reactions.

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There is no indication from the bioinformatics analyses, the *in vitro* data presented on tTAV, and general knowledge of proteins to suggest that tTAV-OX5034 and DsRed2-OX5034 would not be susceptible to degradation in the environment, especially given that microbially produced proteases are not limited to proteinase K and subtilisin A in the environment.

5. Rearing and shipping of OX5034

The applicant provided a description of the manufacturing process and associated standard operating procedures for rearing and shipment of OX5034, which were found to be acceptable to support the EUP (Table 1). Briefly, OX5034 homozygous egg production will take place in the United Kingdom (UK). OX5034 eggs are then shipped to the US where they are prepared for field releases: OX5034 eggs are repackaged for egg deployment in mosquito rearing boxes and some eggs are hatched for packaging of adult males for adult releases. No OX5034 egg production will take place in the US, which means that there will be no maintenance of the OX5034 homozygous colony involving blood feeding or use of tetracyclines.

The OX5034 colony is maintained in Abingdon, UK at Oxitec's insectary, which adheres to biological containment level 2 standards and is licensed by the UK Health and Safety Executive for holding genetically modified organisms in contained use. The OX5034 production area is in a separate containment suite from other *Aedes* spp. strains. The containment suite has a separate changing room and the insectaries are equipped with meshed air conditioning vents and floor drains. OX5034 are kept in at least three levels of containment from non-containment and other containment areas, i.e., the primary container, the insectary, and the changing room. Equipment used in the insectary is dedicated or is decontaminated by freezing for more than 12 hours at $\leq$ -15 °C before being brought into the containment suite. Staff working on OX5034 production are dedicated, which means that staff members who have entered other *Aedes* spp. rearing areas on the same day are not permitted to access the OX5034 production suite. To maintain the OX5034 colony, females are reared in this facility to produce eggs. This process requires the use of tetracyclines in the larval diet to allow female OX5034 survival to adulthood and blood feeding of females to enable them to lay eggs. Adult female mosquitoes are fed with defibrinated horse blood in a heated membrane feeding system. The blood is sourced from a closed British herd. The herd is under veterinary care, tested for certain equine viruses before admission to the farm, and each blood batch is tested by the supplier for bacterial sterility. From the facility in the UK, OX5034 eggs will be shipped to two location in the US for field release preparation, one insectary in Texas and one in Florida. Both facilities adhere to biological containment level 2 standards and are located in the counties in which field releases will take place (Section G experimental program review, available on www.regulations.gov in docket EPA-HQ-OPP-2019-0274). No egg production will take place in the US facilities, and no other third-party mosquito strains will be reared in Oxitec's field trial laboratories.

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6. Characterization of OX5034

a. Insecticide susceptibility

The OX5034 strain was tested for its susceptibility to several insecticides including the larvicide temephos (organophosphate) and the four adulticides permethrin (pyrethroid), deltamethrin (pyrethroid), malathion (organophosphate), and propoxur (carbamate). The insecticide susceptibility studies presented in MRIDs 50698718 and 50973405 are based on the standardized WHO threshold assays for the detection of insecticide resistance in *Ae. aegypti* (temephos) and anopheline mosquitoes (four adulticides) at a single discriminating dose that is specific to each pesticide active ingredient (WHO 2016, 2018). These assays allow for the determination of potential resistance of a tested mosquito strain to the active ingredient through observation of mortality, but do not provide quantitative measures of resistance. Temephos susceptibility was furthermore investigated by determining the LC_{50} for OX5034 and related strains.

OX5034 was shown to be susceptible to permethrin (0.75%), deltamethrin (0.05%), and malathion (5%) at the respective discriminating doses. The WHO classifies mortality below 90% as confirmed resistance for these adulticides (WHO 2018). 100% mortality in OX5034 and LWT after 24 hours exposure was demonstrated following permethrin (OX5034 n = 94; LWT n = 100), deltamethrin (OX5034 n = 100; LWT n = 100), and malathion (OX5034 n = 91; LWT n = 100) challenge, showing weaker knockdown effects in OX5034 in all cases immediately after cessation of exposure compared to the LWT strain.

Susceptibility of the OX5034 strain to the larvicide temephos was determined after initial results indicated potential resistance of OX5034 and the LWT strains at the WHO recommended discriminating dose of 0.012 mg/l. Oxitec provided a study in which 100 larvae of the OX5034, LWT, OX513A (which was developed in the same LWT background), and a control strain with known temephos resistance (Cayman wild-type), were challenged at the discriminating dose. While the WHO does not recommend a specific resistance threshold for temephos, in comparison to the Cayman wild-type strain (51.7% mortality), all three Oxitec strains showed higher mortality: OX5034 (100% mortality), OX513A (100% mortality) and the LWT (96.9%) and in addition significantly lower LC_{50} values at the 95% confidence interval: Cayman strain LC_{50} = 0.0108 (0.0094 - 0.0122), LWT LC_{50} = 0.0055 (0.0047-0.0064), OX5034 LC_{50} = 0.0035 (0.0030-0.0047), and OX513A LC_{50} = 0.0031 (0.0029-0.0034). All three strains are therefore not expected to be resistant to temephos and to respond to temephos challenge in a comparable manner.

Genetic analysis of the OX5034 strain further supports the results of the insecticide challenge that indicate that the OX5034 strain is susceptible to pyrethroids. Pyrethroid resistance in *Ae. aegypti* is often correlated with the presence of two single nucleotide polymorphisms (SNPs) in the Voltage-gated sodium channel gene (*VGSC*) that lead to two single amino acid substitutions, V1016I and F1534C, respectively (Estep et al. 2018). These mutations manifest in the so-called knockdown resistance (*kdr*) phenotype by reducing binding of the pyrethroids to the VGSC protein (reviewed in Hemingway and Ranson, 2000). Multiplex PCR analysis of *VGSC* demonstrated the absence of these resistance-associated alleles in OX5034. In *Ae. aegypti*, only one additional polymorphism, V410L has been associated with pyrethroid resistance. However, the two polymorphisms appear to have strong linkage (Haddi et al. 2017, Estep et al. 2018).

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The information presented in the four MRIDs were acceptable to address the insecticide susceptibility of the OX5034 *Ae. aegypti* strain (Table 1). OX5034 was shown to be susceptible to the pesticide active ingredients temephos, permethrin, deltamethrin, and malathion and the strain does not carry pyrethroid resistance-associated *kdr* mutations. It is therefore expected that these four insecticides are effective for controlling OX5034 in the field. Regarding temephos, it is of note that EPA issued a cancellation order affecting all uses in 2011, while allowing for existing stocks to be exhausted (USEPA 2011). As a result, any residual use of temephos is expected to be limited in the US. While OX5034 showed possible resistance to propoxur at the discriminating dose (0.1% propoxur; 89% mortality, n = 101), this adulticide active ingredient has not been registered for use on mosquitoes in the US since the late 1980s, when EPA determined that several outdoor uses of this chemical, including uses on mosquitoes, were not supported by the data (USEPA 1997). Thus, any resistance associated with propoxur will not affect current mosquito control practices.

b. Laboratory colony and arboviruses

Ae. aegypti is a vector of arthropod-borne human illnesses. Arthropod-borne viruses (arboviruses) may be transmitted to humans through infected *Ae. aegypti* females during blood-feeding. In nature, these viruses are primarily maintained through biological transmission between a susceptible vertebrate host and the hematophagous arthropod (WHO 1985). The likelihood of introducing arboviruses into the environment through OX5034 field releases was assessed (MRID 51094403).

OX5034 was developed in 2013 and has since been maintained as a laboratory colony (Unit II.A.5). Introduction of arboviruses into the mosquito colony under these controlled conditions is unlikely to occur. One possible point of entry is through the dietary horse blood provided to females to support egg production. Even then, arboviral infection of the colony would only be conceivable if the blood contains arboviruses to which *Ae. aegypti* is susceptible and the viral titer is high enough to elicit female infection. The dietary horse blood used in the production of OX5034 is purchased from a single supplier in the UK (Unit II.A.5). Donor horses are kept in a closed herd that is under veterinary care. Before a horse is eligible to become a donor, it is tested for equine infectious anaemia and equine viral arteritis viruses and each blood batch is tested for bacterial sterility. These animal husbandry practices and quality control standards by the supplier do not eliminate but reduce the likelihood of introducing contaminated blood into the colony.

Ae. aegypti is commonly recognized as the principal vector of the yellow fever virus (YFV), dengue viruses (DENV-1, DENV-2, DENV-3, DENV-4), chikungunya virus (CHIKV), and Zika virus (ZIKV) (Souza-Neto et al. 2019). *Ae. aegypti* is therefore susceptible to infection by these arboviruses in nature. While some studies have demonstrated that *Ae. aegypti* can be experimentally infected with other arboviruses that are of human health significance, transmission from infected mosquitoes to other hosts, such as humans and wildlife, was found to be unlikely (Long et al. 2011, Muturi et al. 2011, Serra et al. 2016, Joubert and O'Neill 2017, Brustolin et al. 2018, Chapman et al. 2018, Wiggins et al. 2018, Pezzi et al. 2020). None of the four arboviruses for which *Ae. aegypti* is a major vector naturally occur in the UK. The most recent data in the European Centre for Disease Prevention and Control's Surveillance Atlas for Infectious Diseases record no locally acquired cases of DENV, YFV, CHIKV and ZIKV in the UK (ECDPC 2017). While some human infections were recorded, all were acquired while traveling abroad (Public Health England 2014, ECDPC 2017). Additionally, the UK has currently no known established

populations of *Ae. aegypti*, which, as the primary vector of these arboviruses, would support their natural transmission cycles and establishment in the UK. Since 2011, Public Health England runs a nationwide mosquito surveillance project for invasive mosquitoes, including surveillance for *Aedes* spp. at ports of entry (Vaux et al. 2019). These efforts have on occasion identified the presence of few *Ae. aegypti* individuals, but have not found any established populations of invasive *Aedes* spp. mosquitoes (Medlock et al. 2018). For example, in 2014, a single male *Ae. aegypti* was found in Merseyside, North West England, but follow-up surveys determined that there was no established, self-sustaining population (Dallimore et al. 2017). Two species of *Aedes* spp. are present in the UK, *Ae. cinereus* and *Ae. vexans* (Medlock and Vaux 2009). While *Ae. vexans* has been shown to be able to transmit some arboviruses in the environment, it is only sporadically found in the UK and was therefore discounted from eco-epidemiological considerations (Medlock et al. 2005). Importantly, because none the four arboviruses for which *Ae. aegypti* is the principle vector, nor *Ae. aegypti* itself, is found in the UK, their presence in the horse blood provided to the mosquito colony is unlikely.

In summary, the conditions under which OX5034 is reared are unlikely to result in the presence of arboviruses in the colony. This assessment is based in part on the rearing location and the source of the dietary blood provided to female mosquitoes. Consequently, if invasive *Aedes* spp. or arboviruses for which *Ae. aegypti* is the principal vector become established in the UK, or if the production colony were to be moved outside of the UK, arbovirus status of the colony would need to be reconsidered.

c. Fecundity

The OX5034 strain was evaluated for fecundity through an egg clutch analysis. Three experimental cages each contained 100 immature OX5034 homozygous males and 200 immature LWT females. One cage of 100 immature LWT males and 200 immature LWT females was set up as a control. The cages were left for 12 days to allow pupae to emerge as adults, mature, and mate. Females were then blood fed twice over a three-day period. Two days after the second blood feeding, an oviposition substrate was provided and was then removed two days later. Egg collection happened over four gonotrophic cycles and oviposition papers from the 2nd and 3rd gonotrophic cycles were photographed and analyzed using ImageJ software to estimate total egg number.

The study found that OX5034 homozygous males mated to LWT females produced smaller egg clutch sizes than LWT males mated to LWT females over the two gonotrophic cycles assessed. Specifically, the study found for OX5034 homozygous males mated to LWT females, the average number of eggs from the 2nd gonotrophic cycle was 30 ± 4.7 eggs and 40 ± 11.1 eggs from the 3rd gonotrophic cycle. This was compared to LWT males mated to LWT females, where the average number of eggs from the 2nd gonotrophic cycle was 53 eggs and 54 eggs from the 3rd gonotrophic cycle. The smaller egg clutch sizes from OX5034 matings may indicate a fitness cost to the OX5034 transgene.

