Appendix A

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

February 9, 2015

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

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

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

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

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

Thank you,

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

Appendix B

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

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

Appendix C

OXITEC

INTERNAL RESEARCH REPORT

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

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

3. **Title**: Investigating the tetracycline dose response of *Aedes aegypti* OX513A

4. **Statement Of Data Confidentiality Claims:**

   Confidential Business Information (CBI) has been deleted from this report

5. **Statement Concerning Good Laboratory Practices:**
   This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

6. **Authors:**

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

7. Associated Personnel:

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

8. Test Facility:

This research was performed at Oxitec's research facility located at:
46a Western Avenue,
Abingdon,
Oxfordshire,
OX14 4RU
United Kingdom

9. Objectives:

The objective of this study was to determine the lowest concentration of tetracycline that allows greater survival of *Aedes aegypti* heterozygous for the OX513A construct than when reared in the absence of tetracycline.

10. Summary:

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

11. Introduction

First developed in 2002 at Oxford University, the *Aedes aegypti* RIDL strain OX513A was originally produced by transforming the Rockefeller strain using a *piggyBac*-based construct (Phuc et al., 2007). This strategy allowed the integration of a repressible, dominant lethal system in the *Ae. aegypti* genome whereby, in the absence of tetracycline, >95% of the heterozygous progeny of mating between homozygous RIDL males and wild-type females (and of RIDL females with wild-type males) die during immature stages. This is due to the accumulation of high intracellular levels of tTAV protein produced by a positive feedback loop. However, if tetracycline is added to the larval rearing medium, tTAV expression is repressed, allowing for normal development to adulthood.

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

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

12. **Methods**

Strains

The following strains were used for this experiment:

OX513A (bi-sex lethal RIDL strain): In the absence of tetracycline (tet), this strain expresses high levels of the tTAV protein during immature stages through a positive feedback loop, resulting in high mortality. However, when reared in the presence of high enough tetracycline concentrations, expression of tTAV is repressed, allowing normal development to adulthood (Phuc et al., 2007). OX513A is in the Latin genetic background described below.

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

Insect Rearing

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

Tetracycline Concentrations

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

[1] Assessment included counting the total number of dead pupae, non-viable adults (dead adults on the water, dead adults on the floor of the cage and non-flying adults) and functional adults (flying adults).

Statistical Analysis

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

13. RESULTS

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

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

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

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

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

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

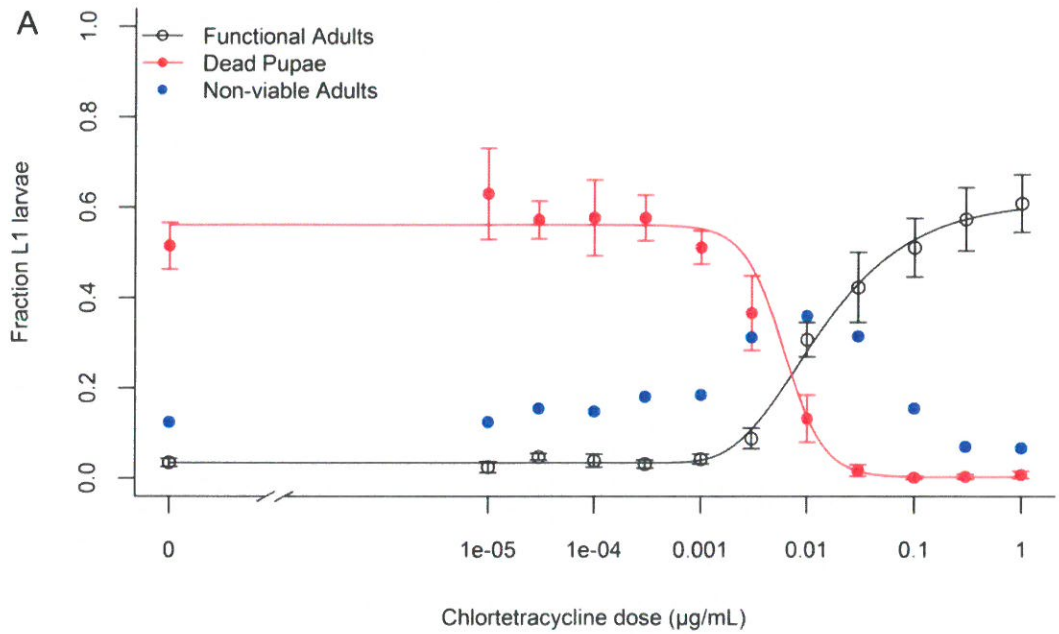

Figure 1: OX513A heterozygous larvae reared at different tetracycline concentrations. Proportion of first instar larvae represents the fraction of individuals reaching the indicated stage based on 200 starting individuals in each repeat. Points represent means from Table 1, and lines represent the model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

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

[3] Non-viable adults are a composite of dead adults on the water of the eclosion container, dead adults on the floor of the cage and non-flying adults; all these classes are considered to have zero fitness as *Aedes aegypti* court and initiate mating on the wing, and flight ability is required in the field to avoid predators and to find mate, hosts and oviposition sites.

14. **Discussion and Conclusions:**

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

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

15. **Literature:**

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

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

Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J., Pape, G., Fu, G., Condon, K.C., Scaife, S., Donnelly, C.A., et al. (2007). Late-acting dominant lethal genetic systems and mosquito control. BMC Biology 5, 11.

Le-Minh, N., S. J. Khan, et al. (2010). Fate of anitbiotics during municipal water recycling treatment processes. *Water Research* **44**: 4295-4323.

Locatelli, M.A.F., Sodre, F.F., and Jardim, W.F. (2011) Determination of antibiotics in Brazilian surface waters using liquid chromatography-electrospray tandem mass spectrometry. *Arch Environ Contam Toxicol*, 60 (385-393).

Appendix D

OXITEC

INTERNAL RESEARCH REPORT

1. **Reference Number**: PH-2013-5-V3a

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

3. **Title**: Investigating the effects of larval rearing temperature on the phenotype of *Aedes aegypti* OX513A.

4. **Statement Of Data Confidentiality Claims:**

   Confidential Business Information (CBI) has been deleted from this report

5. **Statement Concerning Good Laboratory Practices:**
   This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

6. **Authors:**

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

7. Associated Personnel:

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

8. Test Facility:

This research was performed at Oxitec's research facilities located at:

43b Western Avenue,
Abingdon,
Oxfordshire,
OX14 4RU
United Kingdom

71 Innovation Drive,
Abingdon,
Oxfordshire,
OX14 4RQ
United Kingdom

9. Objectives:

The objectives of this study were to determine;

a) If the penetrance of the phenotype of OX513A heterozygotes varies when reared at temperatures different than the laboratory standard.
b) If OX513A has altered survival at temperatures outside of *Aedes aegypti's* natural range, compared to wild-type.

10. Summary:

Aedes aegypti larvae, heterozygous for the OX513A construct, were reared at five temperatures ranging between and including 9°C and 37°C. Larvae were reared in the absence of tetracycline, which as a dietary supplement allows survival of OX513A individuals. Latin wild-type (WT) larvae, the background strain of the OX513A strain, were reared under the same conditions as a control. Five repetitions were conducted for each temperature point. We found that OX513A larvae and Latin WT larvae died before pupation when reared at 9°C and 37°C. This demonstrates that the presence of the OX513A insertion does not extend the viable temperature conditions for *Ae. aegypti* such that they can develop to functional adults at these temperatures. No evidence was therefore found to indicate that OX513A might be able to spread beyond the current temperature-bounded range of wild *Ae. aegypti*. OX513A larvae reared at intermediate temperatures within this range did not show a higher than expected proportion (<5%) of individuals surviving from L1

to functional adult (range 0-2%). Together, these studies demonstrate the phenotype of OX513A is stable over the range of temperatures that larvae are likely to encounter in the field and that they will not be able to expand the habitable geographic range of *Ae. aegypti*.

11. Introduction:

First developed in 2002 at Oxford University, the *Ae. aegypti* RIDL strain OX513A was originally produced by transforming the Rockefeller strain using a *piggyBac*-based construct (Phuc *et al.*, 2007). This strategy allowed the integration of a repressible dominant lethal system in the *Ae. aegypti* genome whereby, in the absence of tetracycline, >95% of the heterozygous progeny of a mating between RIDL males with wild-type females die (and of RIDL females with wild-type males) due to the accumulation of high intracellular levels of tTAV protein produced by a positive feedback loop. However, if tetracycline is added to the larval rearing medium, tTAV expression is repressed, allowing for normal development to adulthood.

Recently, OX513A has been used in the first open field release of transgenic mosquitoes into the environment (Harris *et al.*, 2011). Due to the high penetrance of the lethal phenotype expressed by mosquitoes inheriting the OX513 construct, the overwhelming majority of the offspring of released transgenic males will die before reaching maturity. However, under laboratory conditions, a small percentage of these offspring (<5%) survive to produce functional adults.

Here we attempt to ascertain the effect that rearing larvae at different temperatures has on the OX513A phenotype, and ultimately the percentage of flying adults surviving without tetracycline. The temperatures used in this experiment were chosen based on the reported habitable temperature range of *Ae. aegypti* (e.g., Hemme *et al* 2009, Richardson *et al* 2011). Consequently, we tested temperatures of 9°C and 37°C which represent temperature points somewhat beyond the lower and upper bounds of the reported habitable temperature range. Within the reported habitable range we tested 18°C, 24°C and 30°C, temperatures larvae are likely to encounter in the field. The null hypotheses tested were that

1. there is no difference between OX513A individuals and the wild-type comparator in respect of survival outside the temperature range permissive for egg-to-adult development and
2. the penetrance of the lethal phenotype of OX513A is not temperature dependent (<5% at all temperatures)

12. Methods

Strains

OX513A (bi-sex lethal RIDL strain): In the absence of tetracycline, this strain expresses high levels of the tTAV protein during immature stages through a positive feedback loop, resulting in high mortality. However, when reared in the presence of tetracycline, expression of tTAV is repressed, allowing survival (Phuc et al., 2007).

Latin wild-type: the background strain of OX513A, collected from Chiapas, Mexico in 2007.

Insect Rearing

L1 larvae were counted into pots (16 oz deli pots, Robertson packaging, UK, SICC65), 200 larvae per pot, 200 ml deionised water. Five repeats of OX513A and Latin WT were set up for each of the five temperatures. Larvae were fed according to a standard feeding regime of finely ground Tetramin© fish flakes (Tetra GmbH, Germany). Due to the expected differential development rate of larvae between temperatures, pots were not fed if food remained from the previous day.

Temperatures were maintained using incubators for the 9°C, 18°C and 37°C experiments and heat mats (Habistat, UK) for the 24°C and 30°C experiments. Evaporation from the pots was compensated for by adding deionised water as required to maintain pots at starting levels (200mL). Water temperatures were monitored using Thermochron iButtons (Maxim, UK).

Live pupae were counted and placed into cages (15x 15 x 15 cm, Bugdorm-Megaview, Taiwan) which in turn were placed into the relevant incubator or onto the heat mat. Dead larvae and dead pupae in the pots were counted and discarded. Pots were discarded when there were fewer than six larvae (3% of starting L1s) remaining.

Adults were provided with 10% sucrose solution *ad libitum*. Adult cages were assessed[1] for emergence three days after the last pupa was added.

Statistical Analyses

Data were analysed using R (Version 2.15.0) in R Studio (Version 0.97.237). Parametric significance tests were carried out using ANOVA and post-hoc testing using the Tukey HSD method, using the multcomp package. Non-parametric data was tested for significance using Kruskal-Wallis rank sum test. Confidence intervals were bootstrapped.

13. RESULTS

OX513A heterozygous larvae and Latin WT larvae were reared at five different temperatures ranging from 9°C to 37°C. Each temperature had five repeats of each strain with 200 larvae per repeat, reared in the absence of tetracycline.

The results displayed in Table 1 show that all OX513A larvae and Latin WT larvae reared at 9°C and 37°C died before pupation.

Strain	Temperature	Dead larvae	Total eclosion	Flying adults
OX513A	9°C	100.0%	0.0%	0.0%
	37°C	100.0%	0.0%	0.0%
Latin	9°C	100.0%	0.0%	0.0%

[1] Assessment included counting the total number of dead pupae, non-viable adults (dead adults on the water, dead adults on the floor of the cage and non-flying adults) and functional adults (flying adults).

WT	37°C	100.0%	0.0%	0.0%

Table 1. Developmental fate of heterozygous OX513A and Latin WT *Ae. aegypti* larvae reared at temperatures outside the normal range for *Ae. aegypti*. All larvae reared in the absence of tetracycline.

Table 2 shows the results for OX513A larvae reared at the intermediate temperatures of 18°C, 24°C and 30°C. There was no deviation from the expected proportion of flying adults (<5%), in fact, the percentages were very low with 18°C having no flying adults.

A very high proportion (95.5%) of OX513A larvae reared at 30°C died prior to pupating; this was consistent between repeats but not seen in the Latin WT (Table 2).

OX513A also showed a variation in the total eclosion[2] between these intermediate temperatures. 18°C and 30°C had a significantly lower total eclosion compared to the standard laboratory rearing temperature of 24°C (p=0.000 for both temperatures). This indicates that OX513A larvae do less well, i.e. die earlier in development, if reared at either higher or lower temperatures than nominal (24°C). Latin WT's total eclosion did not vary significantly between 18°C, 24°C and 30°C (p=0.912).

Strain	Temperature	Dead larvae	Total eclosion	Flying adults
OX513A	18°C	27.6% (24.8%-30.3%)	0.8% (0.3%-1.4%)	0.0% (0.0%-0.0%)
	24°C	39.0% (36.3%-42.3%)	16.2% (13.9%-18.5%)	1.0% (0.4%-1.7%)
	30°C	95.5% (94.2%-96.7%)	2.0% (1.2%-2.9%)	2.0% (1.2%-2.9%)
Latin WT	18°C	18.7% (16.3%-21.1%)	71.2% (68.4%-74.0%)	59.6% (56.5%-62.6%)
	24°C	30.0% (27.2%-32.9%)	69.4% (66.6%-72.2%)	68.3% (65.4%-71.2%)
	30°C	30.1% (27.4%-32.9%)	67.0% (64.2%-69.8%)	65.8% (62.9%-68.6%)

Table 2. Developmental fate of heterozygous OX513A and Latin WT *Ae. aegypti* larvae reared at temperatures within the normal habitable range for *Ae. aegypti*. All larvae reared in the absence of tetracycline. Confidence intervals in parentheses.

