UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

SUBJECT: Summary of the Data and Information Related to Vectorial Capacity Presented for the New Product OX5034 (EPA File Symbol: 93167-EUP-E) Containing the Tetracycline-Repressible Transactivator Protein Variant (tTAV-OX5034), a Variant of the Modified *Discosoma* spp. DsRed2 Protein, 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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I. BACKGROUND

Oxitec Ltd., (Oxitec or the applicant) requests an Experimental Use Permit (EUP) under FIFRA section 5 for a new end-use product containing the new active ingredient tetracycline-repressible transactivator protein variant (tTAV-OX5034) protein, the new inert ingredient DsRed2-OX5034 protein, 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-occuring *Ae. aegypti* populations under field conditions.

The applicant requests a 24-month EUP for a cumulative annual test area of 6,600 acres. The area is divided into multiple test and control plots within Monroe, Co., Florida and Harris, Co., Texas. Under the EUP, Oxitec is planning to test the efficacy of the product by deploying eggs, pupae, and adults homozygous for the OX5034 trait.

OX5034 is described as a species-specific female larvicide, or "male-selecting" larvicide, that results in all-male progeny in the absence of tetracycline in the larval diet due to a female-specific self-limiting gene. With continued field releases of OX5034 homozygous males, the *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 wild *Ae. 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 wild female would not inherit the self-limiting gene but would still inherit OX5034 strain genetics. This results in both male and female mosquitoes with OX5034 strain genetics.

Additionally, a recent paper examining the applicant's 1st generation product, OX513A, found evidence of introgression after releases in Brazil of males containing the self-limiting tTAV gene (Evans et al. 2019). Although that paper investigated OX513A mosquitoes, the 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 higher larval survival rates (approx. 5% in OX513A versus 50% in OX5034). Therefore, given that introgression of OX5034 strain genetics will occur during releases, it is pertinent to examine potential associated risks for humans and the environment. Traits associated with a disease vectoring species such as *Ae. aegypti* that may carry risk if introgressed into a wild population are likely to be linked to vectorial capacity, including vector competence, fecundity, and longevity.

The U.S. Food & Drug Administration regulates mosquito-related products that are intended to reduce the viral/pathogen load within a mosquito or are intended to prevent mosquito-borne disease in humans or animals (USFDA, 2017). EPA, on the other hand, regulates products intended to reduce the population of mosquitoes as pesticides, such as OX5034. Under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), EPA evaluates pesticide products to ensure that the products do not cause unreasonable adverse effects on humans or the environment. Consistent with the FIFRA standard, EPA is reviewing several factors that could

contribute to the ability of the OX5034 mosquito to serve as a disease vector from the perspective that an *increased* ability to vector disease could result in a risk to humans or the environment.

Oxitec submitted data and information on OX5034 vector competence, fecundity, and lifespan to address concerns related to the vectorial capacity of OX5034 mosquitoes relative to wild-type mosquitoes already present in the environment.

II. SUMMARY

Aedes aegypti is a known vector for the viruses causing yellow fever, dengue, chikungunya, and Zika. Because introgression of OX5034 strain genetics into the local *Ae. aegypti* population is expected, an understanding of the ability of the OX5034 mosquito to serve as a disease vector is necessary in estimating the potential risk associated with such introgression.

Vectorial capacity is influenced by a number of traits impacted by gene-environment interactions and is confounded by both intrinsic and extrinsic variables (reviewed in Beerntsen et al. 2000). The applicant stated that during releases of OX5034 mosquitoes, the local population is expected to decline and therefore should have a reduced vectorial capacity due to decreased population density. Although this is true, the potential risk from introgressed strain genetics is expected to manifest post-releases. 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 strain genetics due to inundation by OX5034 mosquitoes during releases. This creates the potential to result in an increased frequency of OX5034 strain genetics in the recovered population, thereby altering the population genetics/traits of the local mosquito population.

The applicant mentioned that natural immigration of *Ae. aegypti* happens frequently as the species is known to hitchhike on human modes of transportation such as cars, trucks, and boats. Due to this natural immigration, the applicant stated that vector competence traits could also introgress through these mosquitoes. Although this is true, the continuous, inundative releases proposed in the EUP are likely to result in gene flow rates far higher than expected from natural immigration, thus necessitating evaluation.

EPA considered three traits influencing vectorial capacity to determine whether introgression of OX5034 strain genetics into the wild mosquito population might pose an increased risk: vector competence, fecundity, and longevity. Discussion of each of these traits along with data and information provided by the applicant can be found below. Discussion is also provided on the concepts of "hybrid vigor," in which the crossbreeding of two different genetic backgrounds results in offspring that is superior to both its parents, and the opposite scenario "outbreeding depression," in which the offspring are less viable.