As the LWT strain was used to produce the OX5034 strain, EPA also evaluated how results from both laboratory matings compared to reports on wild *Ae. aegypti* clutch sizes in order to determine how the strain's background genetics may impact fecundity. The below values are from a literature search provided by Oxitec:

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CDC National Center for Emerging and Zoonotic Infectious Diseases: 100 eggs/clutch
(https://www.cdc.gov/dengue/resources/factSheets/MosquitoLifecycleFINAL.pdf)

Cold Spring Harbor Protocol (Clemons et al. 2010): 100-150 eggs/clutch

Singapore National Environment Agency: 100 eggs/clutch
(https://www.nea.gov.sg/dengue-zika/prevent-aedes-mosquito-breeding/aedes-mosquito)

Metabolic relationship between female body size, reserves and fecundity of Aedes aegypti (Briegel 1990): 26-118 eggs/clutch

The values provided above by the applicant are also in line with a literature search conducted by EPA (Steinwascher 1984, Harrington et al. 2001, Goindin et al. 2015, Manorenjitha and Zairi 2015, Isoe et al. 2019). The clutch sizes of the LWT and OX5034 mosquito matings are within the range expected for *Ae. aegypti*.

d. Longevity

The longevity of OX5034 mosquitoes was also evaluated with and without a tetracycline analogue dietary antidote and the results were compared against LWT mosquitoes. OX5034 males (homozygous and hemizygous), OX5034 homozygous females, and LWT males and females were reared and mated for two days prior to being isolated for longevity analysis. All adults were provided with 10% sucrose solution *ad libitum* and females received two blood meals (day 7 and 17). To determine longevity, dead adults from all cages were removed daily and counted.

When reared with the dietary antidote, OX5034 homozygous females have shorter lifetimes than LWT females. Specifically, OX5034 homozygous female mosquitoes and LWT female mosquitoes were found to have median survivals of 42 days and 56 days, respectively. When reared without the dietary antidote, the study found similar longevity between OX5034 hemizygous males with median survival of 44 days and LWT males with median survival of 50 days. Conversely, the study found shorter lifetimes in OX5034 homozygous males with a median survival of 24 days compared to LWT males with a median survival of 49 days. The reduced lifespans of OX5034 homozygous males may indicate a fitness cost to the OX5034 transgene, but that cost is likely related to zygosity, as the OX5034 hemizygous males did not show reduced longevity.

As the LWT strain was used to produce the OX5034 strain, EPA also evaluated how results from both LWT and OX5034 analyses compared to reports on wild *Ae. aegypti* longevity in order to determine how the strain's background genetics may impact lifespan. A literature search indicated that median survival rates reported by the applicant are within the range reported in similar lab studies measuring *Ae. aegypti* longevity when provided with food sources:

Superior reproductive success on human blood without sugar is not limited to highly anthropophilic mosquito species (Braks et al. 2006):

57.18 ± 4.26 days, female, sugar + blood meal
38.09 ± 7.09 days, female, blood meal

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Parity and longevity of Aedes aegypti According to temperatures in controlled conditions and consequences on dengue transmission risks (Goindin et al. 2015):

9-51 days, female, sugar + blood meal, 24 °C
14-56 days, female, sugar + blood meal, 27 °C
19-40 days, female, sugar + blood meal, 30 °C

The adaptation of field collected Aedes aegypti (L.) and Aedes albopictus (Skuse) in laboratory conditions (Manorenjitha and Zairi 2015):

51.7 ± 1.22 days, male, sugar
51.7 ± 1.31 days, female, sugar
45.1 ± 1.25 days, female, sugar + blood meal

The longevity of the LWT strain mosquitoes and the OX5034 mosquitoes are within the range expected for *Ae. aegypti*.

7. Analytical detection methods

Information was provided that showed that homo- and hemizygous OX5034 *Ae. aegypti* larvae expressing the DsRed2-OX5034 fluorescent protein can be visually identified using the fluorescence screening protocol (MRIDs 50889401 and 50889427). The current enforcement analytical method has been demonstrated to be a reliable qualitative indicator of DsRed2-OX5034 presence, e.g., determination of OX5034 inheritance pattern in Unit IV.5.

B. Human Health Assessment

1. Toxicological profile

Oxitec conducted studies to support a conclusion of no unreasonable adverse effects for humans as a result of the experimental permit to release *Ae. aegypti* OX5034 male mosquitoes. A summary of the submitted data is provided in Table 4 and within the risk assessment below. The applicant also submitted scientific rationales to fulfill the acute toxicity data requirements for the proposed EUP. Full review of the rationales is contained within the DERs. Some of the references cited in this assessment were included in rationale provided by the applicant within the MRIDs cited. Other references were included from the open literature that pertained to specific topics discussed below.

Table 4. Summary of OX5034 Human Health Data.

Study type/ Title	OPPTS Guideline No.	Results Summary and Classification	MRID #
Acute oral toxicity	870.1100	This assessment was based on the information provided by the applicant as part of the scientific rationales, information on the product provided in other parts of the application, and information from the scientific literature. The information provided on the hazard assessment of tTAV-OX5034 and DsRed2-OX5034 is, on its own, inadequate to support the	50889402
Acute inhalation toxicity	870.1300		50889403
Acute eye irritation	870.2400		50889404
Primary dermal irritation	870.2500		50889405

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Acute dermal toxicity	870.1200	waiver requests. However, exposure to OX5034, tTAV-OX5034, DsRed2-OX5034 and the genetic material encoding them through the dermal, oral, pulmonary, and ocular routes of exposure is expected to be negligible. Therefore, overall the waiver requests for the acute oral toxicity, acute inhalation toxicity, acute dermal toxicity, primary eye irritation, and primary dermal irritation are acceptable. The statement on the occurrence of hypersensitivity incidents is acceptable. **Classification: Acceptable.**	51086001
Hypersensitivity	N/A		50889406
The self-limiting phenotype, penetrance, longevity and egg clutch size of *Aedes aegypti*, OX5034; Evaluation of field penetrance of OX5034 in open release field trials in Indaiatuba, São Paulo State, Brazil; Supplemental information in support of the study	N/A	For penetrance, both laboratory and field studies were performed. Laboratory crosses of OX5034 mosquitoes and wild-type mosquitoes found that no OX5034 females reached the adult stage, confirming 100% penetrance of the OX5034 phenotype. An additional study using field collected eggs from OX5034 releases in Brazil also found complete penetrance of the OX5034 phenotype. The field study indicates that the OX5034 phenotype still results in female lethality even when the OX5034 rDNA is placed in a different genetic background. Finally, the study confirmed the ability of fluorescent screening to determine presence/absence of the OX5034 rDNA by performing PCR-based genotyping. **Classification: Acceptable.**	50889417 50889423 50889428
Dose response of hemizygous *Aedes aegypti* OX5034 to tetracyclines and effects of environmental exposure to tetracyclines	N/A	The dose-response of OX5034 mosquitoes to a range of tetracycline analogues required to rescue female OX5034 mosquitoes was evaluated. A literature review of tetracycline levels resulting from industrial or household tetracycline usage found that the environmental concentrations of tetracycline analogues appear to be below the levels required to fully rescue adult females capable of maintaining flight. A local survey of environmental concentrations in the EUP treatment areas and investigation into tetracycline degradates would improve the study. **Classification: Supplemental.**	50889415
Bioinformatics analysis for risks of allergenicity and toxicity of tTAV-OX5034 and DsRed2-OX5034 (*in silico* and literature review)	N/A	The study provided bioinformatics analysis on the allergic and toxic potential of the tTAV-OX5034 and DsRed2-OX5034 proteins that were based on protein homology to known allergens and toxins and provided a literature review of the organisms from which these proteins (and their individual sequence components) were derived. The data presented in this study were determined to be supplemental to the overall evaluation of toxicity and allergenicity of the two proteins. No determination of the allergic or toxic potential for tTAV-OX5034 and DsRed2-OX5034 has been made at this time. **Classification: Supplemental.**	50889420
Protein digestibility and environmental degradation of OX5034 tTAV-OX5034 and DsRed2-	N/A	Two studies were presented in this MRID: A bioinformatics-based assay showed that, based on the overall charge density of the tTAV-OX5034 (both cleaved and uncleaved variants of the F2 transcript) and DsRed2-OX5034, these proteins are unlikely to	50889421

OX5034 proteins by proteases, and likelihood of these proteins crossing a cell membrane		cross cell membranes. The analysis was classified as acceptable. *In silico* analyses predict that both the cleaved and uncleaved version of the tTAV-OX5034 variant as well as DsRed2-OX5034 are susceptible to degradation by several proteases present in the human gastric system and the environmental protease proteinase K. These studies were classified as acceptable. The *in vitro* assay results confirmed some, but not all of the results obtained from the *in silico* analyses. Due to uncertainties in some of the *in vitro* data, the study was overall classified as supplemental. **Classification: Supplemental.**	

2. Mammalian toxicity and allergenicity assessment

a. Toxicity assessment

Toxicity of the OX5034-expressed proteins was evaluated through protein sequence comparison with known toxins that are deposited in the NCBI databases using the BLASTp program coupled with the limiting keywords "toxin" and "toxic." The current study relied on protein sequence identity with known toxins deposited in the NCBI databases and utilized the Codex Alimentarius guidelines (Codex Alimentarius 2003) of 35% protein identity to determine significant matches. It is expected that this approach is likely to have uncovered relevant results because the search was conducted as a global sequence alignment, used a conservative identity threshold, and evaluated the literature associated with those proteins that shared greater than 35% sequence identity. At this level of identity, proteins are generally expected to have similar tertiary structures (Abagyan and Batalov 1997, Sillitoe et al. 2015), which may reflect similar biological activities. This analysis was coupled with subsequent review of the literature associated with the identified NCBI entry. This latter step is important as sequences identified through BLASTp are obtained from several sources, including translation from annotated coding regions in GenBank, RefSeq, and third-party annotation (TPA), as well as records from SwissProt, Protein Information Resource (PIR; website: https://pir.georgetown.edu/), Protein Research Foundation (PRF), and Protein Data Bank (PDB) and therefore not every significant match with a protein deposited in NCBI may also be biologically relevant. Separately, the company also conducted a literature search to evaluate the toxic potential of the source organisms for the individual components of the tTAV-OX5034 and DsRed2-OX5034 proteins.

i. tTAV-OX5034

The tTAV-OX5034 protein is a chimeric fusion of the minimally altered *Aeadsx* splicing module from *Ae. aegypti*, UBQ from *D. melanogaster*, linker sequences, and tTAV. The latter is itself a fusion of the tetracycline repressor (TetR) from *E. coli* and the viral tegument protein 16 (VP16) from the herpes simplex virus-1 (HSV-1; Unit II.A.1). Using the search criteria outlined above, tTAV-OX5034 showed significant homology to other tetracycline-controlled transactivator (tTA) proteins in the NCBI databases but not to known toxins. A literature review using "tTAV" as a key word revealed that the protein and its components are generally well tolerated when expressed *in vivo*. For example, the TetR domain has been successfully expressed in mice, demonstrating a certain tolerance to this part of the tTAV-OX5034

protein (Schonig et al. 2013). Furthermore, rabbits survived immunization with the VP16 antigen to produce the polyclonal antibody (AbCam; MRID 50889419), which demonstrates a certain level of mammalian tolerance to that part of the tTAV-OX5034 protein via injection. Some adverse effects have been observed in transgenic mice that expressed tTA proteins, which manifested in emphysema-like symptoms in the lungs and neuronal loss (Sisson et al. 2006, Han et al. 2012). However, these effects are generally thought to be the result of differential gene expression mediated by the tTA protein and there is no indication from the literature that these effects are due to an inherent toxicity of the protein (Han et al. 2012).

The modified Aeadsx protein, which comprises the N-terminus of tTAV-OX5034, is endogenous to *Ae. aegypti* and thus is not expected to be cytotoxic when expressed in insects. UBQ was fused in between Aeadsx and tTAV to facilitate release of the active tTAV variant from the full-length protein subsequent to translation. While this particular UBQ protein sequence was derived from *D. melanogaster*, UBQ is structurally and functionally regarded as one of the most highly conserved proteins in Eukaryota and therefore is expected to be well tolerated by these organisms (Hochstrasser 2009, Zuin et al. 2014).

Due to the lethal nature of the Tet-OFF gene circuit engineered into the OX5034 mosquito, it is difficult to distinguish between adverse effects that are the result of transcriptional squelching and adverse effects that may be the result of tTAV-OX5034 toxicity. However, it remains relevant to note that male adult OX5034 mosquitoes express the tTAV-OX5034 mRNA and can complete their life cycle (Units II.A.3 and 4.b).