14. **Discussion and Conclusions:**

Determining the effect, if any, of larval rearing temperature on the penetrance of the OX513A phenotype is relevant to risk assessment as we expect insects in the wild to experience a variety of temperatures. There are two separate issues: first, does OX513A show differential penetrance at temperatures other than those typically used in laboratory culture, and second, does the presence of the OX513A gene preferentially allow the engineered insects to colonise areas that were previously uninhabitable to wild *Ae. aegypti*.

[2] Total eclosion is the percentage of L1 larvae which eclose to adults including flying function adults, dead adults on the surface of the water, dead adults on the surface of the cage and non-flying adults.

The data presented here show that at the larval rearing temperatures of 9°C and 37°C, somewhat outside the permissive range for wild type *Aedes aegypti* development, neither OX513A nor Latin WT were able to survive to pupation, demonstrating that field-released OX513A would not represent an establishment hazard to *Ae. aegypti*-free areas outside the current temperature-limited range of the species.

The data also show that at the range of intermediate temperatures tested there was no significant difference in the penetrance of OX513A (proportion of L1 larvae developing into functional adults). In other words, across the range of normal habitable temperatures, <5% functional adults were observed, showing that OX513A has a consistent penetrance of the lethality phenotype. This demonstrates that the penetrance of the OX513A lethal trait will not be adversely affected by the temperature of the larval habitats in the receiving environment.

15. **Literature:**

Harris, A.F., Nimmo, D., McKemey, A.R., Kelly, N., Scaife, S., Donnelly, C.A., Beech, C., Petrie, W.D., and Alphey, L. (2011). Field performance of engineered male mosquitoes. Nature Biotechnology 29, 1034-1037.

Hemme, R.R., Tank, J.L., Chadee D.D., and Severson, D.W. (2009). Environmental conditions in water storage drums and influences on *Aedes aegypti* in Trinidad, West Indies. Acta Tropica 112, 56-66.

Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J., Pape, G., Fu, G., Condon, K.C., Scaife, S., Donnelly, C.A., et al. (2007). Late-acting dominant lethal genetic systems and mosquito control. BMC biology 5, 11.

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

Appendix E

INSECTICIDE TESTING FACILITY

Final Study Report

Reference Number: ITF/2011/001

Study Title: Evaluation of insecticide susceptibility status of RIDL strain of *Aedes aegypti.*

Test organism: *Aedes aegypti* Strain OX513A RIDL

Distribution: (1) Master study File, (2) Oxitec Ltd. (3) Head of Insecticide Testing Facility, (4) Head of Vector Group,

Author:	**Study Director:**
Print Name: John Gilmour	**Print Name:** Hilary Ranson
Date: 5th April 2011	**Date:** 5-4-11

Revision History:

Revision	Change Description	Author	Approved by	Date
01	Original version	J. Gilmour		5/4/11

Test Facility: Insecticide Testing Facility, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA

Client: Oxitec Limited, 71 Milton Park, Oxford OX14 4RX

Brief Summary: The *Aedes aegypti* Strain OX513A RIDL is fully susceptible to WHO discriminating doses of 4 of the 5 insecticides tested. 53% survival after bendiocarb exposure was observed. (Note bendiocarb 'resistance' has been detected in several alternative laboratory reference strains and this result suggests, rather than indicating resistance, that the bendiocarb discriminating dose is not appropriate for *Aedes aegypti*). Neither of the knock-down resistance (kdr) mutations, associated with resistance to pyrethroids and DDT (1016 and 1534) was present in the *Aedes aegypti* OX513A RIDL strain.

1.0 Responsible Personnel

All personnel involved in the completion of this study are listed below:

1.1 Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA:

(i) Author: John Gilmour B.Sc, MBA, Head of ITF.

(ii) Study Director: Hilary Ranson PhD Head of Vector Group

(iii) Ms Lori Flood B.Sc: Laboratory Technician

(iv) Ms Grace Matthews B.Sc: Insectary Technician

1.2 Oxitec Limited, 71 Milton Park, Abingdon, Oxford OX14 4RX:

(i) Andrew McKemey (PhD) Technical Development Manager

2.0 Laboratory QA Statement

All investigations were carried out to standard operating procedures at the Insecticide Testing Facility (ITF), Liverpool School of Tropical Medicine (LSTM), Pembroke Place, Liverpool L3 5QA. This facility is not currently GLP accredited. All laboratory equipment used has been verified to function properly and has been calibrated either internally or externally as appropriate. All study staff have been appropriately trained in performance of the required experimental procedures. Experimental data and the final report are reviewed by the Head of ITF for accuracy, completeness and clarity and then approved by the Head of the Vector Group.

3.0 Introduction

Oxitec Ltd is a British company using modern biotechnology to develop transgenic insect strains that can be used to control pests of both public health and agriculture. A key part of this development work is the characterization of these transgenic insect species for insecticide resistance. LSTM has appropriate expertise and experience in insecticide testing and genetic characterization. LSTM offers a suitable testing service which includes genotyping as well as bioassays to clients such as Oxitec Ltd.

Viable eggs from *Aedes aegypti* Transgenic Strain OX513A RIDL were provided by Oxitec Ltd. A susceptible laboratory strain (*Aedes aegypti* New Orleans) was provided by LSTM. LSTM has agreed to characterise the transgenic insect strain provided both with respect to bioassay against five insecticides and genotyping for the presence of two mutations associated with insecticide resistance.

4.0 Aim

The aim of this study was to characterise *Aedes aegypti* Strain OX513A RIDL with respect to

(i) Resistance against the following insecticides: temephos, permethrin, deltamethrin, bendiocarb and malathion.

(ii) Genotyping for 2 knock-down resistance (kdr) mutations associated with resistance to insecticides: 1016, and 1534.

5.0 Study Schedule

All work on this study was carried out between 4th January 2011 and 5th April 2011 at the LSTM facilities in Liverpool.

6.0 Test Strain / Reference Strain

6.1 Test strain: *Aedes aegypti* Strain OX513A RIDL

6.2 Reference strain: *Aedes aegypti* Strain New Orleans.

7.0 Test Systems

7.1 Bioassays:

(i) Larval bioassay was carried out with temephos as follows: 100 larvae from *Aedes aegypti* Strain OX513A RIDL were set up as 4 x25 mosquitoes in 250 ml water containing 0.012mg/l temephos and left overnight. Mortality was scored after 24 hours.

(ii) Adult mosquito bioassays were performed using the WHO cone test as described in WHO bulletin (2006).

7.2 Genotyping Assays:

Genotyping for the two kdr mutations was carried out using

(i) HOLA (1016 mutation), method according to A. Lynd *et al* (2005)

(ii) Tetraplex PCR (1534 mutation), method according to A. F. Harris *et al* (2010)

8.0 Experimental Procedures

8.1 Bioassays

Eggs provided were reared by ITF at LSTM and the following bioassays were carried out both with the transgenic line and with the susceptible strain:

(i) 4th instar larvae were tested using a 24 hour exposure to a discriminating dose of temephos (0.012mg/l)

(ii) 2-3 day old female adults were tested using a 1 hour exposure to the following insecticides
- 0.75% permethrin
- 0.05% deltamethrin
- 0.1% bendiocarb
- 0.8% malathion

All bioassays were performed on a minimum of 100 individuals from the RIDL OX513A strain and 100 from the susceptible New Orleans strain (for bendiocarb and pyrethroids only).
All assays were performed according to standard WHO procedures.
Mortality was recorded 24 hours after exposure. Control bioassays in which mosquitoes were exposed to the carrier only were performed simultaneously. In any cases where control mortality exceeded 5% the results of the days assays were discarded.

8.2 kdr genotyping:

10 Individuals from the *Aedes aegypti* Strain OX513A RIDL colony were genotyped for the two kdr mutations associated with pyrethroid and DDT resistance: 1016 and 1534 using either the HOLA method or tetraplex PCR method.

10 individuals were considered to be sufficient to detect any mutations present as the material provided was representative of a highly derived colony.

9.0 Results

Results obtained with the bioassays are presented in table 1 below and results obtained with the genotyping assays are given in table 2:

Table 1: Mosquito mortality recorded 24 hours after exposure to insecticide

Insecticide	No tested	No Alive	No Dead	% mortality		No tested	No Alive	No Dead	% mortality
	OX513A					New Orleans Strain			
Temephos	102	0	102	100		n/d	n/d	n/d	n/d
Permethrin	100	0	100	100		63	0	63	100
Deltamethrin	100	0	100	100		41	0	41	100
Bendiocarb	200	106	94	47		100	49	51	51
Malathion	100	0	100	100		n/d	n/d	n/d	n/d

Mortality recorded in all control bioassays was 0% and so all results were considered to be valid.

Table 2: Results of kdr genotype tests.

Sample name	Tetraplex (1534)	Hola (Val1016Ile)
RIDL 1	Wild type	Wild type
RIDL 2	Wild type	Wild type
RIDL 3	Wild type	Wild type
RIDL 4	Wild type	Wild type
RIDL 5	Wild type	Wild type
RIDL 6	Wild type	Wild type
RIDL 7	Wild type	Wild type
RIDL 8	Wild type	Wild type
RIDL 9	Wild type	Wild type
RIDL 10	Wild type	Wild type

10.0 Discussion

Results obtained with the WHO cone tests indicate susceptibility of the *Aedes aegypti* Strain OX513A RIDL to discriminating doses of temephos, permethrin, deltamethrin and malathion. Significant survival to 0.1% bendiocarb was however noted. However, high survival rates after 1 hour exposure to 0.1 % bendiocarb exposure to were also observed in the susceptible New Orleans strain. These results taken together indicate that the bendiocarb discriminating dose is not appropriate for *Aedes aegypti* rather than indicating actual resistance to bendiocarb in the OX513A strain.

Results obtained with genotyping against the knock-down resistance (kdr) mutations, associated with resistance to pyrethroids and DDT (1016 and 1534) indicated that neither of these mutations are present in the *Aedes aegypti* OX513A RIDL strain.

11.0 Conclusions

The *Aedes aegypti* OX513A RIDL strain is fully susceptible to WHO discriminating doses of temephos, permethrin, deltamethrin and malathion. However 53% survival after bendiocarb exposure was observed. It should however be noted that apparent bendiocarb 'resistance' has been detected in several alternative laboratory reference strains. It is concluded that these results suggest that the bendiocarb discriminating dose is not appropriate for *Aedes aegypti* and does not indicate actual resistance to bendiocarb. Neither of the tested kdr mutations (1016 or 1534) associated with resistance to pyrethroids and DDT was present in the Aedes aegypti OX513A RIDL strain.

12.0 Cross Referenced Methods

12.1 "A simplified high-throughput method for pyrethroid knock-down resistance (kdr) detection in *Anopheles gambiae*", A. Lynd *et al* Malaria Journal **4**, pp16-21 (2005).

12.2 "Pyrethroid Resistance in *Aedes aegypti* from Grand Cayman" Angela. F. Harris *et al.* Am. J. Trop. Med. Hyg. **83**(2), pp 277-284 (2010).

12.3 "Guidelines for testing Mosquito adulticides for Indoor residual spraying and treatment of Mosquito nets" WHO/CDS/NTD/WHOPES/GCDPP/2006.3 World Health Organization 20 Avenue Appia CH-1211 Geneva 27 Switzerland (2006).

13.0 Archiving

All DNA samples tested will be archived for a period of 1 year in secure storage at -70°C in LSTM. The Final Study Report and raw data will be archived for a period of 5 years in a secure location at LSTM.

14.0 Final Study Report Approvals

14.1 Author: 5/4/2011

John Gilmour B.Sc., MBA
Head of Insecticide Testing Facility
LSTM
Liverpool

14.2 Study Director

Hilary Ranson PhD
Head of Vector Group
LSTM
Liverpool

Appendix F

OXITEC

1. **Reference Number**: PH-2013-3-V3a

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

3. **Title**: Assessment of heterozygous OX513A individuals surviving without provision of tetracycline in the diet: Longevity and Fecundity

4. **Statement Of Data Confidentiality Claims:**

   Confidential Business Information (CBI) has been deleted from this report

5. **Statement Concerning Good Laboratory Practices:**
   This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

6. **Authors:**

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

7. **Associated Personnel:**

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

8. **Test Facility:**

This research was performed at Oxitec's research facility located at:
46a Western Avenue
Abingdon, Oxfordshire,
OX14 4RU
United Kingdom

9. **Objectives:**

The objective of this study was to assess the fitness of the small fraction of heterozygous OX513A individuals that reach adulthood following rearing in the absence of tetracycline.

10. **Summary:**

This study assessed the longevity of adult male and female OX513A *Aedes aegypti*. The homozygous OX513A strain, used for field trials in Brazil, was outcrossed to wild-type of Latin background to generate heterozygous eggs. These eggs were hatched and reared in the absence of the antibiotic tetracycline that is required for survival of most individuals. Emerged, flying adults were collected and housed in single-sex groups. The longevity of these individuals was assessed over a period of over 12 weeks alongside that of non-transformed insects of the same background reared with tetracycline (1 µg/mL) in the rearing water, and wild-type individuals. Rearing in the absence of tetracycline mimics the conditions heterozygous offspring of OX513A males will encounter in the wild. The 1 µg/mL dose was selected because it is the minimum dose needed to provide good survival of OX513A heterozygotes (See PH-2013-4-v1), yet well over the amounts animals might encounter in the field (Le-Minh et al., 2010; Locatelli et al., 2011). Longevity of homozygous OX513A individuals reared on the standard tetracycline dose of 30 µg/mL was also assessed. These experiments therefore examine the longevity of the two types of OX513A female most plausibly present in the field – homozygous females inadvertently co-released with homozygous males, and heterozygous progeny of released males that have mated with wild females and survive as a consequence of incomplete penetrance of the lethal trait. The lifespan of OX513A homozygotes and heterozygotes was found to be significantly reduced relative to wild type comparators. Since longevity is an important component of vectorial capacity, shorter lifespan implies reduced vectorial capacity, especially for heterozygous females reared without tetracycline (median lifespan 2 days relative to wild type 68 days). This reduction in longevity also implies that the mean fitness of heterozygous OX513A males and females reared without tetracycline is even lower than one would estimate simply by considering survival to adulthood.

11. Introduction:

Aedes aegypti RIDL strain OX513A was developed at Oxford University in 2002 by injection of the OX513A *piggyBac*-based construct into the Rockefeller strain (Phuc et al., 2007). The dominant-lethal phenotype of this strain, when reared without tetracycline, results in death of immature heterozygous progeny at a rate of approximately 95%. The basis of this phenotype is the accumulation of the tTAV protein via a positive feedback loop that can be broken by the binding of tetracycline to tTAV itself. When homozygous males bearing this trait are released into the environment to mate with wild females, matings are unproductive as the progeny have no access to tetracycline, and the population is suppressed.