Vector competence

Vector competence refers to the intrinsic ability of a vector to acquire, maintain, and transmit an infection. Vector competence is associated with several anatomic barriers to productive vector

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infection in the mosquito, including a midgut infection barrier, a midgut escape barrier, and a salivary gland barrier. A pathogen must be able to move past each of these barriers for disease transmission to be possible. Although vector competence can be measured in a number of ways, a common practice is to measure the presence of the pathogen in either the heads or salivary glands of the mosquito as a proxy for the saliva (Souza-Neto et al. 2019). However, it should be noted that the actual transmission rate is likely to be lower than the dissemination rate determined from head or salivary glands due to the salivary gland escape barrier.

Although mosquito genetics play a role in vector competence (Beerntsen et al. 2000), pathogen genetics are also a significant factor (Souza-Neto et al. 2019). Additionally, vector competence is impacted by a number of non-genetic factors such as larval habitat/competition, temperature, humidity, and vector microbiota (Beerntsen et al. 2000, Lefevre et al. 2013, Souza-Neto et al. 2019).

Due to environmental factors, viral strain variation, and mosquito population genetic variation, vector competence can vary significantly within a mosquito species both geographically and temporally. For example, a study measuring vector competence for DENV-2 among 24 collections of *Ae. aegypti* from Mexico and the United States found a wide range of results. The vector competence of tested populations, as measured by the percentage of mosquito heads infected with the virus, varied significantly from 24% to 83% (Bennett et al. 2002). A similar study measured variation in *Ae. aegypti* competence for ZIKV transmission and found that presence of the virus in the saliva 14 days post infection varied across geographically distinct populations from 8-51% (Garcia-Luna et al. 2018).

Oxitec provided two case studies of vector competence data for Florida (one of the proposed EUP states) using data summarized by Souza-Neto et al. 2019. The applicant only included studies where comparable viral loads were tested. Vector competence was reported as infection rate (IR), which was measured by the percentage of mosquitoes that tested positive for the virus. However, different studies measured IR across different body parts (e.g., midgut, head, legs), which limits the ability to compare across studies.

Case Study 1: Neighboring Vector Populations Differ Significantly.

The applicant compared infection rates of chikungunya in *Ae. aegypti* from five Florida counties: St. Lucie, Manatee, Monroe, Palm Beach, and Vero Beach (Table 1). Only infection doses of 6 log10 and greater were considered by the applicant. As previously stated, different studies measured IR in different body parts.

Table 1. Infection rates of *Aedes aegypti* with CHIKV virus strains in five Florida counties. IR= infection rate, dpi= days post-infection. Table modified from MRID 50973401 to only show infection doses of 6 log10 and greater as considered by the applicant, and to include additional information on how IR was measured, data from Souza-Neto et al. (2019).

Aedes aegypti origin	Virus genotype/strain	Infection dose	IR, 2-3 dpi	IR, 5-7 dpi	IR, 10-15 dpi	IR, No dpi given	Body part "IR" was measured

Indian River / St. Lucie County	CHIKV	8 log10	37	71	28		legs
Manatee County	CHIKV	8 log10	90	20	54		legs
Monroe County	CHIKV	8 log10	71	68	60		legs
Palm Beach	CHIKV LR2006-OPY1, La Reunion	6.1 log10		18.8			legs (pledget blood feeding)
Palm Beach	CHIKV LR2006-OPY1, La Reunion	6.1 log10		57.7			legs (membrane blood feeding)
Vero Beach	CHIKV 06.21	7.5 log10		100	100		head
Vero Beach	CHIKV 05.115	7.5 log10				100	head

Despite the limitations of the meta-analysis, the values from St. Lucie, Manatee, and Monroe counties were measured in the same study using the same methodology and can therefore provide information of value. In the study by Alto et al. (2017), mosquitoes were collected across the three Florida counties as well as the Dominican Republic. The study found that the dissemination rate, measured as the percent of infected legs from the total number engorged with blood, varied significantly across the counties. Similarly, the study found that transmission rate, as measured by the percent of mosquitoes with infected saliva from the total number of mosquitoes with infected legs, also differed significantly across counties (Alto et al. 2017). The study therefore demonstrates that different geographic populations of *Ae. aegypti* in the state of Florida can indeed vary significantly in their vector competence. This degree of variation across a single state would make it difficult to identify a meaningful comparator strain if the applicant were to pursue vector competence studies.

Case Study 2: Vector Competence in a Single Location Varies with Virus Serotype.