In summary, the information presented in this study, supplemented with information from other parts of the application and the published scientific literature, support the finding that the individual components of the tTAV-OX5034 protein are well tolerated in several eukaryotic organisms. In addition, the two variants of the tTAV-OX5034 protein, uncleaved and cleaved, share no significant protein homology to known toxins. Lastly, there is no indication in the literature to suggest that the source organisms of the various proteins that comprise the tTAV-OX5034 protein have toxic characteristics.

ii. DsRed2-OX5034

Using the search criteria outlined above, the DsRed2-OX5034 protein showed no significant homology to known toxins but shared significant similarity with other fluorescent proteins. Fluorescent proteins have many applications in molecular cell biology, including as gene expression markers, protein tags, and for investigating protein-protein interactions *in vivo*. To that end, in addition to OX5034 *Ae. aegypti*, other transgenic organisms stably expressing DsRed2 and related protein derivatives have been created including other insects (Simmons et al. 2011), fungi (Nahalkova and Fatehi 2003), plants (Nishizawa (Nishizawa et al. 2006), mice (Ryu et al. 2013), and rats (Sato et al. 2003). The successful expression of these related proteins speaks to a certain level of tolerance by eukaryotic organisms, including mammals. Rabbits can also be immunized with the DsRed-Express antigen for the production of polyclonal antibody, which indicates mammalian tolerance to this DsRed protein variant through injection (TaKaRa - Clontech; MRID 50889419).

However, cytotoxic effects have also been observed in association with the heterologous expression of fluorescent proteins such as those discussed above, including variants of DsRed2. Adverse effects

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manifested in the failure of obtaining stably expressing mammalian transformants and reduced growth of transformed mammalian cell lines [(Hadjantonakis et al. 2002) (DsRed); (Koelsch et al. 2013) (GFP); (Zakrzewska et al. 2014) (DsRed2)]. Only a few studies could be found in the literature that explored the potential cytotoxic mechanisms for DsRed variants [(Zhou et al. 2011) (DsRed and DsRed-Express2)] and many of those effects are attributed to protein tetramerization and aggregation resulting from the constitutive expression of these proteins *in vivo* (Yanushevich et al. 2002, Strack et al. 2008).

The DsRed2 protein variant served as the blueprint for the development of DsRed2-OX5034. DsRed2 itself was originally created in search of a DsRed1 variant with reduced aggregation properties and low cytotoxicity. Three N-terminal amino acid substitutions (R2A, K5E, and K9T) in DsRed1 increased protein solubility and generally reduced cytotoxicity (Yanushevich et al. 2002). DsRed2-OX5034 is virtually identical to this DsRed2 variant, but in addition contains a bipartite NLS and linker sequences at its N-terminus and C-terminus (resulting in a protein that is 15% larger). The N-terminal NLS was originally identified in the SV40 large T-antigen in humans, and as such is unlikely to cause mammalian toxicity concerns by itself (Kalderon et al. 1984). The C-terminal NLS is very similar in sequence, but it is unclear from the study whether it is a native or a synthetic NLS. Given that the composition of the N-terminus appears to be an important determinant for the tendency of DsRed2 to aggregate when expressed *in vivo*, it is not possible to anticipate the behavior of DsRed2-OX5034 and to what extent this may affect the cytotoxic characteristics of the protein. It is relevant to note that these adverse effects were observed in the context of *in vivo* expression (both transient and constitutive), which represents a uniquely high exposure scenario for these transgenic organisms and cell lines, especially when the protein is expressed constitutively. *In vivo* expression of proteins can also disrupt normal cellular function, which in turn may have contributed to the observed adverse effects.

In summary, while no determination has been made on DsRed2-OX5034 toxicity, two lines of evidence that commonly support the hazard evaluation of proteins suggest that DsRed2-OX5034 is not toxic: first, there is no evidence from the literature indicating that *Discosoma sp.*, from which DsRed2-OX5034 was derived, has toxic characteristics and secondly, DsRed2-OX5034 itself does not share significant homology to known toxins. In addition to the characteristics of the source organism and the DsRed2-OX5034 protein itself, the characteristics of proteins that are closely related to DsRed2-OX5034 in sequence were considered. While some DsRed protein variants cause cytotoxic effects when expressed *in vivo*, there is no evidence to suggest that this group of related proteins is inherently cytotoxic. This conclusion is supported by their broad use as fluorescence markers in eukaryotic systems *in vivo*, including mammals. Additionally, a certain level of tolerance to some DsRed2 variants via the injection route of exposure is evidenced by the successful production of antibodies in rabbits, a process that usually requires several rounds of injections, either subcutaneous, intradermal, intramuscular, intraperitoneal, or intravenous route (Leenaars and Hendriksen 2005).

b. Allergenicity assessment

Bioinformatics analyses of the primary protein sequence of tTAV-OX5034 and DsRed2-OX5034 were performed using three databases, AllergenOnline (AOL; Food Allergy Research & Resource Program, University of Nebraska), COMPARE (Comprehensive Protein Allergen Resource, Health and Environmental Sciences Institute (HESI)), and the NCBI Entrez Protein database using "allergen" as a restricting keyword. These bioinformatics analyses commonly help determine the potential for IgE cross-

reactivity. AOL and COMPARE are curated databases for putative and known food and non-food allergens, including mosquito allergens, while the NCBI Entrez Protein database is not restricted to allergenic proteins. In addition, a literature search was also conducted to evaluate the allergic potential of the source organisms for the individual components of the tTAV-OX5034 and DsRed2-OX5034 proteins.

i. tTAV-OX5034

tTAV-OX5034 did not show significant homology with any allergenic proteins. The uncleaved form of the tTAV-OX5034 protein (which is expressed in adult males MRID 50889401) is a chimeric fusion protein of the minimally altered *Aeadsx* splicing module from *Ae. aegypti*, the tetracycline repressor (TetR) from *E. coli*, the viral tegument protein 16 (VP16) from the herpes simplex virus-1 (HSV-1), UBQ from *D. melanogaster*, and linker sequences. A literature review using keywords related to "*E. coli*" and "Herpes" coupled with the keywords "allergen" and "allergy," respectively, did not uncover any studies that indicate that these source organisms have allergenic characteristics. Only *Ae. aegypti* is a known source of allergens, as female saliva contains several allergenic proteins. Given that tTAV-OX5034 is a chimeric fusion protein comprised of several proteins from various source organisms and contains only part of the endogenous Aeadsx protein, it is questionable whether the allergic properties of *Ae. aegypti* are biologically relevant for evaluating the likelihood of tTAV-OX5034 allergenicity.

ii. DsRed2-OX5034

Specific to DsRed2-OX5034, the study failed to identify and address the significant sequence identities between the DsRed2 protein (as expressed in OX513A; EPA File Symbol 93167-EUP-R) and four predicted protein variants of the GFP-like protein Akane from *Scleronephthya gracillima* previously identified as part of the evaluation of EPA File Symbol 93167-EUP-R. The two expert panels curating the AOL and COMPARE databases initially evaluated the Kato *et al.*, 2017 publication and added the GFP-like protein variants to their respective databases (AOL version v18A and COMPARE version February 01, 2018). However, all four sequences were subsequently removed after both expert panels reevaluated the publication and independently concluded that the data presented in the study did not meet the criteria for adoption as demonstrated allergens in the databases. Letters from both AOL and COMPARE panels explaining their respective decisions to remove the GFP-like protein Akane were previously provided by Oxitec as part of the application for the OX513A *Ae. aegypti* product, as well as the proposed application of OX5034 *Ae. aegypti* (Goodman et al. 2016, Baumert et al. 2018, Goodman 2018, Hoffman-Sommergruber 2019) (MRID 50889426). A discussion of the data published in Kato *et al.*, 2017 remains relevant for the OX5034 application as DsRed2-OX5034 and the four variants of the GFP-like protein share a level of protein sequence identity that would trigger the need for further characterization of allergenicity under Codex (see Figure 1A, Appendix). Evaluation of the information provided by the applicant in MRID 50889426 was not finalized and no determination on the allergenic potential of DsRed2-OX5034 has been made at this time. There is no indication in the literature to suggest that *Discosoma sp.*, from which DsRed2-OX5034 was derived, has allergenic characteristics.

c. Mammalian toxicity and allergenicity conclusion

The risk that a pesticide poses to human health is a function of hazard and exposure. As discussed in Unit II.B.3.b, the most significant exposure to tTAV-OX5034 and DsRed2-OX5034 would be expected

from biting females if they were present in the environment. Due to the lack of females, i.e., negligible exposure, the risk from OX5034 is considered negligible and thus, the hazard data were not necessary to support the finding of no unreasonable adverse effects for humans.

In addition to information characterizing the exposure to OX5034, Oxitec provided information characterizing the hazard of tTAV-OX5034 and DsRed2-OX5034. Mammalian toxicity and allergenicity of transgenic proteins is commonly evaluated in association with the hazard assessment of plant-incorporated protectants (PIPs) engineered into food plants and is based on a weight-of-evidence approach. These assessments often consider a combination of data obtained from animal testing, several *in vitro* analyses of the protein, and prediction of protein behavior using bioinformatics tools. While OX5034 is not proposed for food use, the same established risk assessment framework for the assessment of toxicity and allergenicity was considered to evaluate tTAV-OX5034 and DsRed2-OX5034. Given the differences between dietary exposure and intradermal exposure, i.e., from blood-feeding females, some of the information elements that commonly characterize the hazard for PIPs in food plants may weigh differently on the assessment of OX5034. To what extent they are different and how, if at all, this difference may affect the hazard assessment remains to be determined. Relatedly, the Agency has not determined whether additional data would be needed to support the hazard evaluation for proteins, such as tTAV-OX5034 and DsRed2-OX5034, that may pose intradermal exposure. Below we discuss the mammalian toxicity and allergenicity data provided by Oxitec and highlight specific considerations for exposure of proteins through the intradermal route.

The assessment of protein toxicity is commonly comprised of a combination of acute oral toxicity data generated in rats, supplemented with homology searches of the protein sequence to known proteinaceous toxins. In support of the toxicity assessment of tTAV-OX5034 and DsRed2-OX5034, Oxitec provided bioinformatics and literature analysis on the potential toxic characteristics of the transgenic proteins, related proteins, and the source organisms of the transgenic proteins. This information did not indicate that either protein is inherently toxic. Mammalian acute toxicity studies for tTAV-OX5034 and DsRed2-OX5034 was not provided. At this time the Agency has not determined whether the results of the presented protein homology and literature-based assessments alone are sufficient to support the hazard assessment of tTAV-OX5034 and DsRed2-OX5034.

EPA uses the Codex Alimentarius guidelines to evaluate proteins for their allergic potential (Codex Alimentarius 2003). The Codex guidelines are based on a weight-of-evidence approach, which generally considers homology to known allergens, allergic characteristics of the source organism from which the protein was derived, proteolytic susceptibility of proteins to gastrointestinal proteases, and protein glycosylation. This information commonly informs the hazard assessment of proteins through the oral and inhalation routes of exposure. Oxitec provided some, but not all of the data commonly associated with the allergenicity assessment of dietary proteins, including homology to known allergens and a literature review of the allergenic characteristics of the source organisms from which the proteins, and/or their individual components were derived. These data did not indicate any allergenicity concerns for tTAV-OX5034 and the evaluation of the information provided for DsRed2-OX5034 has not been finalized. Other data, including the protein digestibility were not adequate to demonstrate lability under these conditions and some, such as protein glycosylation, were not provided (MRID 50889421; Table 4).

Human exposure to *Ae. aegypti* is primarily mediated through females who rely on a blood meal from a vertebrate source to support egg production (Valzania et al. 2019). For feeding to occur, the proboscis must break the skin barrier (epidermis) to reach the blood vessels in the dermal layer. During the probing phase, female mosquitoes inject a few nanoliters of saliva intradermally (Hopp and Sinnis 2015). Female saliva contains anti-hemostatic, anti-inflammatory, and immunosuppressive factors that counteract physiological responses at the bite site and facilitate feeding (Ribeiro and Francischetti 2003, Calvo et al. 2006, Choumet et al. 2012, Ribeiro et al. 2016). As such, the route of exposure to proteins that are secreted into the saliva of biting female mosquitoes is more accurately described as intradermal. Given the differences in the proteolytic environment between the human gastric system and the dermis, the question arises to what extent the same hazard assessment approach, and Codex guidelines for food safety, are applicable.