The use of OX513A has recently been shown to be effective as a genetic SIT strategy in an open field trial (Harris et al., 2012). The high penetrance of heterozygous lethality means that very few heterozygous progeny emerge as functional adults[1] – less than 5% under laboratory conditions. Models suggest that RIDL-based control of *Aedes aegypti* should be effective so long as the average fitness of heterozygous progeny is less than 10% relative to wild type (Phuc et al., 2007). The average fitness of OX513A heterozygous progeny in the field is predicted to be lower than indicated by laboratory based studies based on the presumed rigours of life in natural habitats, as compared with the protected environment of the laboratory, but risk assessments should assume that a non-zero fraction of heterozygous females will reach adulthood in the wild. Additionally, sex-separation methods designed to allow only the release of males are very good (>99% accurate), but not perfect, and a small number of homozygous females are expected to be released over the course of a control programme.

Of particular interest for risk assessment is the ability of OX513A females to serve as disease vectors which is heavily dependent on their ability to live long enough to take at least two bloodmeals, separated by the extrinsic incubation period (EIP) of flaviviruses such as dengue, estimated to range from 7-15 days, depending on environmental temperature (Chan and Johansson, 2012). Evaluation of both the longevity of both relevant types of adult female was therefore conducted in this study: heterozygous OX513A reaching adulthood without the provision of tetracycline, and homozygous OX513A reared with tetracycline. The null hypotheses being tested is that OX513A-bearing individuals are not more long-lived than their wild type comparators. The assessment of homozygous OX513A females will help establish the risks associated with accidental release of females as part of the control programme. The fitness of the heterozygous offspring also relates to the potential efficacy of such a programme – modelling indicates that the predicted efficiency and effectiveness of the method would be substantially reduced it the net fitness of offspring were >10% that of wild type. Lifespan is a major component of fitness. Additionally, an assessment of the fecundity of surviving OX513A heterozygous females that are reared without tetracycline is presented; fecundity is another element of biological fitness.

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[1] Progeny types include dead larvae, dead pupae, non-viable/non-functional adults (dead adults on the water (of the weigh boat), dead adults on the floor of the cage and non-flying adults) and functional adults (adults capable of flight); all these classes except the last are considered to have zero fitness as *Aedes aegypti* court and initiate mating on the wing, and flight ability is required in the field to avoid predators and to find mates, hosts and oviposition sites.

12. **Methods:**

Strains

The following strains were used for this experiment:

Latin Wild-type (LWT): This non-transgenic strain was originally collected in the region of Chiapas (Mexico) and was transferred to Oxitec from Mexico's Institute of Public Health in 2006 (D. Nimmo, personal communication).

OX513A Latin1 (bi-sex lethal RIDL strain): In the absence of tetracycline, this transgenic strain expresses high levels of the tTAV protein during immature stages through a positive feedback loop, resulting in high mortality. However, when reared in the presence of tetracycline, expression of tTAV in this strain is repressed, allowing for high survival levels (Phuc *et al.*, 2007). Individuals of this strain can be identified during immature stages by the expression of the fluorescent protein DsRed2 in their bodies in a characteristic punctate pattern driven by the Actin5C promoter. The particular strain used for this study was generated by introgressing the original OX513A strain into the aforementioned Latin WT genetic background.

Insect Rearing

All strains were reared under standard insectary conditions: 27°C [+/- 1°C], 70% [+/- 10%] relative humidity, 12 h:12 h light:dark cycle. Larvae were fed finely ground Tetramin© fish flakes (Tetra GmbH, Germany) on the standard diet and reared at a density of 1 larva/mL. Adults were provided with 10% sucrose solution *ad libitum*. Strains were reared with the addition of chlortetracycline (tetracycline) to the rearing water at concentrations of 0, 1 or 30 µg/mL, as described.

Fecundity Study

All larvae for this study were reared in the absence of tetracycline. New larval rearing trays and food were used to avoid inadvertent introduction of tetracycline. Adult females were mated to LWT males 4 days post eclosion. Males were allowed to cohabitate with females for 2 days, after which they were removed. A blood meal was offered 7 days post eclosion. Two days post-bloodmeal, females were transferred to entomological tubes with wetted cotton wool in the bottom to promote individual egg laying. Females were discarded and eggs were counted 3 days post transfer of the females to entomological tubes. Eggs were vacuum hatched in pure water 5 days post laying, and L1 larvae counted.

Longevity Study

New larval rearing trays and food were used to avoid inadvertent introduction of tetracycline in tetracycline-free experiments. Pupae were picked and sexed daily. Each 15 cm^3 cage received 25 male or female pupae, all picked on the same day to allow near-synchronous eclosion unless otherwise noted. Females were mated for two days, and the males then removed. Two blood meals of defibrinated horse blood (TCS Biosciences Ltd., UK) were provided on days 7 and 17 with eggs collected on a wet filter paper (Whatman, UK) 4 days later (Figure 2). In addition to 10% sucrose solution, adults were also provisioned with pure water *ad libitum*. Dead adults were removed from cages daily and counted. Cages were rotated in the insectary daily to control for environmental factors based on position in the insectary; sugar and water feeders were replaced every two weeks.

Statistical Analyses

Data were analysed using the RStudio software package version 0.97.237 (RStudio, USA) running version 2.15.0 of the R statistical software. For longevity analysis, the Survival Analysis package, survival (2.37-2), was used to plot Kaplan-Meier curves and test for significance. Normality and homoscedasticity were tested using Shapiro-Wilk and Bartlett-box tests, respectively. Parametric significance tests were carried out using ANOVA, and post-hoc testing was performed using the Tukey HSD method. Average egg-laying numbers were compared using a student's t-test, and hatch rates were compared using the Mann-Whitney *U* test.

13. Results

Fecundity of OX513A

A total of 18 heterozygous OX513A females and 22 LWT females laid eggs. Each strain had one egg clutch that did not hatch, so 17 OX513A egg clutches and 21 LWT egg clutches were examined as part of this study. Only the first gonotrophic cycle was observed. As shown in Figure 1, the mean clutch size for OX513A was 69.9 eggs (S.D. 13.9) and for LWT it was 54.8 eggs (S.D. 12.4). Analysis by t-test revealed a significant difference between average values (p=0.001), indicating that the OX513A strain lays a larger egg clutch during the first gonotrophic cycle, compared to its wild-type background. The mean hatch rates were 92% (S.D. 14) and 82% (S.D. 18) for OX513A and LWT, respectively. Statistical testing did not reveal any significant difference between these values (p=0.089). It should be noted the OX513A strain has been intensively mass reared for over 100 generation equivalents while the LWT strain has not, which may explain the differences observed.

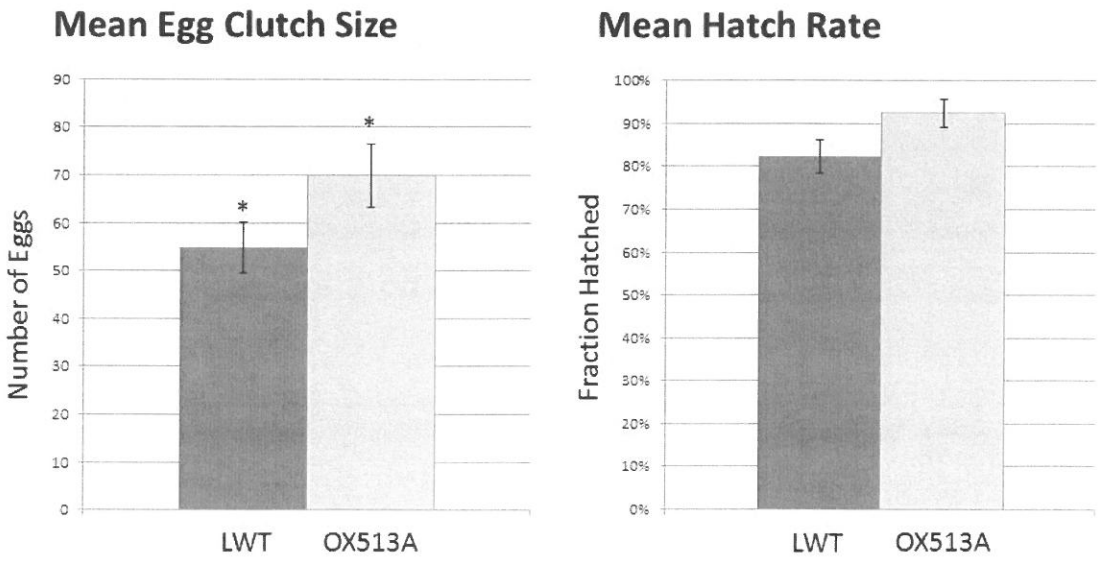

Figure 1: Fecundity study results. * p=0.001. The 95% confidence interval is shown for egg clutch size, and the standard error of the mean for hatch rate.

Longevity of OX513A

Three longevity comparisons were carried out: Heterozygous OX513A without tetracycline in the rearing water, heterozygous OX513A reared with 1 µg/mL tetracycline provided in the rearing water, and homozygous OX513A reared with 30 µg/mL tetracycline provided in the rearing water, each compared to a LWT cohort reared under the same conditions. For

both studies where tetracycline was provided, 25 pupae of each sex were placed into a cage on Day -2, and counting commenced on Day 1 (Figure 2).

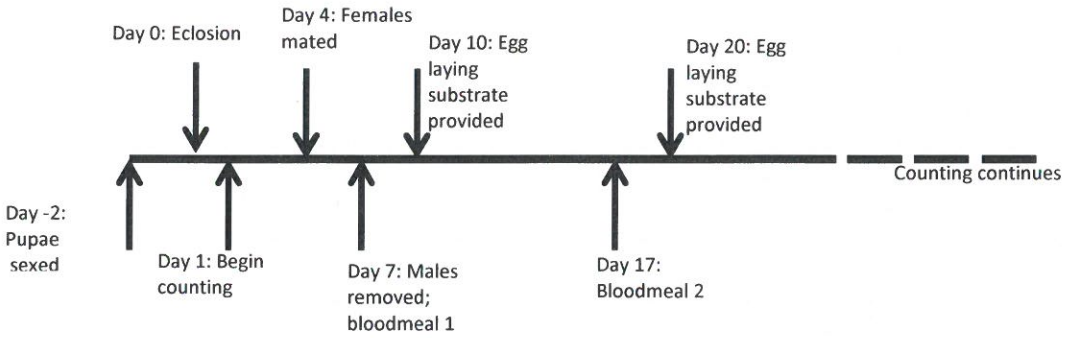

Figure 2: SFFigure 2: Schematic of female longevity experiments. Males were allowed to eclose and then counted each day, starting on Day 1.

Due to anticipated and observed death of the majority of OX513A heterozygous pupae, large, steep-sided eclosion containers (16 oz deli pots, Robertson Packaging, UK, SICC65) containing hundreds of pupae were put into cages for these trials so that the few flying adults could leave the surface of the water and thereby separate from the larger number of non-flying individuals unable to leave the container. In total, 87 females eclosed and were able to exit the eclosion container from a starting cohort of 4000 L1 individuals. The total number of flying adults, male and female, represents 4.4 % of starting L1 larvae. Not all motile females are included in this study as 6 eclosed too late to be used, and females unable to fly on the morning of Day 1 were also excluded. For this reason, the cohort sizes in these cages range from 17 to 30.

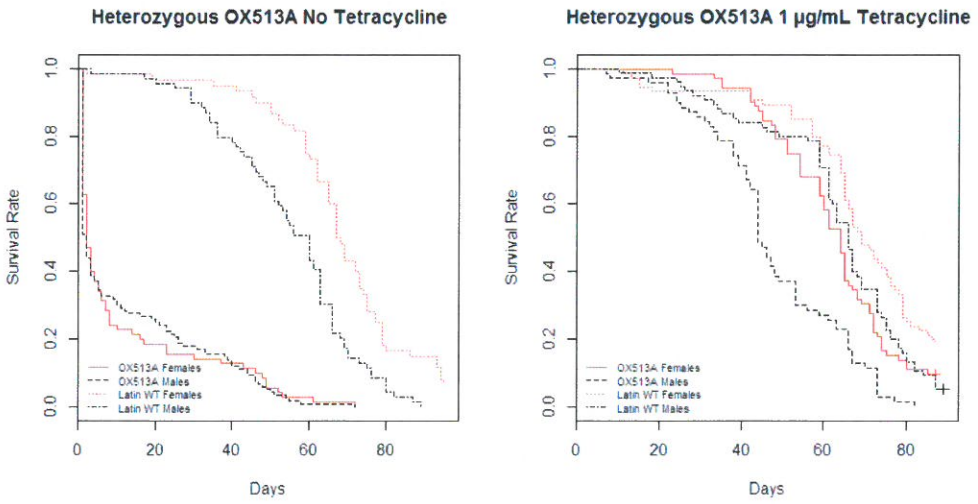

Figure 3: Survival curves for heterozygous OX513A reared without tetracycline or with 1 µg/mL in the larval rearing water.

Substantial mortality was observed within the first few days post-eclosion for the OX513A heterozygous males and females reared without tetracycline (Figure 3), although a small fraction (~20%) do survive long enough to take two blood meals and some produced two clutches of eggs. Very little mortality was observed in the week post-eclosion in the LWT strain for both males and females, which contrasts strongly with the OX513A strain (p-

value=0). Median survival of both OX513A males and females is 2 days compared to LWT males and females with median survival of 60 and 68 days, respectively.

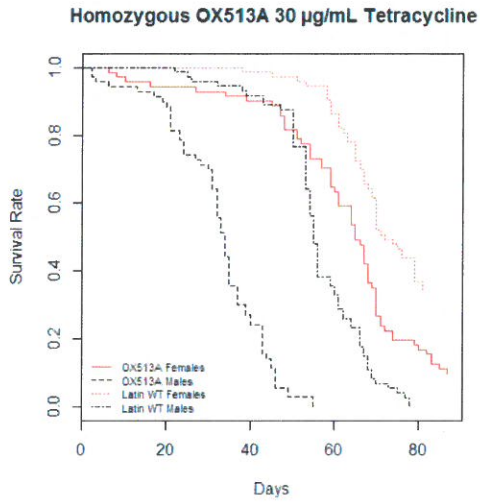

Figure 4: Survival of homozygous OX513A individuals that were reared with 30 µg/mL tetracycline compared to LWT reared under the same conditions.

Survival of heterozygous OX513A is imperfectly rescued by the provisioning of 1 µg/mL in the larval rearing water, as evidenced by comparing their survival curves to those of their LWT counterparts ($p < 0.001$ for males and $p = 0.005$ for females). Median survival times for wild-type and OX513A heterozygous males were 66 days and 44 days, respectively. Median survival of OX513A heterozygous females was 64 days, and median survival of LWT females is 69 days.