Oxitec also submitted an analysis of studies that took place in Key West, Florida to demonstrate variability in competence by serotype. Here, different dengue strains and serotypes were tested. Again, IR was measured across different mosquito body parts and some measurements from the same mosquito sample were reported as independent measurements. For example, the two measurements for DENV-2/US/BID-V1041/2006 infection dose 7.1 ± 1.2 log10 as reported in Table 2 were taken from the same study, where row 6 reports IR measured in the midgut and row 7 reports the dissemination rate as measured by viral presence in the legs of infected mosquitoes from row 6. The two measurements for DENV-1/US/BID-V852/2006 infection dose 6.8 ± 0.5

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log10 were reported in the same way (Table 2, rows 1 and 2). Measurements for vector competence for DENV-1 BOLKW010 infection dose 3.7 log10 in rows 4 and 5 contain values from Richards et al. (2012), some of which were misreported specifically: (1) the reported measurements are from Stock Island rather than Key West (values from both sites were reported in the study); (2) for each row, the two measurements reported under "IR, 21 dpi" were actually taken at 14 dpi; and (3) for each row, the first column under "IR, 21 dpi" reports the IR and the second column under "IR, 21 dpi" reports the dissemination rate. Based on Table 1 in Souza-Neto et al. 2019, the applicant has misreported IR values based on dpi for other cited studies as well.

Table 2. Infection rates of *Aedes aegypti* with DENV1 and DENV2 serotypes in Key West, Florida. IR= infection rate, dpi= days post-infection. Table reproduced from MRID 50973401, data from Souza-Neto et al. (2019). Modified to include row numbers and to include additional information on how IR was measured.

	Aedes aegypti origin	Virus genotype/strain	Infection dose	IR, 5-7 dpi	IR, 10-15 dpi	IR, 21 dpi		Body part "IR" was measured
1	Key West	DENV-1/US/BID-V852/2006	6.8 +/- 0.5 log10	10	6			midgut
2	Key West	DENV-1/US/BID-V852/2006	6.8 +/- 0.5 log10	14	88			legs
3	Key West	DENV-1 BOLKW010	6.3 +/- 0.2 log10			93	80	midgut (1st value), legs (2nd value)
4	Key West	DENV-1 BOLKW010	3.7 log10			89	100	midgut (1st value), legs (2nd value)
5	Key West	DENV-1 BOLKW010	3.7 log10			75	33	midgut (1st value), legs (2nd value)
6	Key West	DENV-2/US/BID-V1041/2006	7.1 +/- 1.2 log10		28	28	28	midgut
7	Key West	DENV-2/US/BID-V1041/2006	7.1 +/- 1.2 log10		12	27		legs

Despite these misreported values, one study reported in Table 2 (rows 1, 2, 6, and 7) can be useful in examining how vector competence differs in the same geographic location across serotypes. In Alto et al. (2014), vector competence of *Ae. aegypti* from Key West was measured after an infectious blood meal with a dose of ~7 log10 of either DENV-1 or DENV-2. When IR was measured as viral presence in the midgut at both 7 and 14 dpi, *Ae. aegypti* in Key West were significantly more susceptible to DENV-2 than DENV-1 (Alto et al. 2014). The same pattern of

greater susceptibility to DENV-2 held when measuring dissemination rate as a percentage of infected mosquitoes with virus present in the legs. This study does indicate that the vector competence of a mosquito population from the same geographic location can vary based on the serotype of the virus.

Case Study Summary

The studies cited by the applicant indicate that vector competence of *Ae. aegypti* in Florida vary by geography and by viral strain, which is in agreement with the larger literature. The applicant also cited the Evans et al. (2019) paper which directly tested vector competence of OX513A and compared the results with that of the Rockefeller lab strain and the local Brazilian strain. That study compared infection rates of DENV-2 and ZIKV in OX513A, and although it did find different infection levels based on virus, it did not find differences in the number of genomic copies of either virus across the different mosquito populations challenged via oral infection (Evans et al. 2019). Oxitec states that due to the controlled variables typical of any lab study, these results are "direct evidence for limited impact of genetic background on vector competence." This argument is inconsistent with that presented by the applicant in Case Study 1, in which lab studies with similar controlled variables were referenced as evidence that *Ae. aegypti* vector competence varies across distinct populations. However, EPA does agree that vector competence is clearly impacted by a number of variables outside of vector genetics.

Due to the complex dynamics involved in disease transmission that are dependent on local ecology, the risk of increased vector competence in the wild population through introgression of OX5034 strain genetics is unlikely to be determined through laboratory competence evaluation studies. In summary, 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 wild mosquito population.

Fecundity

Fecundity, or reproductive capacity, is an important measure to evaluate due to its direct link to population density. An increase in *Ae. aegypti* population density can result in an increase of nuisance biting and disease transmission. Additionally, increased fecundity is a trait that is likely to be selected for in a laboratory setting (Leftwich et al. 2016) so it is a trait of relevance for colony reared mosquitoes like OX5034.