Proteins that are demonstrated to be susceptible to proteolytic degradation in the human digestive system are thought to have a lower likelihood to elicit adverse effects due to reduced exposure. Some proteins injected by mosquitoes have been detected for up to 18 hours in the skin of mice bitten by *Anopheles gambiae* (Choumet et al. 2012) and thus, tTAV-OX5034 and DsRed2-OX5034, if injected, may have the potential to elicit prolonged intradermal exposure. Relatedly, the toxic characteristics of a protein may be different when injected into the skin versus (epi)-dermal exposure, especially in the absence of adjuvants, e.g., snake venoms must generally be injected for toxicity to occur. Mosquito bites furthermore create local inflammatory responses resulting from a combination of the puncturing of the dermis and blood vessels and the presence of naturally occurring allergenic proteins in the mosquito saliva, which may alter the dermal response to a substance at the affected location. Thus, intradermal exposure to a substance is arguably different from dermal exposure. To what extent they are different and how, if at all, this difference may affect the hazard assessment of proteins that pose an intradermal exposure risk remains to be evaluated.

3. Human exposure and human health risk characterization

a. Penetrance of the female-lethal trait

i. Laboratory and field studies confirming 100% penetrance in the absence of tetracycline

In the context of this risk assessment, penetrance refers to the proportion of individuals of a given genotype that show the phenotype typical of that genotype. In the case of penetrance of the self-limiting trait of OX5034 strain, this refers to the proportion of female insects that that die before reaching adulthood. To confirm that the OX5034 self-limiting trait indeed results in 100% female lethality, laboratory crosses were performed (Table 5). Crosses were reared both with the dietary antidote (doxycycline, a tetracycline analogue), which rescues OX5034 females, and without the dietary antidote. Rearing conditions without the dietary antidote are considered field-like conditions.

Laboratory crosses of OX5034 mosquitoes and LWT mosquitoes found that no OX5034 females reached the adult stage when reared without the dietary antidote, confirming 100% penetrance of the OX5034 phenotype. Experimental design resulted in the testing of 500 females homozygous with the OX5034 gene and 1000 females hemizygous with the OX5034 gene. Females hemizygous with the OX5034 gene are genetically comparable to the female offspring that would result in the field from OX5034 male

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releases and therefore, it is important that even with only one copy of the OX5034 gene, 100% female lethality was confirmed.

Table 5. Crosses for penetrance testing. 'OX5034' refers to a homozygous individual (carrying two copies of the transgene). 'LWT' refers to an individual without any copy of the transgene. OX5034 crossed to LWT will produce progeny with one copy of the transgene. LWT crosses act as a negative control, with zero copies of the OX5034 transgene. Doxycycline is a tetracycline analogue. This table has been modified from MRID 50889417 to refer to 'WT' as 'LWT.'

Cross	200 L1 larvae in 200 mL doxycycline (4 µg/mL)	200 L1 larvae in 200 mL dl water
OX5034 ♂ x OX5034 ♀	5 repeats	5 repeats
OX5034 ♂ x LWT ♀	5 repeats	5 repeats
OX5034 ♀ x LWT ♂	5 repeats	5 repeats
LWT ♂ x LWT ♀	5 repeats	5 repeats

Field collected eggs from OX5034 releases outside of the United States (US) were brought into the laboratory, hatched, and reared in the absence of the dietary antidote. The study found complete penetrance of the OX5034 phenotype in the field collected samples, indicating that the OX5034 phenotype still results in female lethality in hemizygous progeny even when the OX5034 trait is expressed in a different genetic background from a geographically distinct population. PCR reactions were also run for a subset of the field collected samples to confirm that surviving females believed to lack the OX5034 cassette based on a lack of fluorescence, did indeed lack the OX5034 cassette (i.e., to confirm that surviving wild-type females were truly wild-type). The results from the studies using mosquitoes from laboratory colonies and from field collections, demonstrate that the OX5034 phenotype is 100% penetrant and that all females containing a copy of the OX5034 trait die prior to adulthood when reared in the absence of a tetracycline analogue.

ii. Environmental sources of tetracycline

Because the presence of tetracycline(s) in the environment may affect survivability of female OX5034 mosquitoes, the likelihood that OX5034 mosquitoes would encounter tetracycline sources at levels high enough for rescue from the lethal phenotype was evaluated. Several lines of evidence including a survey of environmental levels of tetracycline, tetracycline dose-response testing of OX5034 females, and oviposition behavior of *Ae. aegypti*, indicate that the risk of hemizygous OX5034 female mosquitoes emerging in the environment due to high levels of tetracycline is low. Trial site location restrictions using known *Ae. aegypti* dispersal distances to limit exposure to locations with higher probabilities of containing tetracycline would further reduce the likelihood of OX5034 females in the environment to the point where the risk would be considered negligible.

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Tetracyclines in the environment can come from human or animal drugs, or non-drug sources such as in agriculture. The dose-response of OX5034 mosquitoes to a range of tetracycline analogues (doxycycline chlortetracycline, tetracycline, and oxytetracycline) was evaluated to determine what levels are required to rescue female OX5034 mosquitoes (MRID 50889415). These rescue levels were compared to concentrations of the tetracycline analogues typically found in the environment as a result of industrial or household tetracycline usage in the US. Environmental concentrations were obtained through a literature search combining USA with the name of each tetracycline analogue and with relevant terms like wastewater, effluent, ground water, or surface water. In all cases the minimum concentration for each analogue required to rescue OX5034 females capable of maintaining flight is higher than the mean concentrations found in environmental water bodies for the studies reviewed. Because tetracycline analogue levels in the environment were found to be lower than the levels needed for OX5034 rescue, there is a low risk of OX5034 mosquitoes encountering levels of tetracycline high enough to result in the emergence of hemizygous OX5034 females.

The oviposition behavior of *Ae. aegypti* further reduces the likelihood that OX5034 mosquitoes would encounter sources where tetracycline and its analogues may be present. *Ae. aegypti* prefer man-made containers such as gutters, water containers, and tires that hold rainwater or clean still water for their breeding sites (TunLin et al. 1995, Hribar et al. 2001). These containers are unlikely to house significant levels of tetracycline analogues. Female *Ae. aegypti* also lay their eggs at several different sites as opposed to laying them in one breeding container. This oviposition behavior creates a challenge in terms of pest control, but further reduces the likelihood that many eggs would be laid in water containing significant concentrations of tetracycline analogues.

Aquaculture facilities, farms, hospitals, or municipal sewage facilities are likely the only sources that theoretically could introduce sufficiently high levels of tetracycline into the environment to allow survival of OX5034 females, although the literature survey of environmental levels found this to be unlikely. EPA nonetheless considered the likelihood that these potential sources of tetracycline are present or would pose an exposure risk in proposed trial areas based on information specific to the proposed locations. Given that the proposed trial areas are likely to be in developed (urbanized) areas due to preferred *Ae. aegypti* habitat (i.e., *Ae. aegypti* is adapted to humans and urban areas), the presence of livestock or aquaculture is not expected as these are more likely to be in rural environments which would not provide suitable testing locations. However, because Florida is a major producer of citrus and oxytetracycline applications are being used in citrus groves to combat citrus greening, the applicant initially stated that the outer boundary of the trial areas will be greater than 400 m from commercial citrus growing areas to reduce the likelihood that OX5034 mosquitoes could encounter increased levels of oxytetracycline as a result of these applications. Although longer dispersal distances for *Ae. aegypti* have been observed, a compilation of release recapture studies around the world found that most *Ae. aegypti* are recovered within 20 m to 50 m of the release point, with a small percentage found 170 m but generally not more than 200 m from the release point (OECD 2018). Therefore, a restriction of 500 m from citrus growing areas (200 m for released OX5034 males + 200 m for mated *Ae. aegypti* females + 100 m of additional buffer) is more appropriate to provide a conservative buffer zone. These numbers are also in agreement with previous field releases of OX5034 male mosquitoes in Brazil which recorded maximum dispersal distances of 198 meters.

A 2004 survey found that although most mosquito larvae in the Florida Keys were collected from tires, flowerpots, planters, and trivets, some larvae were collected from sewage treatment plants, septic tanks,

and cesspits (Hribar et al. 2004). However, these are not preferred breeding sites and breeding in septic tanks can only occur where the lid is cracked or broken (Burke et al. 2010). Furthermore, since 2004, Key West and surrounding areas in Monroe County have eliminated most septic tanks and use a public sewer line system as the major means of waste disposal. Most of the County is now served by the Cudjoe Regional Wastewater System that includes a deep injection well, which disposes of treated effluent 3,200 feet below the surface, thus excluding exposure of *Ae. aegypti* to effluent (see web links for City of Key West , Monroe County Wastewater Master Plan in references). Each of the Florida Keys Aqueduct Authority Wastewater Districts[2] has its own municipal wastewater treatment facility, which consist of a series of open holding tanks. These open holding tanks could allow access to mosquitoes, although this would not be a preferred breeding site as *Ae. aegypti* prefer to oviposit in clear waters. The likelihood of these tanks containing high enough levels of tetracycline to rescue OX5034 females is also low because tetracycline rapidly undergoes aqueous photolysis in the presence of sunlight. In Harris County, Texas, reclaimed water from all of Houston wastewater plants is discharged directly into a surface waterway, usually one of the area bayous (see web link City of Houston in references). However, bayous are not typical breeding sites for *Ae. aegypti* and any tetracycline present would also undergo aqueous photolysis. The handling of sewage effluent from hospitals in the trial areas is unknown, however these are likely to be closed systems connected to the centralized wastewater treatment systems.

The Agency considers it unlikely that these abovementioned sources would have sufficient levels of tetracycline to rescue OX5034 females based on the literature survey but finds that limiting the proximity of trial site locations from any wastewater treatment facility in either Monroe or Harris County would further reduce that likelihood. Therefore, a restriction of 500 m from a wastewater treatment site (200 m for released OX5034 males + 200 m for mated *Ae. aegypti* females + 100 m of additional buffer) provides a conservative buffer zone.

In addition to the traditional sources of tetracycline discussed above, it has been shown in a laboratory study when OX513A larvae (Oxitec's Generation 1 mosquito) were exclusively fed a chicken-based cat food, some survival to adulthood occurred due to tetracycline contamination (Massonnet-Bruneel et al. 2013). As the trial areas are expected to be in urbanized areas, the presence of pets and their food, such as cat food that may originate from organs/meat from antibiotic treated husbandry animals, is likely. However, cat food is not believed to be a plausible source of tetracycline exposure for OX5034 mosquitoes in the environment as it would require a number of steps: that adequate levels of cat food be found in a container to create a high enough concentration of tetracycline to rescue OX5034 females, that the container also hold adequate levels of water for mosquito development, and that these conditions be maintained over at least 8-10 days for larval and pupal development. In addition, exposure to sunlight would result in aqueous photolysis, so to maintain adequate tetracycline levels the cat food container would have to remain in the shade. For the reasons cited for cat food, other meat-based pet foods are not considered to be plausible sources of tetracycline exposure.

Finally, the Agency also considered whether degradation products of tetracyclines could impact OX5034 female survival. Limited information is available as to environmental concentrations of these degradation products or how the various degradation products could interact with the Tet-OFF system. However, as these degradation products are products of tetracycline, it is logical to infer that sources believed to have the highest concentrations of tetracycline are also those most likely to have any significant level of

[2] Navy, Key Haven, Big Coppitt, Bay Point, Cudjoe Regional, Duck Key, and Layton Long Key.

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degradation product. Therefore, any restrictions on the location trial area boundaries and wastewater treatment facilities or citrus groves would also reduce uncertainty surrounding degradation products.