Survival of the homozygous line reared according to standard procedures was also assessed (Figure 4). Homozygous OX513A males have a reduced fitness compared to their LWT counterparts ($p = 0$). Their median survival is 34 days; median survival of LWT males reared on the same dose of tetracycline is 55 days. The median survival of the LWT females has has been reach at 72 days, but a significant number of females are still alive at the time of this writing. OX513A females have a median survival of 65 days, which is significantly shorter ($p=0.0006$).

14. Discussion and Conclusions:

Understanding the overall fitness of heterozygous OX513A individuals that may survive in the wild contributes to an assessment of the safety and efficacy of a RIDL-based SIT programme.

Longevity: Longevity of three types of OX513A females was assessed: homozygous females reared on 30 µg/ml tetracycline, heterozygous females reared in the absence of tetracycline, and heterozygous females reared on 1 µg/ml tetracycline. In no case was the lifespan of the transgenics longer than that of the wild type comparators, and so the null hypothesis is confirmed. Indeed, the median lifespan of the OX513A females was significantly shorter than that of the wild type comparator in all three cases.

Vectorial capacity: Since the females have to survive at least the extrinsic incubation period (EIP, typically 7-15 days for dengue (Chan and Johansson 2012)) to have any possibility of transmitting the virus, vectorial capacity is very sensitive to vector longevity. OX513A females were found to have a significantly reduced lifespan relative to wild type, especially when heterozygotes were reared in the absence of tetracycline (median survival 2 days vs wild type 68 days). This implies that the vectorial capacity of OX513A females is significantly less than that of wild type females. Environmental factors are thought to reduce daily survival and hence lifespan considerably in the wild relative to the laboratory environment, with possibly as few as 9% of wild females living over 15 days (Joy et al., 2012). Nonetheless, the shorter lifespan observed in these laboratory experiments, particularly for OX513A heterozygotes reared without tetracycline, likely indicates an underlying reduction in fitness that would lead to an equivalent or greater proportional reduction in lifespan under harsher conditions. In practice, these data indicate a very reduced probability that OX513A females will be able to survive long enough to bite humans, and an even further reduced ability to survive the extrinsic incubation period of the virus.

Fitness: Modelling indicates that for efficient use of a RIDL strain of *Aedes aegypti*, such as OX513A, the mean fitness of heterozygous offspring of the released homozygous RIDL males with wild females should be <10% that of wild type (Phuc et al., 2007). Based on survival to functional adults, the fitness of OX513A heterozygotes in this and similar studies is about 4% that of wild type. However, this is likely to be an overestimate as it assumes that these rare survivors are fully fit, i.e. equivalent to wild type. This study found that this is not the case, in particular the longevity of both males and females is significantly lower than that of wild type. Fecundity of OX513A heterozygous females reared in the absence of tetracycline was found to be slightly higher than that of LWT females. This may be a consequence of selection under mass-rearing conditions for early egg production, and does not imply increased per-lifetime female productivity. In any case, the apparent fecundity increase, even if it were maintained throughout the female's lifetime, was nowhere near a large enough effect to outweigh the observed reduction in lifespan; the mean fitness of heterozygous offspring of released homozygous RIDL males with wild females is therefore well below 4% that of wild type, and correspondingly well below the 10% threshold of Phuc et al. (2007). Therefore, the survival of a small proportion of heterozygous individuals is not expected to compromise the efficiency of a mass release program for population control based on systematic releases of OX513A .

As well as programme effectiveness, fitness of transgenic individuals also relates to the potential for the transgene to persist in the environment. Even a modest fitness penalty would be sufficient to ensure the eventual loss of the transgene from a large wild population by natural selection. Here we find that the transgenics have reduced fitness relative to wild type, irrespective of their exposure to tetracycline. The mean fitness of the key class, OX513A heterozygotes reared without tetracycline, is well below 4% relative to wild type; this would lead to extremely rapid elimination of the transgene from a large wild population were releases to stop.

15. **Literature:**

Chan, M., and Johansson, M.A. (2012). The incubation periods of dengue viruses. PloS ONE 7, e50972.

Harris, A.F., McKemey, A.R., Nimmo, D., Curtis, Z., Black, I., Morgan, S.A., Oviedo, M.N., Lacroix, R., Naish, N., Morrison, N.I., et al. (2012). Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. Nature Biotech 30, 828-830.

Le-Minh, N., Khan, S.J., Drewes, J.E., and Stuetz, R.M. (2010). Fate of anitbiotics during municipal water recycling treatment processes. . Water Research 44, 4295-4323.

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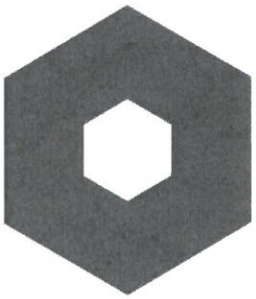
Joy, T.K.,Jeffery Gutierrez, E.H., Ernst, K., Walker, K. R., Carriere, Y., Torabi, M., Riehle, M.A. (2012). Aging field collected *Aedes aeygpti* to determine their capacity for dengue transmission in the Southwestern United States. PLoS ONE. 7,11 (e46946).

Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J., Pape, G., Fu, G., Condon, K.C., Scaife, S., Donnelly, C.A., et al. (2007). Late-acting dominant lethal genetic systems and mosquito control. BMC Biology 5, 11.

Suchman, E.L., Kononko A., Plake, E., Doehling, M., Kleker, B, Black IV, W.C., Buchatsky, L., Carlson, J. (2011). Effects of AeDNV infection on *Aedes aegypti* lifespan and reproduction, Biological Control, *120* (465-473).

Appendix G



OXITEC

INTERNAL RESEARCH REPORT

1. **Reference Number**: PH-2013-2-V2a

2. **Issuing Date**: 21 April 2015

3. **Title**: Ingestion of tetracycline by adult female *Aedes aegypti* does not affect penetrance of the OX513A transgenic phenotype

4. **Statement Of Data Confidentiality Claims**:

   Confidential Business Information (CBI) has been deleted from this report

5. **Statement Concerning Good Laboratory Practices:**
   This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

6. **Authors:**

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

7. Associated Personnel:

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

8. Test Facility:

This research was performed at Oxitec's research facility located at:
46a Western Avenue,
Abingdon,
Oxfordshire,
OX14 4RU
United Kingdom

9. Objectives:

The objective of this study was to test the hypothesis that providing high doses of dietary tetracycline to adult female *Aedes aegypti* (either homozygous OX513A transgenic females mated to wild-type males, or wild-type females mated to homozygous OX513A transgenic males) has no effect in the penetrance of the OX513A lethal phenotype observed in their heterozygous offspring.

10. Summary:

Oxitec's transgenic *Aedes aegypti* strain OX513A has been recently used in open releases of transgenic mosquitoes in the environment and successful suppression of field mosquito populations by the release of genetically sterile males. Because the lethal phenotype displayed by this strain is repressible by the addition of tetracycline to the larval rearing medium, we wanted to establish whether the oral administration of high doses of tetracycline to parental female mosquitoes (either homozygous OX513A females mated to wild-type males, or wild-type females mated to homozygous OX513A males) has any measurable effect on the penetrance of the transgenic phenotype observed in the offspring of said females.

Our data indicate that the penetrance of the OX513A phenotype in heterozygous offspring of female mosquitoes which have ingested high doses of tetracycline is not significantly different from that observed in the offspring of females that were not provided with tetracycline in their diet.

11. Introduction:

First developed in 2002 at Oxford University, the *Aedes aegypti* RIDL strain OX513A was originally produced by transforming the Rockefeller strain using a *piggyBac*-based construct. This strategy allowed the integration of a repressible dominant lethal system in the *Ae.*

aegypti genome whereby, in the absence of tetracycline, >95% of the heterozygous progeny of mating between RIDL males with wild type females (and of RIDL females with wild-type males) die before becoming functional adults due to the accumulation of high intracellular levels of tTAV protein produced by a positive feedback loop. However, if tetracycline is added to the larval rearing medium in sufficient quantities (e.g. to 30 µg/ml), tTAV expression is repressed, allowing for normal development to adulthood (Phuc *et al.*, 2007).

Recently, strain OX513A has been used in the first open release of transgenic mosquitoes in the environment (Harris *et al.*, 2011) and the first successful suppression of field mosquito populations by the release of genetically sterile males (Harris *et al.*, 2012). Due to the high penetrance of the lethal phenotype expressed by mosquitoes inheriting the OX513A construct, the overwhelming majority of the offspring of released transgenic males will die before reaching adulthood. However, under laboratory conditions, a small percentage of these offspring (<5%) might survive to produce flying adults, of which approximately half will be female (Phuc *et al.*, 2007). Although preliminary data suggest that this survival rate is probably much reduced in heterozygous individuals produced in the field (due to the many environmental challenges that mosquitoes face in the wild relative to benign laboratory conditions), it is nonetheless possible that a few female individuals carrying the OX513A construct could make it to adulthood following a field release. Furthermore, although the sex-separation techniques used to eliminate females from the released cohorts are highly efficient (well above 99% female elimination) (Harris *et al.*, 2012), they are not perfect, and therefore the potential exists for a small proportion of OX513A adult females to remain in the male populations released during a field trial.

Tetracycline is an antibiotic used extensively as a therapeutic and/or prophylactic agent in human and veterinary medicine. Therefore, it is possible that a female mosquito could feed on a person or animal that had recently received a dose of tetracycline and carries some level of this antibiotic in the bloodstream. In vertebrates, the concentration of tetracycline in the blood usually reaches a peak 2-6 hours following an oral or injected dose, and then gradually declines due to the body's metabolic activity (Agwuh and MacGowan, 2006). In both humans and livestock, the peak concentration of tetracycline in blood (plasma) following standard therapeutic doses normally remains below 10 µg/ml (Agwuh and MacGowan, 2006; Bimazubute *et al.*, 2011). To the best of our knowledge, the highest concentration of tetracycline recorded in vertebrate blood is ~20 µg/ml (a level observed in pigs that received unusually high intra-muscular doses as part of experimental treatments) (Bimazubute *et al.*, 2011).

Although we are not aware of any evidence suggesting that oral ingestion of tetracycline by a female mosquito results in deposition of active tetracycline in her eggs, we wanted to investigate whether providing an adult female mosquito with tetracycline-containing meals would have any measurable effect on the penetrance of the phenotype of her heterozygous offspring. Therefore, we hypothesized that providing high doses (50-100 µg/ml) of dietary tetracycline to adult female *Ae. aegypti* (either homozygous OX513A females mated to wild-type males, or wild-type females mated to homozygous OX513A males) should have no effect in the penetrance of the OX513A phenotype observed in their heterozygous offspring. To test this hypothesis, we set up crosses using females which had access to either tetracycline-free meals or meals containing high doses of tetracycline, and we evaluated the penetrance of the lethal phenotype on their heterozygous offspring.

Our results suggest that the ingestion of high concentrations of tetracycline by a female mosquito does not affect the penetrance of the lethal OX513A phenotype in her offspring.

12. **Methods:**

Strains

This study was performed using the following *Ae. aegypti* strains:

- **Latin wild-type:** This non-transgenic strain was originally collected in the region of Chiapas (Mexico) and was transferred to Oxitec from Mexico's Institute of Public Health in 2006 (D. Nimmo, personal communication). This strain will be henceforth referred to as WT.

- **OX513A (bi-sex lethal RIDL strain):** In the absence of tetracycline, this transgenic strain expresses high levels of the tTAV protein during immature stages through a positive feedback loop, resulting in high mortality. However, when reared in the presence of tetracycline, expression of tTAV in this strain is repressed, allowing for high survival levels (Phuc *et al.*, 2007). Individuals of this strain can be identified during immature stages by the expression of the fluorescent protein DsRed2 in their bodies in a characteristic punctate pattern driven by the Actin5C promoter. The particular strain used for this study was generated by introgressing the original OX513A strain into the aforementioned Latin WT genetic background.

Insect Rearing

All specimens were reared under standard insectary conditions: 27°C [+/- 1°C], 70% [+/- 10%] relative humidity, 12h: 12h light: darkness cycle. Larvae were fed finely ground Tetramin© fish flakes (Tetra GmbH, Germany) and adults were provided with 10% sucrose solution. To obtain eggs, mated females were provided with defibrinated horse blood (TCS Biosciences Ltd., UK) and given access to wet filter paper (Whatman, UK) as oviposition substrate.

In the case of groups requiring rearing on-tetracycline ('ON-tet'), 30 µg/ml chlortetracycline hydrochloride (Sigma-Aldrich, USA) was added to the larval rearing water.

Crosses

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

Tetracycline-loading

To evaluate the effects of the ingestion of high tetracycline concentrations, selected groups of female mosquitoes (henceforth referred to as 'tet-loaded' groups) received both blood and sugar meals containing a pre-determined dose (either 50 µg/ml or 100 µg/ml) of chlortetracycline hydrochloride.

Experimental design

Table 1 provides a description of the different control and experimental groups set up for this experiment. With the exception of group F, all groups consisted of six repeats containing 200 L1 larvae each. Group F consisted of six repeats containing 85-200 L1 larvae each (see table 1 and annex 1 for details).

Table 1. **Control and experimental groups.**

GROUP	DESCRIPTION	PARENTAL CROSS	TET-LOADING DOSE*	LARVAL REARING MEDIUM
A	Non tet-loaded control	OX513A ♂ vs. WT ♀	None‡	OFF-TET
B	Experimental	OX513A ♂ vs. WT ♀	50 µg/ml	OFF-TET
C	Experimental	OX513A ♀ vs. WT ♂	50 µg/ml	OFF-TET
D	Experimental	OX513A ♂ vs. WT ♀	100 µg/ml	OFF-TET
E	Experimental	OX513A ♀ vs. WT ♂	100 µg/ml	OFF-TET
F†	Rearing control	OX513A ♂ vs. WT ♀	None‡	ON-TET
G†	Rearing control	OX513A ♂ vs. WT ♀	50 µg/ml	ON-TET

*Refers to concentration of tetracycline offered to parental females in both blood and sugar meals.

‡The parental females of groups A and F received only tetracycline-free diets.

†Groups F and G were set-up to control for mortality caused by factors independent from the penetrance of the phenotype (i.e. environmental conditions, manipulation, etc.) and were therefore excluded from statistical analysis.

Within each group, we evaluated the following parameters:

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

Statistical analysis

Data were analysed using the RStudio software package version 0.97.237 (RStudio, USA). Normality was tested using the Shapiro-Wilk method. For normally-distributed data (survival to pupation, adult emergence), parametric significance tests were carried out using ANOVA and, when required, Tukey's honestly-significant-difference (HSD) tests for post-hoc analysis. For non-normally distributed data (number of flying adults), non-parametric testing was performed using the Kruskal-Wallis test, followed by post-hoc analysis using the Nemenyi test (Zar, 1999).