Oxitec provided fecundity data as defined by the number of eggs laid per female mosquito for the OX5034 strain (Table 3). The applicant measured egg clutch size from three cohorts of 100 OX5034-homozygous males mated with 200 Latin wild-type females. The applicant also mated 100 Latin wild-type males to 200 Latin wild-type females. The Latin wild-type is the strain that was used to produce the OX5034 line and so it provides relevant information regarding the background genetics of OX5034. "Wild-type" in this instance, refers to the absence of the self-limiting gene in the Latin strain and is not meant to denote that the strain is from "the wild," as the Latin strain has been maintained by Oxitec in the laboratory since 2006.

Table 3. Number of eggs laid per female measured from a cohort of females. Results show the mean number of eggs laid per female, assuming that all 200 starting females contributed equally to both egg collections. Table reproduced from MRIDs 50973401.

Cross	Number of cages	2nd gonotrophic cycle	3rd gonotrophic cycle
100 OX5034-homozygous males x 200 WT females	3	30 ± 4.7 eggs	40 ± 11.1 eggs
100 WT males x 200 WT females	1	53 eggs	54 eggs

The results of these matings alone are not enough to evaluate how the Latin strain and OX5034 mosquitoes compare in fecundity to actual wild-type mosquitoes. However, the applicant also provided reports on wild *Aedes aegypti* clutch sizes from the literature:

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 Latin strain and OX5034 mosquito matings are within the range expected for *Ae. aegypti*. These results indicate that OX5034 mosquitoes are not more fecund than wild-type *Ae. aegypti* mosquitoes and therefore introgression of OX5034 strain genetics is not expected to increase the fecundity of the wild population.

Longevity

The lifespan of disease vectors impacts the degree of disease transmission with a longer lifespan resulting in the potential for increased transmission. This is due to increased lifespan resulting in additional time for viral incubation and subsequent host feeding. The applicant proposes to assess OX5034 longevity as part of the EUP but also provided data on male OX5034 mosquitoes from 2018-2019 field trials in Brazil. To assess male OX5034 longevity, the applicant marked the OX5034 adult males with a colored powder dye and released them from a fixed location in

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the middle of a BG Sentinel trap network. The traps were collected daily and the released mosquitoes were identified and counted in the laboratory. The applicant found that the released male mosquitoes survived up to 7 days in the field and that the average life expectancy after release was 1.3 days.

The applicant also provided laboratory studies of OX5034 longevity (MRID 50889417). In the laboratory studies, OX5034 males (homozygous and hemizygous) and Latin wild-type strain male mosquitoes were reared and mated for two days prior to being isolated for longevity analysis. The applicant reared male mosquitoes both on and off doxycycline, but only those reared off doxycycline will be reported here as those conditions are more comparable to the field. Adults were provided with 10% sucrose solution *ad libitum*. Dead adults from all cages were removed daily and counted. Homozygous OX5034 males reared off-doxycycline had a median survival of 24 days compared to Latin wild-type males which had a median survival of 49 days. Hemizygous OX5034 males reared off-doxycycline had a median survival of 44 days compared to Latin wild-type males which had a median survival of 50 days. Homozygous OX5034 female mosquitoes and Latin wild-type female mosquitoes were reared on-doxycycline and received two blood meals (day 7 and 17) and were found to have median survivals of 42 days and 56 days, respectively.

Lifespan was found to be significantly higher in the laboratory studies than in the field mark release recapture studies, although this is to be expected given the stable environmental conditions, abundant food, and lack of predators in the lab setting. Additionally, a literature search indicates 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

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

Therefore, there is no indication that OX5034 strain genetics would confer greater longevity if introgressed into the wild *Ae. aegypti* mosquito population.

Hybrid fitness

The Evans et al., 2019 study postulated that releases of the applicant's 1st generation product, OX513A, in Brazil and the resulting introgression of strain genetics into the wild population "very likely [resulted] in a more robust population than the pre-release population due to hybrid vigor." 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 wild mosquito populations under the proposed EUP are suffering from inbreeding depression. Therefore, there is no indication that matings between OX5034 mosquitoes and wild mosquitoes would result in hybrid vigor.

The opposite of inbreeding depression and heterosis would be outbreeding depression. In this instance, genetic incompatibilities like Dobzhansky-Muller incompatibilities result in reduced fitness in hybrids between widely divergent populations or species (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. That being said, there is no indication that the OX5034 strain is so divergent from wild populations as to expect any significant degree of postzygotic isolation and therefore, there is no indication that matings between OX5034 mosquitoes and wild mosquitoes would result in outbreeding depression.

III. CONCLUSION

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 wild 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 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 wild mosquito population.

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Fecundity and longevity of the OX5034 mosquito were also evaluated. Data provided by the applicant combined with literature searches indicate that introgression of OX5034 strain genetics is unlikely to result in the increased fecundity or longevity of wild mosquitoes.

In conclusion, given 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 wild mosquito populations under the applied for EUP.

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