Based on known oviposition preference of *Ae. aegypti* and literature surveys of environmental concentrations of tetracycline analogues indicating levels lower than those shown necessary through dose response testing to rescue OX5034 females, the likelihood that OX5034 mosquitoes would encounter tetracycline levels high enough to result in OX5034 females is low. However, maintaining sufficient distance between trial area boundaries and potential tetracycline sources would further increase confidence that there will be no OX5034 females in the trial areas, and reduce uncertainty surrounding potential degradation products. Based on worldwide release recapture studies (OECD 2018), trial area outer boundaries at least 500 m from these potential tetracycline sources (i.e., citrus groves and wastewater treatment facilities) would greatly reduce remaining uncertainties as this would account for OX5034 male dispersal and mated *Ae. aegypti* dispersal. Therefore, although the likelihood that released OX5034 mosquitoes encounter levels of tetracycline or its analogues high enough to result in the emergence of OX5034 females is low, by further reducing access to these potential tetracycline sources, the likelihood would be reduced to negligible.

b. Human exposure characterization

The risk assessment process for pesticide evaluation is characterized by determining the hazard and exposure to a pesticide product using the basic equation: Risk = Hazard x Exposure. Based on this relationship, a pesticide may not exhibit human health risk if it poses either negligible human health hazards and/or negligible human health exposure. A discussion of the potential for human exposure to OX5034 *Ae. aegypti* and its associated traits is provided below.

i. Dermal exposure

OX5034 *Ae. aegypti* are proposed for environmental releases where they are expected to spread the tTAV-OX5034 and DsRed2-OX5034 traits throughout the local *Ae. aegypti* population. Given this method of pesticide application, the biology of *Ae. aegypti* is an important consideration for evaluating the potential human exposure to OX5034 and associated traits. The dermal route of exposure to tTAV-OX5034 and DsRed2-OX5034 is considered the most relevant were females to be present in the environment, as they could expose humans to both proteins while taking a blood meal (Unit II.B.2.c).

As discussed in Unit II.B.2.c, at the time the review was completed, no determination on the hazard potential of the tTAV-OX5034 and DsRed2-OX5034 proteins was made. Hazard information is not required, however, to support the current risk assessment of OX5034 due to negligible exposure to female *Ae. aegypti* expressing the tTAV-OX5034 and DsRed2-OX5034 proteins and the resulting negligible human health risks from these individuals (Unit II.B.3.a). Negligible exposure to females is based in part on an analysis of potential oxytetracycline sources in the proposed EUP locations and the subsequent recommendation of spatial separation of OX5034 release sites from commercial citrus growing areas and wastewater treatment sites. These parameters reduce the likelihood that OX5034 mosquitoes could encounter increased levels of oxytetracycline, and consequently, reduces the likelihood of emergence of female *Ae. aegypti* carrying the OX5034 traits.

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Dermal exposure to male OX5034 was also considered, as these will be released into the environment. The only plausible route of dermal exposure to male OX5034 is expected to occur via direct (epi-)dermal contact, e.g., upon landing of an adult mosquito on bare skin. This is because males, unlike females, do not feed on human blood, but rather rely on plant carbohydrates for energy synthesis (Foster 1995, Ribeiro et al. 2016). Given the male feeding behavior, the frequency of human interaction with male mosquitoes is expected to be minimal. Even if direct skin contact were to occur, because the tTAV-OX5034 and DsRed2-OX5034 proteins are present within the insect's cells, exposure to these substances is expected to be negligible.

ii. Oral exposure

Oral exposure to OX5034 males is expected to be negligible. OX5034 is not proposed for food use, and therefore oral exposure is only conceivable through accidental ingestion of mosquitoes. As discussed in the dermal exposure section above, exposure to adult female OX5034 is expected to be negligible. The experimental protocol of this EUP proposes releases of both mosquito eggs and male adults to evaluate the effects on the local *Ae. aegypti* population. Eggs will be deployed in rearing boxes from which adult OX5034 males will emerge. The parameters of the egg deployment are unlikely to be conducive for accidental ingestion to occur. The rearing boxes will be physically isolated from the public, or where that is not possible, located out of public view. Handling of the release devices is only required during a time when adult mosquitoes are not present, i.e., during their initial placement and setup and after adults have deployed from the device. Additionally, it is unlikely that at any given time the number of OX5034 males emerging from the rearing box is high enough for accidental ingestion to occur, as mosquitoes are expected to develop somewhat asynchronously, resulting in a staggered release of adult males from the rearing boxes.

Adult male OX5034 releases from vehicles are expected to facilitate dispersal of the mosquitoes in the environment and reduce potential interaction with the applicator to negligible levels. Releases of mosquitoes from containers on foot may result in increased male OX5034 mosquito abundance only at the time and location of application. However, because males do not seek out human interaction and are likely to quickly disperse once released to find shaded areas and look for food and mating opportunities, the likelihood of accidental ingestion, remains low. Together, oral exposure to tTAV-OX5034 and DsRed2-OX5034 is expected to be negligible.

Another conceivable route of exposure to tTAV-OX5034 and DsRed2-OX5034 may be through ingestion of water containing OX5034 eggs or larvae that resulted from the matings of OX5034 males and local females. While *Ae. aegypti* is an anthropophilic species, its oviposition sites are not expected to be a source of potable water in the proposed test areas. *Ae. aegypti* is a container-breeding species that is often found to lay eggs in small bodies of water such as puddles, tires, and plants (e.g., leaf axils of bromeliads; phytotelmata). Any OX5034 mosquitoes that are present in these bodies of water and that are unable to mature due to the expression of the lethal tTAV-OX5034 protein will be exposed to the normal processes of biodegradation. This expectation is supported by the bioinformatics analysis that predicts susceptibility of the tTAV-OX5034 and DsRed2-OX5034 proteins to the environmental protease proteinase K. Based on these considerations, it is expected that tTAV-OX5034, DsRed2-OX5034 are degraded, virtually eliminating this route of exposure.

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iii. Ocular exposure

Ocular exposure to tTAV-OX5034 and DsRed2-OX5034 is unlikely, as they are contained within the cells of the insect, which essentially eliminates these exposure routes. The exposure considerations discussed above (i.e., mode of pesticide application, male mosquito behavior once released) in the primary dermal irritation/ acute dermal toxicity, and acute oral toxicity waiver rationales are equally relevant to evaluate the likelihood of ocular exposure. Based on these assessments, ocular exposure to male OX5034 mosquitoes is unlikely to occur. Even if an OX5034 adult male were to accidentally come in contact with the eye, the physical effect from that accidental contact is expected to be minimal and to not be different from those effects resulting from accidental ocular exposure to a wild-type mosquito.

iv. Pulmonary exposure

The exposure considerations discussed above are equally relevant to evaluate the likelihood of pulmonary exposure. Pulmonary exposure to adult OX5034 males is not expected to occur as they are too large to be inhaled, especially into deeper lung tissues (Gorguner and Akgun 2010). Accidental oral exposure for objects this size is therefore more likely to result in swallowing, i.e., ingestion, rather than inhalation. The circumstances that would allow for the formation and accumulation of airborne particles are not expected to be present at the release sites. Male adult OX5034 will be released into the environment where they seek out females for mating. *Ae. aegypti* oviposition sites will most likely be present outdoors, in areas that are exposed to rain where water can collect. Microbial activity in the environment will facilitate biodegradation of the transgenic proteins, which will facilitate removal of these biotic components in the release area over time. Further, male adult mosquitoes are the only adult life stage that carry the OX5034 traits, but only adult females seek out the presence of humans as they rely on the blood meal for egg production. Access to indoor spaces is expected to be minimal. However, should access be available, the presence of females will not lead to exposure to the tTAV-OX5034 and DsRed2-OX5034 proteins, as these do not carry the OX5034 traits. While it is possible that males may follow females indoors, it is expected to be a rare event that those males would then remain indoors and contribute to dust formation in a significant way. The presence of tTAV-OX5034 and DsRed2-OX5034 proteins in the insect's cells is expected to further reduce the likelihood of pulmonary exposure to overall negligible levels. Information on the charge density of and protein size of tTAV-OX5034 and DsRed2-OX5034 was also considered as part of the exposure evaluation. Neither protein is expected to be able to freely diffuse over cell membranes, which may reduce uptake of these proteins into the lungs. The extent to which these physicochemical characteristics would prevent potential adverse effects is, however, unknown (MRID 50889421).

C. Environmental Fate of the Transgene and OX5034 Background Genetics

1. Introgression data

OX5034 is described as a species-specific female larvicide, or "male-selecting" larvicide, that results in all-male progeny in the absence of tetracyclines in the larval diet due to a female-specific self-limiting gene. With continued field releases of OX5034 homozygous males, the local *Ae. aegypti* population is expected to progressively decline due to the reduced number of females emerging in the area. Specifically, when OX5034 homozygous males are released into the environment and mate with local *Ae.*

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aegypti females, their offspring inherit a single copy of the self-limiting gene. The self-limiting gene kills only female offspring while hemizygous males survive to pass on the OX5034 self-limiting gene further. As the self-limiting gene is inherited in a Mendelian fashion, half of the offspring resulting from a mating between an OX5034 hemizygous male and a local female would not inherit the self-limiting gene but would still inherit OX5034 background strain genetics. This results in both male and female mosquitoes in the local *Ae. aegypti* population with some degree of OX5034 background strain genetics.

Oxitec submitted scientific rationale, experimental data, modeling studies, and a meta-analysis to address introgression and persistence of the OX5034 transgene and OX5034 background strain genetics in the local *Ae. aegypti* population. A summary of these data is provided in Table 6 and within the risk assessment below. A full review of the rationale is contained within the DERs except for the study "Introgression of OX5034 Background Genetics." This study was instead reviewed in the format of an interagency collaborative memo between the EPA and the CDC. Some of the references cited in this assessment were included in rationale provided by the applicant within the MRIDs cited. Other references were included from the open literature that pertained to specific topics discussed below.

Table 6. Status of data submitted to address environmental fate of the OX5034 transgene and OX5034 background genetics.

Study Topic	OPPTS Guideline No.	Results Summary and Classification	MRID No.
OX5034 male-selecting trait decline in a caged population of wild-type *Aedes aegypti*	N/A	Three experimental caged populations (n=200 individuals per generation per cage) were used to determine how long the OX5034 trait would remain in a wild mosquito population once OX5034 releases ceased. The experiment found that the trait reduced by approx. 54% each generation and disappeared from the populations after a maximum of 9 generations. **Classification: Acceptable.**	50889416
Introgression of OX5034 Background Genetics	N/A	A literature review of vectorial capacity and vector competence was combined with a meta-analysis of vector competence data from Florida, data on the OX5034 mosquito (fecundity, longevity), and data from a study on the OX513A mosquito. **Classification: Acceptable.**	50973401
Modelling the Introgression of OX5034 Background Genetics	N/A	The study aimed to model the degree and persistence of OX5034 background genetic introgression into wild field mosquito populations through use of a deterministic, discrete-generation population genetic model. However, several deficiencies and necessary clarifications make it so the models have limited value. Given that the applicant has characterized the mosquito traits of interest (i.e., characterized the potential hazard), information that could potentially be gleaned from modeling the degree of introgression (i.e., the exposure) is not critical to the risk assessment. **Classification: Supplemental.**	50973402

2. Persistence of OX5034 transgene in the environment post-release

The applicant provided an experimental study and a corresponding modeling study to determine the rate at which the introduced OX5034 self-limiting trait will decline in a caged, wild-type *Ae. aegypti* population. The studies are used to indicate how rapidly the OX5034 self-limiting trait will decline and become extinct after the proposed releases of OX5034 male mosquitoes have ceased.

Three replicate caged populations were maintained in the absence of the dietary antidote in the laboratory. Each population was derived from 100 OX5034-hemizygous males crossed to 200 LWT females in a 15 x 15 x 15 cm cage. These populations represent the generation after the final release of OX5034 males under the scenario where all male *Ae. aegypti* mosquitoes remaining in the population are offspring of the released OX5034 males. They also represent the scenario with the highest possible frequency of the OX5034 transgene in the environment because in this scenario there are no wild-type males remaining and all of the males in the population contain a copy of the transgene. During the caged population studies, each subsequent generation was composed of 200 offspring resulting from the previous generation. The frequency of the OX5034 transgene in the population was calculated via proportion of fluorescent pupae for each subsequent generation and graphed over time to show the decline of the OX5034 transgene in the three populations. The applicant also provided a simple stochastic model to predict the decline of the OX5034 transgene where the model assumed complete penetrance of the OX5034 trait and no competitive disadvantage for OX5034 males.