13. RESULTS

For all parameters analysed, numeric data (raw and average) are presented in Annex 1.

Survival to pupation: ANOVA revealed significant differences between groups for this parameter ($F(4,25)=4.57$, $p=0.007$). Post-hoc analysis using Tukey's HSD test indicated a

statistically significant difference ($p<0.01$) in the average survival to pupation between groups B and E (fig. 1). No other significant differences were observed during pair-wise post-hoc testing.

Adult emergence: Statistical testing (ANOVA) revealed no significant differences between groups for this parameter ($F(4,25)=1.616$, $p=0.201$) (fig. 1).

Flying adults: Kruskal-Wallis test indicated significant differences between groups for this parameter ($H(4)=9.929$, $p=0.04164$). Nemenyi post-hoc tests revealed a significant difference ($p<0.05$) in the average number of flying adults between groups C and E (fig. 1). No other significant differences were observed during pair-wise post-hoc testing.

14. Discussion and Conclusions:

No significant differences were observed between the non tet-loaded controls and the experimental groups in any of the parameters examined in this study, supporting the hypothesis that penetrance of the OX513A phenotype in the heterozygous offspring of female mosquitoes which have ingested high doses of tetracycline (50-100ug/mL) is not different from that observed in the offspring of females that did not ingest any tetracycline with their diet.

Post-hoc testing of our results identified significant differences between various tet-loaded groups in two parameters (survival to pupation between groups B and E, and number of flying adults between groups C and E). The fact that no significance was observed when comparing either of those groups to their corresponding non tet-loaded controls suggests that the observed differences are caused by factors unrelated to the ingestion of tetracycline. Although the exact nature of these factors remains to be described, we believe they are probably related to environmental conditions during rearing, and therefore not relevant to the specific objectives of this study.

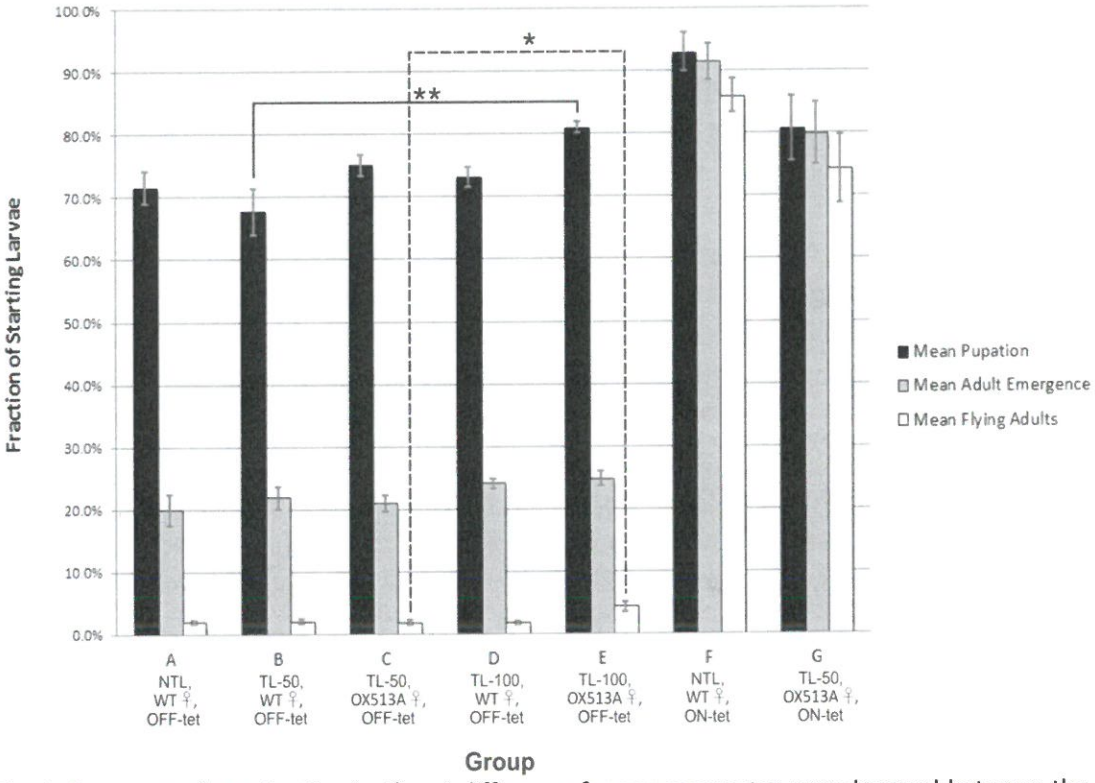


Fig. 1. **Summary of results**. No significant difference for any parameter was observed between the non tet-loaded control group (A) and any of the treatment groups (B-E). Significant differences were only observed in pupation between groups B and E ($p<0.01$), and in the number of flying adults between groups C and E ($0.01<p<0.05$). Values for the ON-tet control groups (F,G) are shown for reference. NTL: Non tet-loaded. TL-50: Tetracycline loaded, 50µg/ml. TL-100: Tetracycline loaded, 100µg/ml. WT ♀: Female of parental cross was wild-type. OX513A ♀: Female of parental cross was transgenic. OFF-tet: Larvae reared without tetracycline. ON-tet: Larvae reared with tetracycline added to the rearing water.

It is important to highlight that the highest dose of tetracycline used in this study is 10-fold higher than the normal concentration found in the blood of humans or animals receiving usual therapeutic doses of tetracycline, and 5-fold higher than the highest dose reported (to the best of our knowledge) from any animal blood. This suggests that during a field release of transgenic mosquitoes, the OX513A phenotype should not be compromised by the presence of individuals (human or animal) receiving tetracycline treatments in the target area, whether on an individual basis in terms of survival of heterozygous progeny from released specimens, or on a population basis in terms of the suppressing effect of systematic mass releases of OX513A mosquitoes.

15. Literature:

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

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

Harris, A.F., McKemey, A.R., Nimmo, D., Curtis, Z., Black, I., Morgan, S.A., Oviedo, M.N., Lacroix, R., Naish, N., Morrison, N.I., *et al.* (2012). Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. Nature biotechnology 30, 828-830.

Harris, A.F., Nimmo, D., McKemey, A.R., Kelly, N., Scaife, S., Donnelly, C.A., Beech, C., Petrie, W.D., and Alphey, L. (2011). Field performance of engineered male mosquitoes. Nature biotechnology 29, 1034-1037.

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Zar, J.H. (1999). Biostatistical Analysis. Prentice-Hall. New Jersey, USA. 663 pp.

Appendix H

FINAL REPORT

STUDY NUMBER: 232SRFR12C1 **TRIAL NUMBER:** SRFR12-001-232XC1

TITLE

A laboratory prolonged toxicity study to determine the effects of ingestion of larvae and pupae of the genetically modified sterile mosquito strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae); according to OECD No. 204 (1984) modified for oral route of exposure.

AUTHOR

E. YTHIER

GUIDELINE

OECD No. 204 (1984) modified for oral route of exposure

SYNTECH RESEARCH TEST FACILITY

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France

TEST SITE

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

SPONSOR

Oxitec Ltd
71, Milton Park
Abingdon, Oxfordshire, OX14 4RX
United Kingdom

Study Initiation Date: 05 OCT 2012

Study Completion Date: 11 MAR 2013 **Total number of pages:** 43

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GLP COMPLIANCE STATEMENT

A laboratory prolonged toxicity study to determine the effects of ingestion of larvae and pupae of the genetically modified sterile mosquito strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae); according to OECD No. 204 (1984) modified for oral route of exposure.

I confirm that I fulfilled the responsibilities of Study Director for the above non-clinical health and environmental safety (regulatory) study. I declare that the objectives laid down in the Study Plan were achieved and the data generated are valid.

Each phase of this study was conducted in accordance with the Principles of Good Laboratory Practices (GLP):
* The OECD Principles of Good Laboratory Practice, N°1, as revised in 1997 [ENV/MC/CHEM (98) 17].
* The application of the OECD Principles of GLP to the Organisation and Management of Multi-site Studies, N°13, 2002 [ENV/JM/MONO (2002) 9].
* The country-specific regulations embodying these principles where appropriate.

These phases were in compliance to GLP with the following exceptions: raw data related to the preparation of ISO reconstituted water. These data were not generated according to GLP principles. These exceptions are considered not to affect the GLP status of the study and the validity of the conclusions drawn.

In addition, I certify that Study Plan and Final Report are conformed to the OECD Principles of GLP and French GLP regulations (« Article Annexe II à l'Article D523-8 du Code de l'Environnement du 16 Octobre 2007 »).

E. YTHIER
Study Director
SynTech Research

11 MAR 2013
Study completion date

QUALITY ASSURANCE STATEMENT

A laboratory prolonged toxicity study to determine the effects of ingestion of larvae and pupae of the genetically modified sterile mosquito strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae); according to OECD No. 204 (1984) modified for oral route of exposure.

Accuracy of transcription from the raw data generated by SynTech Research to the Final Report was checked. The Final Report fully reflects the raw data generated during the study.
The following study specific audits have been carried out by the Quality Assurance personnel at SynTech Research France in accordance with SynTech Research policy and procedures for Good Laboratory Practice.

Table 1: Specific study inspection dates

Type of Inspection	Inspection date	Date of inspection report	Date of dispatch to*
Study Plan verification	04 OCT 2012	05 OCT 2012	05 OCT 2012
Lab-based inspections: Test system, application, assessment and Lab Note Book	25 OCT 2012	09 NOV 2012	09 NOV 2012
Reported data: Final Report, Lab Note Book and raw data	21 DEC 2012	11 JAN 2013	11 JAN 2013

* Study Director, Management and Principal Investigator if relevant

In addition, the following facility and procedure based inspections associated with this type of study have been carried out.

Table 2: Facility and procedure inspection dates

Type of Inspection	Inspection date	Date of inspection report	Date of dispatch to*
Facility: Nîmes (30) (organization, staff, facilities, equipment, documentation)	14 MAY 2012	25 MAY 2012	25 MAY 2012
Facility: La Chapelle de Guinchay (71) (organization, staff, facilities, equipment, documentation)	25-27 JUL 2012	22 AUG 2012	22 AUG 2012
Process (balance & masses calibration)	10 APR 2012	18 MAY 2012	18 MAY 2012
Process (weighing)	30 APR2012	18 MAY 2012	18 MAY 2012
Process (shipping)	22 MAY 2012	24 MAY 2012	24 MAY 2012
Process (archiving)	28 SEP 2012	28 SEP 2012	28 SEP 2012

*Management and relevant personals

Y.TACIK
Test Facility QA
SynTech Research

11 MAR 2013
Date

PRINCIPAL STUDY PERSONNEL

E. YTHIER	Study Director
L. MARTIN	Principal Investigator

REVIEWERS / SUPERVISORS

p.o.

P. ESCHENBRENNER
Test Facility Management
SynTech Research

11 March 2013

Date

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SUMMARY

Report: Ythier, E. (2012): A laboratory prolonged toxicity study to determine the effects of ingestion of larvae and pupae of the genetically modified sterile mosquito strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae); according to OECD No. 204 (1984) modified for oral route of exposure.
Source: SynTech Research France, 613 Route du Bois de Loyse, F-71570 La Chapelle de Guinchay, France
Report No: 232SRFR12C1, issued 11 March 2013
Guidelines: OECD No. 204 (1984) modified for oral route of exposure
Deviations: No deviation
GLP: Yes

Materials and methods:
Guppys *Poecilia reticulata* (Actinopterygii: Poeciliidae), measuring 20 to 26 mm at the start of the test, were orally exposed to mixed larvae and pupae of the genetically modified sterile strain *Aedes aegypti* OX513A over a period of 14 days, in laboratory semi-static conditions.
During the study period, the fish were fed with the transgenic mosquitoes once daily, by incorporating freshly defrosted larvae and pupae into the fish diet, at the rate of 700 g mosquitoes/kg diet. The rate to be tested was determined following a 96-hour non-GLP compliant range-finding study conducted with 20, 100, 300, 500 and 700 g mosquitoes/kg diet, in order to evaluate the maximum rate of insects (mosquitoes) the guppy can ingest (in natural conditions the mean ratio of insects ingested by this species is usually about 50% w/w, i.e. 500 g insects/kg food). Each tested rate was entirely consumed and no adverse effect was observed during the 96-hour range-finding study. The quantity of diet administered daily did not exceed the amount ingested immediately by the fish and was kept constant during the study duration, i.e. 4 per cent of the initial fish weight. A control (non-genetically modified mosquitoes of the same background strain, incorporated to the fish diet at the same rate of 700 g mosquitoes/kg diet) was included to assess the natural mortality rate, appearance, size and behaviour of the test organisms. A toxic reference item (potassium dichromate, applied at a concentration of 100 mg a.s./L) was included to indicate the relative susceptibility of the test organisms and the test system.
Acute and sublethal (appearance, size and behaviour) effects were observed daily during the test period. Data were analysed for significant differences compared to the control group using ANOVA ($p \leq 0.05$) and to determine values for the LR_{50}, ER_{50}, LOER and NOER.

Dates of work: 15 October 2012 - 28 October 2012

Findings (Table 3): Summary of *P. reticulata* mortality, length and weight after 14-day oral exposure to *Aedes aegypti* OX513A

Test item	Genetically modified sterile strain *Aedes aegypti* OX513A		
Test organism	*Poecilia reticulata*		
Test medium	ISO reconstituted water		
Exposure	Daily oral exposure		
Endpoint	14-day mortality [%]	14-day length [mm]	14-day weight [mg]
Control (700 g non-GM mosquitoes/kg diet)	10	22.44	198.3
OX513A (700 g GM mosquitoes/kg diet)	0	23.20	212.9
LR_{50} / ER_{50} [g GM mosquitoes/kg diet]	> 700		
LOER [g GM mosquitoes/kg diet]	> 700		
NOER [g GM mosquitoes/kg diet]	700		

GM = genetically modified

Conclusions:

The study is valid since mean mortality in the control did not exceed 10% during the test period (actual value: 10%), dissolved oxygen concentration was over 60% of the air saturation value throughout the test (actual minimum value: 73.5%) and environmental conditions (T°, pH) remained constant throughout the test.

The potential acute and sublethal effects of ingestion of mosquitoes of the genetically modified sterile strain *Aedes aegypti* OX513A on the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae) were investigated during 14 days in laboratory semi-static conditions.

There was no significant difference between mortality, fish length, weight, appearance and behaviour in the control and the test item, after 14 days. Hence the NOER was found to be 700 g GM mosquitoes/kg diet and the LOER and LR_{50}/ER_{50} were estimated to be > 700 g GM mosquitoes/kg diet.