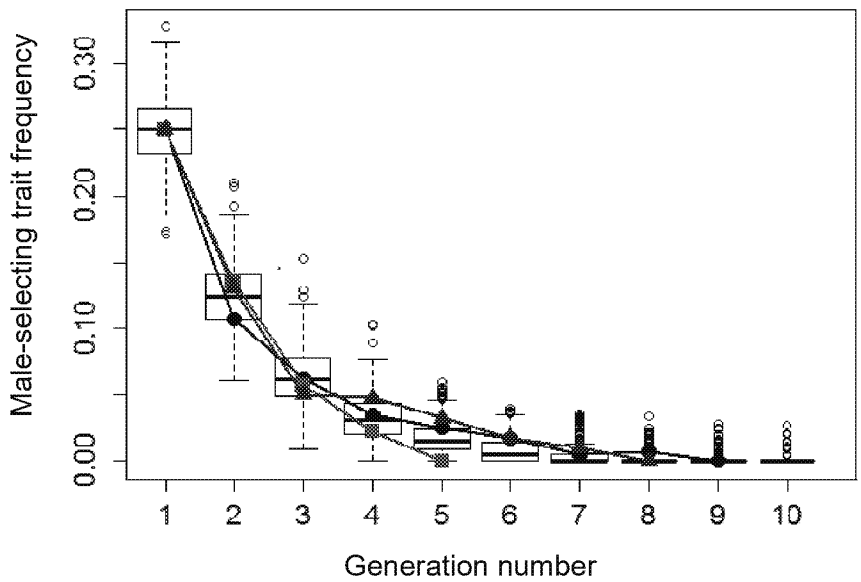

Figure 3. Boxplots showing the results from 500 iterations of a stochastic model simulating the extinction of a male-selecting genetic trait under restrictive conditions. Horizontal bold lines represent generational medians; upper and lower box lines represent first and third quartiles, respectively; outer horizontal lines represent 1.5x the interquartile range; and open circles represent data points over 1.5x above or below the first and third quartiles. Overlaid onto the box plots are lines (red, blue and green) showing male-selecting trait frequency changes from three replicates of caged experiments. Generation 1 represents a post-field release population with a trait frequency of 0.25. Graph and figure legend reproduced from MRID 50889416.

The caged population study found the mean number of generations until disappearance of the OX5034 self-limiting trait across three caged populations to be 7.3 (±1.2 SE) with 9 being the maximum number of generations before trait disappearance (Figure 3). The model and the starting assumptions predicated that the self-limiting trait frequency would halve in each generation due to the 50% fitness cost of the OX5034 trait (i.e., 100% female lethality). This prediction was observed in the experimental cages, with the average frequency of the self-limiting trait decreasing in each generation by 54% (±7.0 SE).

Therefore, upon cessation of the proposed OX5034 male releases, it is expected that the OX5034 transgene would disappear from the environment within 10 generations. Furthermore, it is likely that in the field when OX5034 males compete against local *Ae. aegypti* males, the trait will decline faster than found in the modeling and caged population studies, as additional fitness costs associated with the OX5034 trait have been recorded, like reduced egg clutch size in matings with OX5034 homozygous males (Unit II.A.6.c.; MRID 50889417).

3. Introgression of OX5034 background strain genetics

Because the transgene is inherited in a Mendelian fashion and OX5034 hemizygous males are anticipated to survive and contribute to offspring in subsequent generations, it is expected that although the transgene will eventually disappear from the *Ae. aegypti* populations at the proposed release sites, introgression of OX5034 background strain genetics (i.e., genes other than the transgene) will occur. This is because only half of the offspring (both male and female) of an OX5034 hemizygous male would inherit the transgene, resulting in the other half being "wild-type" but still inheriting other OX5034 background strain genetics. Introgression would result in both male and female mosquitoes in the local *Ae. aegypti* population acquiring some degree of OX5034 background strain genetics, which could increase in frequency after releases have ceased. This is because the releases (if successful in suppressing the population) could result in a population bottleneck once they have ceased, where the founding population is composed of the surviving individuals who likely contain OX5034 background strain genetics due to inundation by OX5034 mosquitoes during releases. This creates the potential to result in an increased frequency of OX5034 background strain genetics in the recovered population, thereby altering the population genetics/traits of the local mosquito population.

A recent paper examining Oxitec's 1st generation product, OX513A, found evidence of introgression of the strain's background genetics after releases in Brazil of males containing the self-limiting tTAV gene (Evans et al. 2019). These findings are relevant to the evaluation of OX5034 because the degree of introgression is likely to be significantly higher than that of the OX513A strain due to differences in larval survival (approx. 5% in OX513A versus 50% in OX5034).

Therefore, given that introgression of OX5034 strain background genetics is expected to occur during releases, it is pertinent to examine potential associated risks for humans and the environment. To do this, EPA evaluated OX5034 mosquitoes for key traits that could increase the ability of mosquitoes to transmit disease, result in larger populations numbers, or result in more robust mosquitoes. As discussed below, based on a combination of laboratory data, meta-analyses, and the large impact of environmental factors on the traits evaluated, EPA finds it is unlikely that the local mosquito population would pose any increased risk to humans or the environment due to releases of OX5034 mosquitoes and introgression of OX5034 background strain genetics under the applied for EUP.

a. Vectorial capacity

As *Ae. aegypti* is a known disease vector, traits associated with vectorial capacity in OX5034 mosquitoes were evaluated. Vector competence, or the intrinsic ability of a vector to acquire, maintain, and transmit an infection, was reviewed for *Ae. aegypti* mosquitoes across different geographies and different viruses. Other traits like insecticide resistance, fecundity and longevity were also reviewed, as insecticide resistance could result in more difficult to control and therefore larger mosquito populations, increased fecundity could result in larger mosquito populations, and increased longevity could increase the likelihood of a mosquito transmitting an infection.

For insecticide resistance, laboratory studies found that OX5034 was susceptible to the pesticide active ingredients temephos (larvicide), permethrin, deltamethrin, and malathion (three adulticides), and the strain does not carry pyrethroid resistance-associated *kdr* mutations (MRIDs 50698718, 50889418, and 509734-05; discussed in Unit II.A.6.a). While the strain showed some resistance to propoxur, this chemical is not approved for uses on mosquitoes in the US and thus any resistance associated with propoxur will not affect current mosquito control practices. For the other traits associated with vectorial capacity, EPA, along with CDC, conducted a review of laboratory data, a meta-analysis, and rationale submitted by the applicant to compare the vectorial capacity of OX5034 mosquitoes to that of wild mosquitoes. A full review of this topic can be found in the accompanying memo (USEPA 2020). The conclusions of this review are below:

Vectorial capacity is influenced by a number of traits impacted by gene-environment interactions and is confounded by both intrinsic and extrinsic variables. Several traits relevant to vectorial capacity were evaluated for the OX5034 mosquito given the expectation of introgression of OX5034 strain genetics into the local mosquito population.

In terms of introgression of alleles related to vector competence, different populations of the same mosquito species can differ in the likelihood of becoming infected, which can also differ by virus type and even by strains of the same virus. However, vector competence is only partially influenced by genetics, with other known influences coming from abiotic factors, nutrition, microbiota, and larval stage competition. Given the potentially limited role of mosquito genetics in vector competence as well as the known temporal and spatial variation of vector competence among mosquito populations, it is not expected that introgression of OX5034 strain genetics would increase the vector competence of the local mosquito populations.

Fecundity and longevity of the OX5034 mosquito were also evaluated (Unit II.A.6.c and Unit II.A.6.d). Data provided by the applicant combined with literature searches indicate that fecundity and longevity of OX5034 mosquitoes is within the range expected for *Ae. aegypti* and therefore introgression of OX5034 strain genetics is unlikely to result in the increased fecundity or longevity of local mosquitoes.

In conclusion, given the data on insecticide resistance, longevity, and fecundity, the large impact of the environment on all traits evaluated, and the complexity of vector competence, EPA believes it is unlikely that the introgression of OX5034 strain genetics would result in increased vectorial capacity of the local mosquito populations under the applied for EUP.

b. Hybrid vigor

EPA also investigated the concepts of "hybrid vigor," in which the crossbreeding of two different genetic backgrounds results in offspring that are superior to both parents, and the opposite scenario "outbreeding depression," in which the offspring are less viable. These topics are also reviewed in the accompanying memo (USEPA 2020), but will be restated below.

The Evans et al., 2019 study postulated that releases of the OX513A mosquito in Brazil and the resulting introgression of strain background genetics into the local population "very likely [resulted] in a more robust population than the pre-release population due to hybrid vigor." However, an Addendum of an Editorial Expression of Concern was published with the study on March 24, 2020 stating that regarding hybrid vigor, "there are no data in the Article to support this point; furthermore, data included in the Article indicate that a number of hybrid individuals rapidly declined post-release." The concept of hybrid vigor, or heterosis, is that deleterious alleles persist in populations and that inbreeding due to drift or population isolation results in reduced vigor from increasing homozygosity of deleterious alleles (Charlesworth and Willis 2009). This vigor is restored by crossing individuals of divergent genotypes, resulting in hybrid vigor through rescue from recessive, deleterious alleles. Hybrid vigor is most commonly reported in crossings within domesticated crops and livestock, which is expected given the intense artificial selection and inbreeding depression that takes place in the development of the parental lines. An instance of hybrid vigor was reported in a malaria vectoring mosquito, *Anopheles coluzzii*, but this was found after crossing two inbred laboratory strains, which again, is unsurprising (Ekechukwu et al. 2015). Although there may be some inbreeding depression in the OX5034 colony due to common lab rearing practices, there is no indication that the local mosquito populations under the proposed EUP are suffering from inbreeding depression. Therefore, there is no indication that matings between OX5034 mosquitoes and local mosquitoes would result in hybrid vigor.

The opposite of inbreeding depression and heterosis would be outbreeding depression. In this instance, genetic differences accrued in widely divergent populations or species that were previously isolated from each other result in genetic incompatibilities in their hybrid offspring that cause reduced hybrid fitness (e.g., Dobzhansky-Muller incompatibilities; Orr 1995). If outbreeding depression were to occur, residual population control from male hybrid offspring containing the self-limiting tTAV gene may be reduced due to decreased hybrid fitness, but some level of population control is still expected from initial OX5034 releases. However, there is no indication that the OX5034 strain is so divergent from local populations as to expect any significant degree of postzygotic isolation (e.g., genetic incompatibilities resulting in inviable hybrids), and therefore, there is no indication that matings between OX5034 mosquitoes and local mosquitoes would result in outbreeding depression.

4. Potential for resistance during field releases

Resistance can evolve in response to the OX5034 releases through two primary mechanisms:
(1) mosquitoes with the OX5034 trait can evolve genetic resistance resulting in increased larval survival rates and (2) local mosquitoes can evolve behavioral resistance to avoid mating with OX5034 males.

OX5034 *Ae. aegypti* contains a dominant lethal trait, which results from the overproduction of the tTAV-OX5034 protein in female mosquitoes, mediated by a positive feedback loop circuit. The overproduction

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of the protein is thought to interfere with gene expression within the insect cell through a mechanism called transcriptional squelching (Gong et al. 2005). The level of tTA expression in Tet-OFF systems was shown to be positively correlated with the level of penetrance of the lethal trait in other insects (Fu et al. 2007). Thus, mutations affecting the efficiency of the OX5034 *Ae. aegypti* gene circuit may result in resistance. Further, genetic backgrounds have been hypothesized to play a role in resistance development through epigenetic effects (Knudsen et al. 2020). However, penetrance of the OX5034 sex-specific lethal trait has been shown to be 100% in OX5034 homo-and hemizygous individuals of the LWT background as well as in hemizygous individuals collected from the field resulting from matings with a genetically distinct background and thus the risk from epigenetic effects is expected to be negligible (Unit II.B.3.a).

Spontaneous mutations naturally occur in organisms and contribute to genetic diversity. Resistance to the lethal trait may develop through mutations of genetic elements that are associated with the function of the positive feedback loop gene circuit, thereby lowering the production of *tTAV-OX5034* below a tolerance threshold. The genetic circuit engineered into OX5034 *Ae. aegypti* exploits the endogenous transcriptional machinery of the mosquito, such as the pre-mRNA splicing machinery. Mutations in these conserved parts of the cellular machinery are expected to carry a significant fitness cost for the individual as other essential functions of the cell would likely also be affected. Additionally, only a subset of mutations in the genetic cassette would have the potential to affect the function of the positive feedback loop in a meaningful way. For example, some mutations may be silent in that they do not change the amino acid sequence of tTAV-OX5034 and others may occur in parts of the *tTAV-OX5034* gene that are unlikely to negatively affect the positive feedback loop, e.g., within linker sequences or the N-terminus of UBQ. The applicant reports that genetic resistance to the OX5034 trait has not been observed in 27 generation equivalence of OX5034 nor as part of the field releases involving over 12 million OX5034 homozygous males and during the EUP, Oxitec will continually monitor for the occurrence of genetic resistance. Considering these lines of evidence together, the likelihood for genetic resistance to occur during the field releases is negligible.

Although uncommon, resistance through assortative mating where local females preferentially mate with local males rather than modified males has been reported in other modified insect release programs (e.g., sterile insect technique programs). In these instances, this has typically been linked to a loss of quality in the mass-reared insects where local females do not mate with "lesser-quality" males, thereby resulting in a reduction of product efficacy. Loss of quality in mass-reared insects can be reversed by increasing genetic diversity through outbreeding. However, there is an instance of resistance via assortative mating in melon flies that was not linked to a decline in mass-reared insect quality (Hibino and Iwahashi 1991, Koyama et al. 2004). Existing variation in female preference coupled with strong selective pressure for females who preferentially mated with local males led to a change in mating preference in this population. In this instance, increasing the number of modified male melon flies overcame the resistance in local females (Koyama et al. 2004). There is evidence of rapid evolution in mating preference of *Ae. aegypti* mosquitoes (Bargielowski et al. 2013, Bargielowski and Lounibos 2014), indicating that the potential for resistance through changes in mating preference is possible for this species. However, if behavioral resistance were to evolve, the impact on humans or non-target organisms would be negligible as this would not result in any new risk or exposure scenario and would instead result in decreased efficacy of the OX5034 releases through lack of successful matings. A reduction in efficacy would not pose an increased risk from nuisance biting or disease vectoring from the local *Ae. aegypti* population because similar mosquito abatement activity will occur in both treated and untreated areas during the proposed EUP.

The continued mosquito abatement activity can also play a role in combating resistance. By using a combination of mosquito control efforts, this creates a scenario in which the target population faces a greater variation in selection pressures (Leftwich et al. 2016). This strategy can give rise to more sustainable pest control over the long term, as the selection pressure for resistance is weaker and presented as a moving target. Additionally, the combination approach ensures that mosquitoes are killed even if they are resistant to one of the approaches applied.

D. Environmental Effects Assessment

1. Ecological effects data

Oxitec submitted scientific rationale to fulfill most non-target organism data requirements for the proposed EUP. The applicant also conducted studies to support a conclusion of no adverse effect to freshwater fish or freshwater invertebrates. A summary of these data is provided in Table 7 and within the risk assessment below, and full review of the rationale is contained within the DERs. Some of the references cited in this assessment were included in rationale provided by the applicant within the MRIDs cited. Other references were included from the open literature that pertained to specific topics discussed below.

Table 7. Status of data submitted to comply with nontarget organism data requirements published in 40 CFR § 158.2060.

Data Requirement	OPPTS Guideline No.	Results Summary and Classification	MRID No.
Avian Acute Oral Toxicity Avian Dietary Toxicity Wild Mammal Toxicity Terrestrial Plant Toxicity (Seedling Emergence) Terrestrial Plant Toxicity (Vegetative Vigor) Nontarget Insect Testing	850.2100 850.2200 850.2400 850.4100 850.4150 880.4350	Rationale submitted provides sufficient information to determine that toxicity of OX5034 mosquitoes to nontarget organisms is not expected for this use based on limited exposure and lack of sequence homology of OX5034 rDNA proteins to known mammalian toxins. Rationale submitted also provides sufficient information regarding the role of *Ae. aegypti* in ecosystem food webs (e.g., food source, pollinator) to determine that indirect impacts of OX5034 mosquitoes are negligible. **Classification: Acceptable.**	50889409 50889410 50889422 50889411 50889412 50889413
Freshwater Invertebrate, LC_{50}	850.1010	In a 96-hour flow through acute toxicity study, American signal crayfish (*Pacifastacus leniusculus*) were fed OX5034 mosquitoes daily at a rate of 700 g mosquito larvae/kg-diet. No mortality or adverse sub-lethal effects were observed. No hazard to freshwater invertebrates is expected. **Classification: Acceptable.**	50698707 50889407

Fish Acute Oral Toxicity, Freshwater	850.1075	In a 14-day semi static-renewal acute toxicity study, guppies (*Poecilia reticulata*) were fed OX5034 mosquitoes daily at a rate of 700 g mosquito larvae/kg-diet. No mortality or adverse effects were observed. No hazard to freshwater fish is expected. **Classification: Acceptable.**	50698708 50889408
Endangered Species Assessment	N/A	A literature review was provided as an analysis of the potential impact of OX5034 *Aedes aegypti* on threatened and endangered species or critical habitat. **Classification: Acceptable.**	50889414

2. Ecological exposure and risk characterization

The pesticidal effect of OX5034 is species-specific as it only effects the reproductive success of *Ae. aegypti* through mating between OX5034 *Ae. aegypti* males and local *Ae. aegypti* females that are already present in the release area. With continued field releases of OX5034 homozygous males, the number of *Ae. aegypti* in the treatment area is thought to progressively decline due to the reduced number of females emerging at each consecutive generation. There is also the potential for OX5034 mosquitoes to be released on an area-wide scale, which could result in population level decline of *Ae. aegypti.* Possible adverse effects to non-target organisms from OX5034 releases are two-pronged: direct effects from oral consumption of OX5034 mosquitoes and indirect effects on ecosystem processes from reduced *Ae. aegypti* populations. Both possibilities are evaluated in this risk assessment. To understand the potential exposure to non-target organisms by OX5034 mosquitoes as well as to understand potential indirect impacts of their continued release, a review of basic *Ae. aegypti* biology is presented.

Originating in sub-Saharan Africa, *Ae. aegypti* is believed to have been introduced to the Americas in the 17th century (Nelson 1986, Powell and Tabachnick 2013). While *Ae. aegypti* historically bred in tree holes and other phytotelmata, it is now well adapted to humans, flourishes in urban areas, and can breed in a number of artificial containers. After mating, female *Ae. aegypti* produce a batch of 100 to 200 eggs and lay their eggs at several different breeding containers. Larval and pupal development occur in these breeding containers, completing the life cycle with adult emergence.

Typical larval habitats include stagnant water with organic matter and can range from tree holes and rock pools to bottle caps and tires (Barrera et al. 2008, Delatte et al. 2008, Jansen and Beebe 2010). *Aedes aegypti* usually uses man-made containers such as gutters, water containers, cans, and tires as breeding sites. The use of these containers as a larval habitat reduces the risk of exposure to non-target organisms, thereby reducing the risk of any direct adverse effect on non-targets. In terms of indirect effects, such as ecosystem impacts from a reduction of the local *Ae. aegypti* population as a result of OX5034 releases, *Ae. aegypti* is a non-native species in the US and has therefore not likely co-evolved with other organisms in the ecosystem and does not represent a keystone species on which other organisms rely for biological processes (Juliano and Lounibos 2005).

Although the likelihood of *Ae. aegypti* playing a major ecological role is low, mosquitoes can play a number of roles in the environment such as pollinator, detritivore, or food source. These roles can also be relatively diverse as the mosquito life cycle spans both aquatic and terrestrial habitats. Larvae live in

water and they can act as food for other aquatic organisms. Mosquito larvae themselves eat microscopic matter like decaying leaves and other organic detritus. As adults, mosquitoes make up part of the diet of some insect-eating animals, such as birds, bats, adult dragonflies, or spiders. However, most mosquito predator species are generalist feeders that do not depend on the presence of any single prey species for survival. In addition to acting as a food source to other organisms, mosquitoes may also act as pollinators due to their consumption of nectar. Each of these points is discussed in further detail in the below subsections.

a. Terrestrial animals and plants

i. Birds and mammals

Birds and wild mammals (e.g., bats) will be exposed to OX5034 mosquitoes primarily through ingestion of OX5034 adults and larvae as prey. Based upon bioinformatic analyses, neither DsRed2-OX5034 or tTAV-OX5034 are known to share significant sequence homology with known toxins (MRID 50889420). Both of these proteins are predicted to be susceptible to several proteases found in the human gastric system (i.e., pepsin, trypsin, chymotrypsin) based upon bioinformatics analysis (MRID 50889420), and thus proteins are expected to be broken down following ingestion. Based upon bioinformatics analyses, both DsRed2-OX5034 and tTAV-OX5034 are also predicted to be susceptible to an environmental protease (i.e., proteinase K) and are thus expected to degrade under field conditions. While several variants of DsRed can sometimes exhibit toxic effects when expressed within living cells, oral consumption and subsequent digestion would result in protein degradation thereby making uptake of the intact protein into cells following ingestion unlikely. Because biting females will not be released, wild birds and mammals will not serve as bloodmeals for mosquitoes carrying tTAV-OX5034 and DsRed2-OX5034 proteins, thus excluding this as an exposure pathway to these proteins. Therefore, direct adverse effects are not expected in birds or wild mammals as a result of release of OX5034 male mosquitoes.

Because OX5034 mosquitoes have the potential to be used on an area-wide scale to suppress local *Ae. aegypti* mosquito populations, it is possible that birds or wild mammals could be indirectly affected through the reduction of *Ae. aegypti* as a food source. Several types of birds including most varieties of swallows, warblers and other songbirds consume mosquitoes among other flying insects. However, the mosquito is likely to form only a small part of the bird diet. Barn swallows for instance, feed at lower heights where mosquitoes are more likely to fly, but due to the small size of the mosquito, they instead tend to prefer larger insects such as flies or dragonflies. 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, reports of foraging studies have not found that mosquitoes constitute a significant portion of the Purple Martin diet (Wiggens 2005), and instead mosquitoes typically do not make up more than 3% of the Purple Martin diet (American Mosquito Control Association). In contrast, one study examining Western Bluebirds, *Sialia mexicana*, in California vineyards found that the most common arthropod prey consumed was *Aedes* (species not identified) among 66 species identified in fecal samples (Jedlicka et al. 2017). Although samples from adults and nestlings indicated that 51% and 49%, respectively, contained *Aedes* as prey, the study did not provide an indication as to the proportion of the diet that *Aedes* comprised. Conversely, stomach content analyses in another study did not note *Aedes* as a food item for Western Bluebirds. Jedlicka et al. (2017) hypothesizes that a difference in habitat (vineyard

vs. woodland) and the generalist nature of bluebird foraging is a likely explanation for the high frequency of detection of *Aedes* in the Western Bluebird diet found in vineyards compared to other studies.

Insectivorous bats are often anecdotally regarded to be a significant predator of mosquitoes and are thought to eat large quantities of mosquitoes. However, in areas where larger, more nutritious insect prey are available, bats do not consume large numbers of mosquitoes as they do not constitute significant calories or nutrients relative to the task of predating upon them (Gonsalves et al. 2013, Wetzler and Boyles 2018). For example, a study examining the fecal pellets for the little brown bat, *Myotis lucifugus*, found that their natural diet was composed of only 1.8% mosquitoes (Whitaker and Lawhead 1992) and the diet of the big brown bat, *Eptesicus fuscus,* was dominated by beetles, moths, and mayflies (Clare et al. 2014). Under certain conditions, such as colder nights where larger insects were less available or when female bats are lactating, Diptera, including mosquitoes and crane flies, may constitute a larger portion of the diet of the southeastern brown bat, *Myotis austroriparius*, in Florida (Zinn and Humphrey 1981). However, the diversity of the diet of this insectivorous bat increased considerably during warmer temperatures (i.e., most spring and summer nights like when OX5034 releases are proposed). In conclusion, as potential bird or wild mammal predators of *Ae. aegypti* mosquitoes are more generalist in nature and do not rely on *Ae. aegypti* as their primary food source, indirect adverse effects such as a reduction in an important food source are not expected as a result of release of OX5034 male mosquitoes.

ii. Nontarget insects

Although the adult stage of *Ae. aegypti* is terrestrial, the larval stage is aquatic and preferred oviposition sites are usually man-made containers such as gutters, water containers, cans, and tires. As such, there is reduced risk of exposure to terrestrial non-target insects and reduced likelihood that terrestrial non-target insects specialize in *Ae. aegypti* larvae as a food source. Dragonflies are known to eat adult mosquitoes; however, they also consume butterflies, moths and smaller dragonflies which serve as significant energy sources. Due to this large variety of food sources and the relative lack of energy provided by mosquito consumption, mosquitoes are likely not an essential part of their diet (Pfitzner et al. 2015).