INTRODUCTION

The test item is a mixture of larval and pupal life stages (collected 7-8 days post hatching) of heterozygous *Aedes aegypti* mosquito strain OX513A from an Asian background expressing a repressible lethality trait (based on the tet-off system (Gossen and Bujard 1992) and DsRed2 fluorescent marker gene).

The objective of the study was to determine potential acute and sublethal effects of ingestion of mosquitoes of the genetically modified sterile strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae) in laboratory semi-static conditions, following oral exposure to larval and pupal life stages of *Aedes aegypti* OX513A over a period of 14 days.

During the study period, the fish were fed with the transgenic mosquitoes once daily, by incorporating freshly defrosted larvae and pupae into the fish diet, at the rate of 700 g mosquitoes/kg diet. The quantity of diet administered daily did not exceed the amount ingested immediately by the fish and was kept constant during the study duration, i.e. 4 per cent of the initial fish weight.

The rate to be tested was determined following a 96-hour non-GLP compliant range-finding study conducted with 20, 100, 300, 500 and 700 g mosquitoes/kg diet, in order to evaluate the maximum rate of insects (mosquitoes) the guppy can ingest (in natural conditions the mean ratio of insects ingested by this species is usually about 50% w/w, i.e. 500 g insects/kg food). Each tested rate was entirely consumed and no adverse effect was observed during the 96-hour range-finding study.

A control (non-genetically modified mosquitoes of the same background strain as the test substance, incorporated to the fish diet at the same rate of 700 g mosquitoes/kg diet) was included to assess the natural mortality rate, appearance, size and behaviour of the test organisms. A toxic reference item (potassium dichromate, applied at a concentration of 100 mg a.s./L) was included to indicate the relative susceptibility of the test organisms and the test system.

Acute and sublethal (appearance, size and behaviour) effects were observed once a day during 14 days. As no adverse effect was observed in the fish group fed with OX513A mosquitoes between 7 and 14 days, whilst control mortality remained at an accepted level (10%), the study duration was not extended.

The study was conducted in accordance with the OECD guideline No. 204 (1984) modified for oral route of exposure. The experimental phase of the study was performed at the test site of SynTech Research France SAS, 1095 chemin du Bachas, 30000 Nîmes, France.

All aspects of the study were carried out according to international Good Laboratory Practice (GLP) guidelines and were based on the international codes of GLP (see References on p.19).

The study encompassed the objectives of Regulation (EC) No. 1107/2009 and was designed to comply with the FAO Guidelines on Producing Pesticide Residue Data from supervised trials, Rome 1990 and “Commission Working Document 7029/VI/95 - Rev. 5, July 1997”.

The study was conducted in accordance with French GLP regulations (“Article Annexe II à l'Article D523-8 du Code de l'Environnement du 16 octobre 2007”). This study is referred to GLP area of expertise No.4: “Environmental toxicity studies on aquatic or terrestrial organisms”.

TEST ITEM *AEDES AEGYPTI* OX513A

Table 4:

Test item code	*Aedes aegypti* OX513A
Physical state, appearance	Mixture of larval and pupal life stages in distilled water
Quantity received / Date of receipt	327.33 g on 31 August 2012
Storage requirement	In its original container, tightly closed, in frozen conditions.
Test item supply	Oxitec Ltd, 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX, United Kingdom

Three batches of the test item were received on 31 AUG 2012 at the test site of Nîmes and identified as SynTech Research No. NI12-303, NI12-304 and NI12-305. The test item was stored deep frozen between -18.4°C and -38.3°C between its receipt and its last use.

CONTROL ITEM *AEDES AEGYPTI* WILD TYPE

Table 5:

Test item code	*Aedes aegypti* Wild Type
Physical state, appearance	Mixture of larval and pupal life stages in distilled water
Quantity received / Date of receipt	203.01 g on 31 August 2012
Storage requirement	In its original container, tightly closed, in frozen conditions.
Test item supply	Oxitec Ltd, 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX, United Kingdom

Two batches of the control item were received on 31 AUG 2012 at the test site of Nîmes and identified as SynTech Research No. NI12-306 and No. NI12-307. The control item was stored deep frozen between -18.4°C and -38.3°C between its receipt and its last use.

REFERENCE ITEM POTASSIUM DICHROMATE

Table 6:

Reference item	Potassium dichromate
Batch No.	102403H
Reference item (nominal conc.)	Potassium dichromate (1000 mg/kg)
Reference item (actual conc.)	Potassium dichromate (999.7 mg/kg)
CAS No.	7778-50-9
Formulation density [g/ml]	1 (solid)
Physical appearance	Orange solid crystals
Storage requirement	Dry, cool and well-ventilated area
Product supply	Merck KGaA, 64271 Darmstadt, Germany

The reference item was received on 03 DEC 2010 at the test facility of La Chapelle de Guinchay (identified as SynTech Research No. CG10-349) and transferred to the test site of Nîmes on 23 MAR 2011 (identified as SynTech Research No. NI11-302). The reference item was stored between 12.6°C and 24.9°C between its receipt and its last use. The material safety data sheet was available on 03 DEC 2010. A retained sample of formulated product used as reference item is kept by SynTech Research (No. CG10-349A).

EXPERIMENTAL PHASE

Study Plan Amendments and Deviations

No Study Plan Amendment and Deviation.

Study organisation

Table 7:

Study Sponsor:	Oxitec Ltd 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX, United Kingdom	
Study Monitor and Sponsor's Representative:	Camilla BEECH	Tel: 44 (0)1235 433549 e-mail: Camilla.Beech@oxitec.com
Test Facility:	SynTech Research France SAS 613 route du Bois de Loyse, 71570 La Chapelle de Guinchay, France	Tel: +33 (0)3 85 36 82 36 Fax: +33 (0)3 85 36 78 97
Management: Study Director: Lead Quality Assurance:	Pierre ESCHENBRENNER Eric YTHIER Yannick TACIK	e-mail: peschenbrenner@syntechresearch.com e-mail: eythier@syntechresearch.com e-mail: ytacik@syntechresearch.com
Test site for experimental phase:	SynTech Research France SAS Aquatoxicology Laboratory 1095 chemin du Bachas F-30000 Nîmes, France	Tel: +33 (0)4 66 70 98 65
Principal Investigator:	Lucie MARTIN	e-mail : lmartin@syntechresearch.com
Item supply:	Oxitec Ltd Merck KGaA	71, Milton Park, Abingdon, Oxfordshire, OX14 4RX, United Kingdom 64271 Darmstadt, Germany

Archiving

After completion of the final report, the Study Director will transfer the following data generated in the study to:

Camilla BEECH
Oxitec Ltd
71, Milton Park, Abingdon,
Oxfordshire, OX14 4RX,
United Kingdom

Data to be transferred will include, but not be limited to:
1. The original study plan, amendments, and deviations
2. The original final report
3. Test item characterisation and certification documentation
4. The original raw data package

Copies of the study plan, raw data, amendments, deviations and final report, as well as all non-study specific data (e.g. log books describing equipment maintenance and calibration) will be stored in the archives of SynTech Research France SAS for ten years. No data will be discarded without the Sponsor's prior written consent.

Test system

The experimental phase of the study was conducted at the Aquatoxicology laboratory of SynTech Research France SAS, 1095 chemin du Bachas, 30000 Nîmes, France.

The fish used for this study were the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae; source: La Grande Rivière, France). The fish were obtained and held in the laboratory for 12 days before they were used for testing. In order to adapt the fish to the test conditions, a fish culture in the test medium was prepared 12 days before start of the test under the following conditions:

- light: 16 hours photoperiod daily
- temperature: 21°C to 25°C
- oxygen concentration: at least 80% of air saturation value
- feeding (diet without mosquitoes - see diet composition below): once daily until 24 hours before the study start

Following a 48-hour settling-in period, mortality was recorded to be < 5% (actual value: 0%) and the batch was accepted to be used for the study.

All organisms used for the study were originated from cultures established from the same healthy stock of fish. At the start of the test, the animals were 20 mm ± 10 and the loading was < 1 g fish/L test medium (actual size values 20 to 26 mm / mean 22.5 mm; actual loading value 0.698 g fish/L; based on 10 organisms randomly sampled in the fish culture the day before the start of the test; see Appendix 2). They were in good health and free from any apparent malformation. The fish were not fed from 24 hours before the test start and during the test period.

Test vessels (= test units) consisted of 4 L glass jars containing 3 L of test medium. During the test period, test units were capped to reduce the loss of water due to evaporation and to avoid the entry of dust into solutions. Each test unit was labelled with the study number and a unique test unit number.

The ISO test medium was used. The composition of the test medium is described in Annex 3 of OECD guideline No. 203. The test medium was made at the test site, using distilled water. The test medium was aerated until oxygen saturation and then stored for 2 days prior to use. The test medium was aerated during the study. The test medium was renewed twice weekly and at the time of each renewal

the test medium temperature, dissolved oxygen and pH were recorded (see Appendix 3). At each renewal, a second series of test vessels were prepared and the test organisms were transferred to them.

The study comprised a control, a toxic reference item and one rate of the test item: 700 g mosquitoes/kg diet. There was one test unit with 10 replicates (= 10 fish) for each test item, control and toxic reference item.

A control (non-genetically modified mosquitoes of the same background strain, incorporated to the fish diet at the rate of 700 g mosquitoes/kg diet) was included to assess the natural mortality rate, appearance, size and behaviour of the test organisms. A toxic reference item was included in the study to demonstrate the susceptibility of the test organism and the sensitivity of the test system. The toxic reference item was potassium dichromate applied at an application concentration of 100 mg a.s./L (the toxic reference group was fed with diet incorporated with non-genetically modified mosquitoes at the rate of 700 g mosquitoes/kg diet).

The items comprised:
- Test item: genetically modified mosquitoes *A. aegypti* OX513A at 700 g mosquitoes/kg diet
- Control: non-genetically modified mosquitoes *A. aegypti* at 700 g mosquitoes/kg diet
- Toxic reference item: potassium dichromate at 100 mg a.s./L (fed with non-genetically mosquitoes *A. aegypti* at 700 g mosquitoes/kg diet)

The diet was administered daily, at the dose of 4% of the initial fish weight. Quantity of administered diet was calculated each day according to the number of living fish. The sequence of feeding was as follows: control group, followed by the test item group and finally the toxic reference item group.
The diet (TetraMin®, used during both holding and exposure periods) consisted of:
- fish and fish derivatives,
- cereals,
- yeasts,
- vegetable protein extracts,
- molluscs and crustaceans,
- oils and fats,
- algae,
- sugars,
- mineral substances.
- components: protein 47%, fat 10%, fiber 3%, vitamins D3 and A, elements Mn, Zn, Fe and Co.

During the holding phase (12 days before fish were used for testing), the diet was administered daily, except during the 24 hours before the study start (exposure phase). The diet was administered without mosquitoes during the holding phase.
During the exposure phase, genetically modified (OX513A) or non-genetically modified (control and reference item) mosquitoes were incorporated to the fish diet at the rate of 700 g mosquitoes/kg diet and the new diet was administered daily.
In both holding and exposure phases, the quantity of diet administered daily did not exceed the amount ingested immediately by the fish and was kept constant during the study duration, i.e. 4 per cent of the initial fish weight. Quantity of administered diet was calculated each day according to the number of living fish.

Twice a week, the temperature, dissolved oxygen and the pH were recorded (see Appendix 3). Test units were kept in controlled environment conditions between 21ºC to 25°C (constant within the range of ± 2ºC; actual values: 20.5-22.4°C) and received 16 hours light (1120-1340 lux) and 8 hours dark cycle. Item groups were placed on separated shelves in the laboratory.

Table 8: Test system summary

Experimental phase location:	Aquatoxicology Laboratory SynTech Research France SAS 1095 Chemin du Bachas 30000 Nîmes, France
Test organism (species):	Guppy, *Poecilia reticulata* Peters (Actinopterygii: Poeciliidae)
Test system:	Items: 3 (1 test item, 1 toxic reference item, 1 control). Test unit: 4 L capped glass jar (one per test item). Test organisms (= replicates): 10 *Poecilia reticulata* in each test unit ; 20 to 26 mm (mean 22.5 mm) and loading 0.698 g fish/L at the start of the test. Test medium: ISO reconstituted water.
Items:	- Test item: genetically modified mosquitoes *A. aegypti* OX513A at 700 g mosquitoes/kg diet - Control item: non-genetically modified mosquitoes *A. aegypti* at 700 g mosquitoes/kg diet - Toxic reference item: potassium dichromate at 100 mg a.s./L (fed with non-genetically mosquitoes *A. aegypti* at 700 g mosquitoes/kg diet)
Number of applications:	1 toxic reference application
Number of feeding:	The fish were fed once daily with prepared diet (4% of the initial fish weight).
Number of renewals:	The test medium was renewed twice weekly.
Replicates:	10 replicates (= fish) for each test item, control and reference item.
Item details:	Item groups were separated from each other in the culturing chamber to avoid contamination between treated/control test units and between treated test units.
Test duration	The duration of the test was 14 days.
Test organism destruction:	At the end of the study, the remaining test organisms were destroyed according to SynTech SOPs.
Test conditions:	Monitoring of environmental conditions was carried out throughout the study, at regular intervals, using calibrated equipment. Organisms were maintained at temperature of 20.5-22.4°C and in 16 hours light cycle (1120-1340 lux).
Guideline:	The study was conducted in accordance with the OECD guideline No. 204 modified for oral route of exposure.

Exposure details

Table 9: item applied concentrations

Item ID	Item	a.s. concentration / L test medium	f.p. concentration / L test medium*	Mosquitoes / kg diet
C101	Control	NA	NA	700 g non-GM mosquitoes / kg diet
T102	OX513A	NA	NA	700 g GM mosquitoes / kg diet
R103	Potassium dichromate	100 mg a.s./L	100.03 mg f.p./L	700 g non-GM mosquitoes / kg diet

* Based on the actual a.s. content of the toxic reference item.
NA = not applicable; a.s. = active substance; f.p. = formulated product; GM = genetically modified.

Assessment details

The test endpoint is acute toxicity. Fish were considered as dead if there is no visible movement and if touching of the caudal peduncle produces no reaction.

Sublethal effects were also recorded. These include all effects observed on the appearance, size and behaviour of the fish that make them clearly distinguishable from the control animals, e.g. different swimming behaviour, different reaction to external stimuli, changes in appearance of the fish, reduction or cessation of food intake, changes in length or body weight.

Food intake was evaluated by verifying if the entire administrated diet quantity was consumed or if remaining diet was found in the test unit 1 hour after its administration.

Each test unit was inspected daily during the exposure period.

Representative samples of the test population were weighed and measured before the test starts. All survivors were weighed and measured at the termination of the test.