EPA also finds the likelihood of adverse effects to insect species should they consume OX5034 mosquitoes to be low. A submitted feeding study with an aquatic invertebrate found that the test organisms were not adversely affected when fed OX5034 larvae and exhibited no signs of toxicity or mortality (see Unit II.D.2.b for additional detail). The lack of adverse effects observed in an aquatic invertebrate from ingesting OX5034 mosquitoes is also expected for terrestrial invertebrates.

The risk of transfer of the OX5034 cassette to other insect species through mating with OX5034 mosquitoes is highly unlikely as mating in mosquitoes is species specific and depends on time of day, swarming behavior, and wing beat/tone matching. *Ae. aegypti* and *Ae. albopictus* share similar mating habitats and behaviors and therefore the risk of transfer of the OX5034 cassette is likely highest between these two species. However, forced laboratory matings between *Ae. aegypti* and *Ae. albopictus* yielded eggs that were not viable (Lee et al. 2009, Nazni et al. 2009). Therefore, the likelihood of transfer of the OX5034 cassette to other insect species is negligible.

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The risk of transfer of OX5034 background strain genetics to local *Ae. aegypti* mosquito populations was also considered. The potential for introgression along with an evaluation of key mosquito traits are discussed in Unit II.C.3 and was determined to be of negligible risk.

iii. Nontarget plants

Although female *Ae. aegypti* mosquitoes take blood meals from humans, mosquitoes of both sexes require plant juices as an energy source. Therefore, OX5034 male mosquitoes are likely to encounter plants during the experimental releases. Floral nectaries are the best-known sources of sugars and amino acids for insect pollinators, but mosquitoes are also known to obtain sugars from extra-floral nectaries, damaged fruits, damaged and intact vegetative tissues, and honeydew (Clements 2000). *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 (Martinez-Ibarra et al. 1997). However, given that DsRed2-OX5034 and tTAV-OX5034 are expressed in OX5034 tissues within the confines of its chitinaceous exoskeleton, both proteins are unavailable to plants, therefore resulting in negligible exposure.

There is limited information on the pollination of plant species by mosquitoes in general, though related *Aedes* spp. are known pollinators of the orchid species *Platanthera obtusata*, which is widely distributed across the Pacific Northwest, around the Great Lakes, and in parts of New England (Thien 1969, Gorham 1976). There are no reports that *Ae. aegypti* is a pollinator for any plant species but a laboratory study demonstrated that *Ae. aegypti* is attracted to the scent of the orchid *P. obtusata* and thus is physiologically capable of being a pollinator for this plant (Lahondere et al. 2020). It is important to note that *P. obtusata* is found outside of the experimental permit area, although even if it were found in the experimental permit area, it is highly unlikely that *P. obtusata* would be reliant on *Ae. aegypti* as a primary pollinator 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 (Patiny 2012). Therefore, due to the lack of dedicated pollinator activity by *Ae. aegypti*, any reduction in the local mosquito population as a result of OX5034 male releases is not expected to adversely impact plant populations.

b. Aquatic animals and plants

Aquatic organisms which feed on mosquitoes are regarded as generalized predators. Aquatic invertebrate predators, such as larvae from the Coleoptera (beetles), Diptera (flies), Hemiptera (true bugs), and Odonata (dragonflies) orders are known to prey on mosquito larvae (Shaalan and Canyon 2009). Mosquitoes themselves are known to prey on the larvae of other species. For example, *Toxorhynchites splendens*, also known as the elephant mosquito or mosquito eater, consume larvae of other mosquitoes as well as other aquatic organisms (Amalraj et al. 2005). In addition to arthropods, nematodes can also prey upon mosquito larvae. The nematodes from the family Mermithidae are generalist parasitoids infecting a number of mosquito species (Paily and Balaraman 2000, Achinelly and Micieli 2013). Because *Ae. aegypti* usually use man-made containers such as gutters, water containers, cans, and tires as breeding sites, there appears to be no specific predator that preys upon this species but rather predators that are generally opportunistic and feed on larvae if and when they encounter them. Therefore, it is unlikely that

a reduction in *Ae. aegypti* would adversely impact nontarget organisms. To evaluate the direct impact on nontarget aquatic invertebrate organisms through oral consumption of OX5034, a submitted study tested the potential toxicity of OX5034 mosquitoes to an aquatic invertebrate. A feeding study examined the American signal crayfish (MRID 50698707) and found no apparent or measurable toxicity to the test organisms when fed OX5034 mosquitoes over a 96-hour test period. Given the limited timeframe and acreage associated with the experimental permit, and the lack of toxicity observed through a feeding study, EPA finds the direct risk from OX5034 mosquitoes to aquatic invertebrates to be low. However, as many of the aquatic insects that may consume OX5034 larvae are larvae themselves and thus more susceptible to even low-level toxins, additional certainty regarding the lack of toxicity to aquatic insect larvae could be gained through a larval feeding study prior to a Section 3 registration.

In terms of aquatic vertebrates, a submitted study tested the potential toxicity of OX5034 mosquitoes fed to guppies (MRID 50889408) to evaluate the direct impact of oral consumption of OX5034 mosquitoes on nontarget aquatic vertebrate organisms. The study found no apparent or measurable toxicity over the 14-day test period, indicating that the likelihood for adverse effects to nontarget aquatic vertebrates from consumption of OX5034 mosquitoes is low. While it is not known that any aquatic vertebrates have evolved to specifically target *Ae. aegypti* mosquitoes as a major portion of their diet, in some instances, mosquitoes can constitute a significant source of prey. For example, amphibians have the capacity to consume large quantities of mosquito larvae, and a study showed that in the laboratory, 200-400 3rd instar larvae of *Culex* species per day could be consumed by salamander species. However, these numbers were seen when the *Culex* mosquitoes were the only food source and there was no prey choice (DuRant and Hopkins 2008). In the field, the Tiger Salamander, *Ambystoma tigrinum*, was found to readily consume mosquito (*Culicidae*, species not identified) larvae based on 26% of analyzed stomach samples containing remnants of larvae (Brodman and Dorton 2006). However, it is important to note that *Ae. aegypti* is but one of hundreds of species of mosquitoes and it does not appear that the salamanders noted above feed significantly on *Ae. aegypti* larvae. Mosquito larvae are also eaten by a number of fish including guppies, bass, catfish, bluegills and even goldfish. The most effective species for eating mosquito larvae are the mosquito fish, *Gambusia affinis* and *Gambusia holbrooki*, which are specialized predators. Due to the preferred larval habitat of *Ae. aegypti* mosquitoes, exposure to vertebrate predators is expected to be limited, therefore also limiting the role *Ae. aegypti* play in the predator diet.

Due to a lack of toxicity observed in feeding studies and the generalist nature of most predators that may feed on *Ae. aegypti*, direct and indirect adverse effects are not expected in aquatic nontarget organisms as a result of release of OX5034 male mosquitoes.

c. Microbes

EPA also considered the possibility of the spread of antibiotic resistant bacteria in the environment from the release of OX5034 mosquitoes. The tTAV-OX5034 expression is made female-specific by inclusion of a splicing module that has been linked to the tetracycline-off (Tet-OFF) system. The Tet-OFF system activates tTAV-OX5034 expression in females in the absence of a tetracycline analogue resulting in a lethal phenotype, therefore all OX5034 females die in the absence of tetracycline analogues. However, if a suitable tetracycline analogue is added to the larval rearing medium in sufficient quantities, tTAV-OX5034 expression is repressed, allowing for normal development of females to adulthood.

Although tetracycline analogues will be used in the OX5034 colony in the UK, no tetracyclines will be used in US facilities used to produce OX5034 male adults for release or in the release devices for field deployment of OX5034 mosquito eggs (e.g., rearing boxes). Because antibiotics are not used in the facilities or release devices for OX5034 males, there is no selective pressure for antibiotic resistant bacteria to evolve, thereby greatly reducing the likelihood that OX5034 males for release would carry antibiotic resistant bacteria.

However, as the OX5034 colony in the UK will be reared using antibiotics, the presence of antibiotic resistant bacteria in the mosquito microbiome of the OX5034 colony is possible. Therefore, an additional consideration is whether vertical transmission of antibiotic resistant bacteria could occur and OX5034 male mosquitoes for release could acquire antibiotic resistant bacteria through this route. While OX5034 eggs shipped to the US could have some bacteria on their surface, any bacteria that survived shipping would likely comprise only a negligible portion of the OX5034 microbiome. This is largely due to the fact that, although some organisms directly acquire their gut microbiota from their parents, mosquitoes predominantly acquire their gut microbiota from their environment as larvae (Strand 2018). Furthermore, as the environment during development of OX5034 males in the US for release does not contain antibiotics, there is a lack of selective pressure to maintain any antibiotic resistant bacteria that may have been on the eggs shipped from the OX5034 colony.

Therefore, due to the lack of antibiotics used in release devices or in US facilities used to produce OX5034 male mosquitoes for release, coupled with the fact that mosquitoes generally acquire their microbiome from their environment, the risk that released OX5034 male mosquitoes would spread antibiotic resistant bacteria in the environment is very low.

3. Impacts on endangered species

EPA has determined that no adverse effects are anticipated for nontarget organisms as a result of the experimental permit to release OX5034 mosquitoes. Therefore, since adverse effects are not anticipated to nontarget organisms, a "No Effect" determination is also made for direct and indirect effects to federally listed endangered and threatened species, and for their designated critical habitats.

III. HUMAN HEALTH & ENVIRONMENTAL RISK CONCLUSIONS

EPA has reviewed the OX5034 manufacturing process detailing the production and quality assurance processes used in the development and manufacture of OX5034 mosquitoes, associated standard operating procedures, and other pertinent information characterizing OX5034 mosquitoes on a genetic and phenotypic level. EPA determined this information to be adequate to support a finding of no unreasonable adverse effects to man and the environment during the proposed EUP.

EPA has determined that there will be no unreasonable adverse effects for humans as a result of the experimental permit to release *Ae. aegypti* OX5034 male mosquitoes provided such releases do not take place within 500 m of commercial citrus growing areas or wastewater treatment sites due to considerations regarding the impact of environmental sources of tetracyclines on female OX5034 mosquito survival. A compilation of release recapture studies around the world found that most

Ae. aegypti are recovered within 20 m to 50 m of the release point, with a small percentage found 170 m but generally not more than 200 m from the release point. Therefore, a restriction of 500 m from potential sources (200 m for released OX5034 males + 200 m for mated *Ae. aegypti* females + 100 m of additional buffer) provides a conservative buffer zone. The human health assessment considered data provided on the mammalian toxicity and allergenicity of the tTAV-OX5034 (active ingredient) and DsRed2-OX5034 (inert ingredient) proteins and the potential routes through which humans may be exposed to these substances as a result of OX5034 application. While no determination has been made on the potential of either protein to pose mammalian hazard, the human health risk was found to be negligible, as exposure to female mosquitoes carrying these traits was determined to be negligible given that the penetrance of the tTAV-OX5034 lethal trait was shown to be 100% in female mosquitoes and the restrictions on access to potential tetracycline sources.

EPA has determined that there will be no unreasonable adverse effects for humans or the environment due to introgression of OX5034 background strain genetics into the local *Ae. aegypti* population. EPA evaluated OX5034 mosquitoes for key traits that could increase the ability of mosquitoes to transmit disease, result in larger populations numbers, or result in more robust mosquitoes. Based on a combination of laboratory data, meta-analyses, and a review of the scientific literature, EPA finds it is unlikely that the local mosquito population would pose any increased risk to humans or the environment as a result of releases of OX5034 mosquitoes and introgression of OX5034 background strain genetics.

EPA has also determined that no unreasonable adverse effects are anticipated for non-target organisms as a result of the experimental permit to release *Ae. aegypti* OX5034 male mosquitoes. No direct adverse effects due to consumption of OX5034 males by non-target organisms is expected based on acute oral toxicity studies and bioinformatics analyses. There are also no indirect adverse effects anticipated from reduction in *Ae. aegypti* as a food source should the release of OX5034 mosquitoes successfully reduce the local *Ae. aegypti* population. In the case of *Ae. aegypti*, their status as invasive species and their oviposition choice behavior makes it less likely that they serve an integral role in newly invaded ecosystems. Additionally, *Ae. aegypti* are regularly subjected to other control methods such as insecticide treatment and source reduction and it is therefore unlikely any predator species or plant is dependent on *Ae. aegypti* presence.

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