The mortality was determined according to the following expression:
Mean mortality (%) = 100 x [(T-L)/T]
L = number of living organisms, T = total number of organisms

The results were corrected for control mortality according to Abbott (1925):

$$M\% = \left(\frac{Mt - Mc}{100 - Mc}\right) \times 100$$

where M% = corrected mortality
Mt = % mortality in the test or toxic reference item group
Mc = % mortality in the control

The statistical evaluation (NOEC/LOEC determination) was conducted with the software Minitab® Release 14.

Table 10: Assessments details and dates

Study Plan timing	Actual date	Action
Day before exposure	14 OCT 2012	Length / weight of representative samples of the test population.
First day of exposure	15 OCT 2012	Application of the reference item and first oral exposure (feeding). Assessment (O_2 / temperature / pH).
Once daily during exposure period	15 to 28 OCT 2012	Assessment (mortality / sublethal effects / food intake).
Twice weekly during exposure period	18, 22, 25 OCT 2012	Test medium renewal. Assessment (O_2 / temperature / pH) on the fresh and aged test medium.
Last day of exposure	28 OCT 2012	Length / weight of all surviving test organisms.

RESULTS

Validity criteria:

The experimental phase of this study is valid, because:
- Mean mortality in the control did not exceed 10% during the test period (actual value: 10%).
- dissolved oxygen concentration was over 60% of the air saturation value throughout the test (actual minimum value: 73.5%) and environmental conditions (T°, pH) remained constant throughout the test (see Appendix 3).

A summary of the results is given below and the individual data are shown in Appendix 2.

Mortality:

Table 11: *P. reticulata* 14-day mean mortality

Item ID	Item	14-day mean mortality [%]
C101	Control (700 g non-GM mosquitoes/kg diet)	10
T102	OX513A (700 g GM mosquitoes/kg diet)	0
R103	Potassium dichromate (100 mg a.s./L)	100*

* Item group significantly different from the control (ANOVA plus Dunnett's after Log transformation, see Appendix 2).
a.s. = active substance; GM = genetically modified.

The mean mortality was 10% in the control and 100% in the toxic reference item. There was no significant difference between mortality in the control and the test item, after 14 days (ANOVA plus Dunnett's, 95% confidence level).

Abbott (1925) corrected mortality:

Table 12: *P. reticulata* 14-day Abbott corrected mean mortality

Item ID	Item	14-day Abbott corrected mean mortality [%]
C101	Control (700 g non-GM mosquitoes/kg diet)	0
T102	OX513A (700 g GM mosquitoes/kg diet)	- 11.1
R103	Potassium dichromate (100 mg a.s./L)	+ 100*

* Item group significantly different from the control (ANOVA plus Dunnett's after Log transformation, see Appendix 2).
a.s. = active substance; GM = genetically modified.

Corrected mortality in the reference item group was 100%. There was no significant difference between corrected mortality in the control and the test item, after 14 days (ANOVA plus Dunnett's, 95% confidence level).

The NOER was found to be 700 g GM mosquitoes/kg diet and both LOER and LR_{50} were estimated to be > 700 g GM mosquitoes/kg diet.

Food intake, body length and weight:

During the exposure period, the entire administrated diet quantity was consumed by the fish in both control and test item. No remaining diet was found in the test units 1 hour after administration.

The day before the start of the test, 10 representative samples of the test population were randomly sampled and were weighed and measured. The animals were 20 to 26 mm (mean 22.5 mm) and 95.5 to 371 mg (mean 206.8 mg; loading 0.698 g fish/L; see Appendix 2). All survivors in control and test item groups were weighed and measured at the termination of the test (see Table 13 below and Appendix 2).

Table 13: *P. reticulata* 14-day body length and weight

Item ID	Item	14-day mean length [mm]	14-day mean weight [mg]
C101	Control (700 g non-GM mosquitoes/kg diet)	22.44	198.3
T102	OX513A (700 g GM mosquitoes/kg diet)	23.20	212.9

GM = genetically modified.

There was no significant difference between fish length and weight in the control and the test item, after 14 days (ANOVA plus Dunnett's, 95% confidence level). Hence the NOER was found to be 700 g GM mosquitoes/kg diet and both LOER and ER_{50} were estimated to be > 700 g GM mosquitoes/kg diet.

Other observed biological effects:

No abnormal behaviour or appearance was observed among the fish in the test item, 14 days after exposure to the test item, in comparison to the control.

DISCUSSION AND CONCLUSION

The study evaluated potential acute and sublethal effects of ingestion of mosquitoes of the genetically modified sterile strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae) in laboratory semi-static conditions, following oral exposure to larval and pupal life stages of *Aedes aegypti* OX513A over a period of 14 days.

During the study period, the fish were fed with the transgenic mosquitoes once daily, by incorporating freshly defrosted larvae and pupae into the fish diet, at the rate of 700 g mosquitoes/kg diet. The quantity of diet administered daily did not exceed the amount ingested immediately by the fish and was kept constant during the study duration, i.e. 4 per cent of the initial fish weight.

Acute and sublethal (appearance, size and behaviour) effects were observed once a day during 14 days.

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

Test item	Genetically modified sterile strain *Aedes aegypti* OX513A		
Test organism	*Poecilia reticulata*		
Test medium	ISO reconstituted water		
Exposure	Daily oral exposure		
Endpoint	14-day mortality [%]	14-day length [mm]	14-day weight [mg]
Control (700 g non-GM mosquitoes/kg diet)	10	22.44	198.3
OX513A (700 g GM mosquitoes/kg diet)	0	23.20	212.9
LR_{50} / ER_{50} [g GM mosquitoes/kg diet]	> 700		
LOER [g GM mosquitoes/kg diet]	> 700		
NOER [g GM mosquitoes/kg diet]	700		

GM = genetically modified

Conclusion

The potential acute and sublethal effects of ingestion of mosquitoes of the genetically modified sterile strain *Aedes aegypti* OX513A on the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae) were investigated during 14 days in laboratory semi-static conditions.

There was no significant difference between mortality, fish length, weight, appearance and behaviour in the control and the test item, after 14 days. Hence the NOER was found to be 700 g GM mosquitoes/kg diet and the LOER and LR_{50}/ER_{50} were estimated to be > 700 g GM mosquitoes/kg diet.

REFERENCES

Code de l'Environnement. Article Annexe II à l'Article D523-8 du Code de l'Environnement du 16 octobre 2007.

Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonization of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

OECD guideline No. 203. Fish, Acute Toxicity Test (Adopted 17 July 1992). 9 pp.

OECD guideline No. 204. Fish, Prolonged Toxicity Test: 14-day Study (Adopted 4 April 1984). 9 pp.

OECD Principles of Good Laboratory Practice (as revised in 1997). Series on Principles of GLP and Compliance Monitoring, No. 1. ENV/MC/CHEM(98)17 and No. 13. ENV/JM/MONO (2002)9. Organisation for Economic Co-operation and Development, Paris, France which are accepted by regulatory authorities throughout the European Community, the United States of America (FDA and EPA) and Japan (MHW, MAFF and MITI) on the basis of intergovernmental agreements.

Regulation (EC) No.1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC.

Appendix 1
Study Plan 232SRFR12C1

(16 pages)

SynTech Research

STUDY PLAN

STUDY NUMBER: 232SRFR12C1 **TRIAL NUMBER:** SRFR12-001-232XC1

STUDY TITLE:
A laboratory prolonged toxicity study to determine the effects of ingestion of larvae and pupae of the genetically modified sterile mosquito strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae); according to OECD No. 204 (1984) modified for oral route of exposure.

STUDY DIRECTOR:
Eric YTHIER

DATE:
05 October 2012

TEST FACILITY:
SynTech Research France SAS
613 route du Bois de Loyse
71570 La Chapelle de Guinchay, France

SPONSOR:
Oxitec Ltd
71, Milton Park
Abingdon, Oxfordshire, OX14 4RX
United Kingdom

APPROVED BY:

Study Director:
SynTech Research France
05 oct 2012
Eric YTHIER

Test Facility Management:
SynTech Research France
05 oct 2012
Pierre ESCHENBRENNER

Test Facility Lead Quality Assurance:
SynTech Research France
05 OCT 2012
Yannick TACIK

Study Monitor
Sponsor's Representative:
Oxitec Ltd
08 oct 2012
Camilla BEECH

Proposed study timetable

Experimental Starting Date (first exposure):	October 2012
Laboratory experimental completion date:	November 2012
Final Report Issue:	December 2012

Total number of pages: 16

TABLE OF CONTENTS

1. PURPOSE OF THE STUDY

The objective of the study is to determine potential acute and sublethal effects of ingestion of mosquitoes of the genetically modified sterile strain *Aedes aegypti* OX513A towards the guppy *Poecilia reticulata* (Actinopterygii: Poeciliidae) in laboratory semi-static conditions, following oral exposure to larval and pupal life stages of *Aedes aegypti* OX513A over a minimum period of 14 days.

During the study period, the fish will be fed with the transgenic mosquitoes once daily, by incorporating freshly defrosted larvae and pupae into the fish diet, at the rate of 700 g mosquitoes/kg diet. The quantity of diet administered daily will not exceed the amount ingested immediately by the fish and will be kept constant during the study duration, i.e. 4 per cent of the initial fish weight.

The rate to be tested was determined following a 96-hour non-GLP compliant range-finding study conducted with 20, 100, 300, 500 and 700 g mosquitoes/kg diet, in order to evaluate the maximum rate of insects (mosquitoes) the guppy can ingest (in natural conditions the mean ratio of insects ingested by this species is usually about 50% w/w, i.e. 500 g insects/kg food). Each tested rate was entirely consumed and no adverse effect was observed during the 96-hour range-finding study.

A control (non-genetically modified mosquitoes incorporated to the fish diet at the same rate of 700 g mosquitoes/kg diet) will be included to assess the natural mortality rate, appearance, size and behaviour of the test organisms. A toxic reference item (potassium dichromate, applied at a concentration of 100 mg a.s./L) will be included to indicate the relative susceptibility of the test organisms and the test system.

Acute and sublethal (appearance, size and behaviour) effects will be observed once a day during 14 days. If adverse effects in the fish group fed with OX513A mosquitoes increase between 7 and 14 days, whilst control mortality remains at an accepted level (i.e. ≤10%), the study duration will be extended to 21 or 28 days (depending on effects between 14 and 21 days) maximum.

The biological part of the study will be performed in the Aquatoxicology laboratory of SynTech Research France SAS and the method will be based on the OECD guideline n°204 modified for oral route of exposure. All aspects of the study will be carried out according to international Good Laboratory Practice (GLP) guidelines, and will be based on the following guidelines and international codes of GLP:

- OECD guideline n°204. Fish, Prolonged Toxicity Test: 14-day Study (Adopted 4 April 1984). 9 pp.

- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonization of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

- OECD Principles of Good Laboratory Practice (as revised in 1997). Series on Principles of GLP and Compliance Monitoring, No. 1. ENV/MC/CHEM(98)17 and No. 13. ENV/JM/MONO (2002)9. Organisation for Economic Co-operation and Development, Paris, France which are accepted by regulatory authorities throughout the European Community, the United States of America (FDA and EPA) and Japan (MHW, MAFF and MITI) on the basis of intergovernmental agreements.

The study will encompass the objectives of Regulation (EC) No 1107/2009 and will be designed to comply with the FAO Guidelines on Producing Pesticide Residue Data from supervised trials, Rome 1990 and "Commission Working Document 7029/VI/95 - Rev. 5, July 1997".

The experimental phase of this study will be conducted in accordance with French GLP regulations ("Article Annexe II à l'Article D523-8 du Code de l'Environnement du 16 octobre 2007"). This study will be referred to GLP area of expertise n°4: "*Environmental toxicity studies on aquatic or terrestrial organisms*".

2. STUDY ORGANISATION

Study Sponsor:	Oxitec Ltd 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX, United Kingdom	
Study Monitor and Sponsor's Representative:	Camilla BEECH	Tel: 44 (0)1235 433549 e-mail: Camilla.Beech@oxitec.com
Test Facility:	SynTech Research France SAS 613 route du Bois de Loyse, 71570 La Chapelle de Guinchay, France	Tel: +33 (0)3 85 36 82 36 Fax: +33 (0)3 85 36 78 97
Management: Study Director: Lead Quality Assurance:	Pierre ESCHENBRENNER Eric YTHIER Yannick TACIK	e-mail: peschenbrenner@syntechresearch.com e-mail: eythier@syntechresearch.com e-mail: ytacik@syntechresearch.com
Test site for experimental phase:	SynTech Research France SAS Aquatoxicology Laboratory 1095 chemin du Bachas F-30000 Nîmes, France	Tel: +33 (0)4 66 70 98 65
Principal Investigator:	Lucie MARTIN	e-mail : lmartin@syntechresearch.com
Item supply:	Oxitec Ltd Merck KGaA	71, Milton Park, Abingdon, Oxfordshire, OX14 4RX, United Kingdom 64271 Darmstadt, Germany

3. GLP PRINCIPLES

3.1. GLP compliance

This study will be performed according to the procedures described in this study plan and in accordance with OECD Principles of GLP and Compliance Monitoring No.1 revised [(ENV/MC/CHEM(98)17] and Monitoring No.13 [(ENV/JM/MONO(2002)9]. The Study Director will be responsible for compliance with the relevant national GLP regulations.

3.2. Quality Assurance

Inspection of laboratory phase of the study, including the report, will be the responsibility of the Test Facility Quality Assurance. Study plan and one critical laboratory phase will be inspected (minimum). All inspection and audit findings will be reported to the Study Director and line management as appropriate, on completion of each audit. The final report will be audited to determine that it reflects the procedures adopted and the raw data generated and that it meets GLP requirements.

3.3. Standard Operating Procedures

Study procedures will follow the applicable SOPs of the respective test facilities/sites, unless they conflict with study plan requirements, which always override standard procedures.

4. ITEM DESCRIPTION

It is the responsibility of the Study Director to request timely dispatch of the relevant test and reference items, Material Safety Data Sheets and Certificate(s) of Analysis to the appropriate study personnel. These are ordered from the Sample Dispatch Co-ordinator.

4.1 Test item

The test item is a mixture of larval and pupal life stages (collected 7-8 days post hatching) of heterozygous *Aedes aegypti* mosquito strain OX513A from an Asian background expressing a repressible lethality trait (based on the tet-off system (Gossen and Bujard 1992) and DsRed2 fluorescent marker gene).
The test item will be supplied frozen (-15°C) in distilled water. Test item not used will be returned to the Sponsor or discarded by the contract test site following local regulations and after agreement with the Sponsor.

Test item code	*Aedes aegypti* OX513A
Physical state, appearance	Mixture of larval and pupal life stages in distilled water
Quantity received / Date of receipt	327.33 g on 31 August 2012
Storage requirement	In its original container, tightly closed, in frozen conditions.
Test item supply	Oxitec Ltd, 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX, United Kingdom

4.2 Toxic reference item

The Material Safety Data Sheets will be provided by the product supplier to the Study Director before the laboratory phase is commenced. Toxic reference item not used in the study will be stored in the test facility until the use-by date stated on the container label.

Reference item	Potassium dichromate
Batch No.	102403H
Active substance (nominal conc.)	Potassium dichromate (1000 g/kg)
Active substance (actual conc.)	Potassium dichromate (999.7 g/kg)
Active substance CAS number	7778-50-9
Physical state, appearance	Solid, orange crystalline powder
Storage requirement	Cool, dry and well-ventilated place. In the original container.
Reference item supply	Merck KGaA, 64271 Darmstadt, Germany

5. TEST SYSTEM

5.1 Test organisms

The fish used for this study will be the guppy *Poecilia reticulata*. The source and the maintenance details of the fish before use in the study will be recorded in the raw data and included in the final report.

In order to adapt the fish to the test conditions, they will be held in the laboratory for at least 12 days before they will be used for testing. A fish culture in the test medium will be prepared at least 7 days before start of the test under the following conditions:

- light: 12 to 16 hours photoperiod daily
- temperature: 21°C to 25°C
- oxygen concentration: at least 80% of air saturation value
- Feeding (diet without mosquitoes - see diet composition below): once daily until 24 hours before the test will start

Following a 48-hour settling-in period, mortalities will be recorded and the following criteria will be applied:

- mortality > 10%: rejection of the entire batch
- mortality between 5% and 10%: acclimatisation continued for 7 additional days
- mortality < 5%: acceptance of the batch

At the start of the test, the animals will be 2 cm ± 1. They will be in good health and free from any apparent malformation. The fish will not be fed from 24 hours before the test is started.

Individuals for use in the study will be selected without conscious bias but any that are obviously unhealthy or damaged will be discarded. Procedures for the assignment of individual test organisms to test groups will be recorded in the raw data and described in the study report.

5.2 Test medium

The ISO test medium (reconstituted test water) will be used. The composition of the test medium is described in Annex 2 of OECD guideline n°203. The test medium will be made at the test site, using distilled water. The test medium will be aerated until oxygen saturation and then stored for 2 days prior to use. The total hardness, the pH and the conductivity will be recorded. The test medium will be renewed twice weekly. At each renewal, a second series of test vessels will be prepared and the test organisms will be transferred to them.

5.3 Test units

Test vessels (= test units) will consist of 4 L glass jars containing 3 L of test medium. Test units will be thoroughly cleaned before each use.
During the test period, test units will be capped to reduce the loss of water due to evaporation and to avoid the entry of dust into solutions. Aeration can be used.
Each test unit will be labelled with the study number and a unique test unit number.

5.4 Diet

The diet (TetraMin®, to be used during both holding and exposure periods) will consist of:

- fish and fish derivatives,
- cereals,
- yeasts,
- vegetable protein extracts,
- molluscs and crustaceans,
- oils and fats,
- algae,
- sugars,
- mineral substances.
- components: protein 47%, fat 10%, fiber 3%, vitamins D3 and A, elements Mn, Zn, Fe and Co.

During the holding phase (at least 12 days before fish are used for testing), the diet will be administered daily, except during the 24 hours before the test (exposure phase) is started. The diet will be administered without mosquitoes during the holding phase.

During the exposure phase, genetically modified (OX513A) or non-genetically modified (control and reference item) mosquitoes will be incorporated to the fish diet at the rate of 700 g mosquitoes/kg diet and the new diet will be administered daily.

In both holding and exposure phases, the quantity of diet administered daily will not exceed the amount ingested immediately by the fish and will be kept constant during the study duration, i.e. 4 per cent of the initial fish weight. Quantity of administered diet will be calculated each day according to the number of living fish.

5.5 Test groups

The study will comprise a control, a toxic reference item and one rate of the test item: 700 g mosquitoes/kg diet. There will be one test unit with 10 replicates (= 10 fish) for each test item, control and toxic reference item.

A control (non-genetically modified mosquitoes incorporated to the fish diet at the rate of 700 g mosquitoes/kg diet) will be included to assess the natural mortality rate, appearance, size and behaviour of the test organisms. A toxic reference item will be included in the study to demonstrate the susceptibility of the test organism and the sensitivity of the test system. The toxic reference item will be potassium dichromate applied at an application concentration of 100 mg a.s./L and should result in a cumulative 14-day mean mortality > 50% (the toxic reference group will be fed with diet incorporated with non-genetically modified mosquitoes at the rate of 700 g mosquitoes/kg diet).

The items will comprise:

- Test item: genetically modified mosquitoes *A. aegypti* OX513A at 700 g mosquitoes/kg diet
- Control: non-genetically modified mosquitoes *A. aegypti* at 700 g mosquitoes/kg diet
- Toxic reference item: potassium dichromate at 100 mg a.s./L

The diet will be administered daily, at the dose of 4% of the initial fish weight. Quantity of administered diet will be calculated each day according to the number of living fish.

The sequence of feeding will be as follows: control group, followed by the test item group and finally the toxic reference item group. Twice a week, the temperature, dissolved oxygen and the pH will be recorded.

6. EXPERIMENTAL PHASE LOCATION AND CONDITIONS

The experimental phase of the study will be carried out at the Aquatoxicology laboratory of SynTech Research France SAS, 1095 chemin du Bachas, 30000 Nîmes, France. Test units will be maintained under controlled environment conditions during the tests. Conditions will be recorded and all environmental data will be included in the report.

7. EXPERIMENTAL PROCEDURES

Study number:	232SRFR12C1	**Trial number:**	SRFR12-001-232XC1
Experimental phase location:	Aquatoxicology laboratory SynTech Research France SAS 1095 Chemin du Bachas 30000 Nîmes, France		
Test organism (species):	Guppy *Poecilia reticulata* Peters (Actinopterygii: Poeciliidae)		
Test system:	Test medium: ISO reconstituted water. Items: 3 (1 test item, 1 toxic reference item, 1 control). Test unit: 4 L capped glass jar. Test organisms (= replicates): 10 *Poecilia reticulata* (2 cm ± 1) in each test unit (maximum loading: 1 g fish /L of solution).		
Items:	- Test item: genetically modified mosquitoes *A. aegypti* OX513A at 700 g mosquitoes/kg diet - Control item: non-genetically modified mosquitoes *A. aegypti* at 700 g mosquitoes/kg diet - Toxic reference item: potassium dichromate at 100 mg a.s./L (fed with non-genetically mosquitoes *A. aegypti* at 700 g mosquitoes/kg diet)		
Number of application:	1 toxic reference application.		
Number of feeding:	The fish will be fed once daily with prepared diet (4% of the initial fish weight).		
Number of renewals:	The test medium will be renewed twice weekly.		
Replicates:	10 replicates for each test item, control and toxic reference item.		
Study duration:	14 to 28 days, depending on effects after 14 and 21 days.		
Test organism destruction:	At the end of the study, the remaining test organisms will be destroyed according to SynTech SOPs.		
Test conditions:	Monitoring of environmental conditions will be carried out throughout the trial, either at regular intervals or continuously, using calibrated equipment. Organisms will be maintained between 21°C to 25°C (constant within the range of ± 2°C), in 12 hours to 16 hours light cycle.		
Guideline:	The study will be conducted in accordance with the OECD guideline n°204 modified for oral route of exposure.		

Exposure details:

The actual concentration of toxic reference item potassium dichromate (999.7 g a.s./kg) will be used when preparing the solution and when calculating the deviation percentage. Full details of dose preparation procedures will be recorded in the raw data and presented in the study report.

Item ID	Item	a.s. concentration / L	f.p. concentration / L*	Mosquitoes / kg diet
C101	Control	NA	NA	700 g non-GM mosquitoes / kg diet
T102	OX513A	NA	NA	700 g GM mosquitoes / kg diet
R103	Potassium dichromate	100 mg a.s./L	100.03 mg f.p./L	700 g non-GM mosquitoes / kg diet

* Based on the actual concentration of the toxic reference items. a.s. = active substance; f.p. = formulated product; NA = not applicable; GM = genetically modified. A variation of ±10% is acceptable.

Test conditions:

Test units will be maintained under controlled environment conditions during the test: a culturing chamber will be used, in which the test medium temperature will be maintained between 21°C to 25°C and constant within the range of ± 2°C. The test units will receive 12 hours to 16 hours light cycle. Oxygen concentration will be maintained over 60% of the air saturation value. Aeration can be used.
The study will be carried out without adjustment of pH. Conditions will be recorded and all environmental data will be included in the report.
Short-term deviations from these temperature and light conditions when handling the test units (less than two hours) are not expected to have an adverse effect on results and will not be reported as deviations from the study plan, according to SynTech SOPs.
pH of the solutions will be measured twice weekly. The pH should be in the range of 6 to 8.5.

Validity criteria:

The study will be invalid and will be repeated if:
- the mortality in the control is more than 10% during the test period.
- the conditions are not maintained constant throughout the test.
- the dissolved oxygen concentration falls under 60% of the air saturation value throughout the test.

Assessment details:

The test endpoint is acute toxicity. Fish will be considered as dead if there is no visible movement and if touching of the caudal peduncle produces no reaction.

Sublethal effects will be also recorded. These include all effects observed on the appearance, size and behaviour of the fish that make them clearly distinguishable from the control animals, e.g. different swimming behaviour, different reaction to external stimuli, changes in appearance of the fish, reduction or cessation of food intake, changes in length or body weight.

Food intake will be evaluated by verifying if the entire administrated diet quantity is consumed or if remaining diet is found in the test unit 1 hour after its administration.

Each test unit will be inspected daily during the exposure period.

Representative samples of the test population will be weighed and measured before the test starts. All survivors will be weighed and measured at the termination of the test.

Study schedule:

Timing*	Action
Day before exposure	Length / weight of representative samples of the test population.
First day of exposure	Application of the reference item and first oral exposure (feeding). Assessment (O_2 / temperature / pH).
Once daily during exposure period	Assessment (mortality / sublethal effects / food intake).
Twice weekly during exposure period	Test medium renewal. Assessment (O_2 / temperature / pH) on the fresh and aged test medium.
Last day of exposure	Length / weight of all surviving test organisms.

* Acceptable tolerance: ± 1 day.

8. BIOLOGICAL DATA ANALYSIS

Results will be analysed with the statistical software Minitab® Release 14 (ANOVA test plus Dunnett's) to determine any significant differences.

Results will be corrected for control using an adaptation of Abbott's formula (1925) as follows:

$$M\% = \left(\frac{Mt - Mc}{100 - Mc} \right) \times 100$$

where Mt = % mortality in the test or toxic reference item
Mc = % mortality in the control

9. AMENDMENTS TO THE STUDY PLAN

The Study Director must approve all amendments to this study plan before implementation. The Study Monitor will be notified before the implementation of the amendment. The Sponsor will sign the amendment. Amendments will contain the following information:

1. A detailed description of the amendment.
2. The reasons for the amendment.
3. The signatures of the Study Director, Management, Sponsor and Lead Quality Assurance.
4. Impact of the amendment on the study.
5. The date upon which the amendment was signed.

10. STUDY PLAN DEVIATIONS

Any deviation from the study plan will be identified in writing and communicated to the Study Director and Study Monitor as soon as possible. The Study Monitor will receive the draft deviation statement before signature. The final deviation statement will then be signed by the Study Director. Any statement regarding a study plan deviation will include a description of the deviation, the reason for the deviation, the date of occurrence and its anticipated effect on the outcome of the study.

11. DATA REPORTING

The draft report will be sent to the Study Monitor for review. The report will be in the standard SynTech Research format and will include but not be limited to the following:

1. Study title and number
2. Name and address of the Test Facility and study initiation and termination dates
3. Name of Study Director, Study Monitor and all personnel involved in the study
4. Objectives and procedures stated in the study plan, including amendments and deviations to the study plan
5. Quality Assurance Statement listing procedures audited, data and reports reviewed, the respective inspection dates, and the dates the findings were reported to the Study Director and Study Director's management
6. Study Director's signature
7. Good Laboratory Practice Compliance Statement signed by the Study Director
8. Complete identification of test item identified by name, source, lot or batch number, characteristics (purity etc.) as provided by the Sponsor
9. Description of test site, including location, etc
10. Description of the experimental design and all procedures used during the conduct of the study, including test item preparation, administration to the test system, environmental parameter monitoring and data collection
11. Description of testing conditions, including temperature and test item rate
12. Description of any statistical procedures conducted (e.g. analysis of variance)
13. An exact description of any adverse effects of the test item on the test system

14. A description or presentation of all transformations, calculations, or operations performed on the data, along with a summary of the statistical analyses and a statement of the conclusions drawn from the analyses and calculations
15. A description of all circumstances that may have adversely affected the quality or integrity of the data.
16. Location where the final report and raw data are to be archived
17. A copy of the GLP compliance certificate for the Testing Facility during the study
18. A copy of the original Study Plan and any amendments
19. Information on the test organisms

12. RECORDS TO BE MAINTAINED

Records to be maintained and provided in the raw data by the Study Director include, but are not limited to the following:

1. The original study plan, any amendments and deviations
2. A list of all study participants and their signatures and initials
3. A list of equipment used in the study
4. A list of SOPs followed
5. SOP deviations, if any, and their impact on the study
6. Test item Material Safety Data Sheet and Certificate of Analysis
7. Test and reference item receipt and use records
8. Items preparation and application records
9. Test organism receipt details, where applicable
10. Environmental data collected during study
11. All original data collection sheets
12. Written correspondence between the Sponsor and Test Facility

13. RETENTION OF RECORDS

After completion of the final report, the Study Director will transfer the following data generated in the study to: Camilla BEECH
Oxitec Ltd
71, Milton Park, Abingdon,
Oxfordshire, OX14 4RX,
United Kingdom

Data to be transferred will include, but not be limited to:

1. The original study plan, amendments, and deviations
2. The original final report
3. Test item characterisation and certification documentation
4. The original raw data package

Copies of the study plan, raw data, amendments, deviations and final report, as well as all non-study specific data (e.g. log books describing equipment maintenance and calibration) will be stored in the archives of SynTech Research France SAS for ten years.

14. ARCHIVING

An aliquot of the test item will be retained by the test facility until at least the expiry date of the batch used in this study. An aliquot of toxic reference item will be archived at SynTech Research France SAS until expiry date of the product. Test items not used in the study will be returned to the Sponsor or discarded by SynTech Research following local regulations. No data will be discarded without the Sponsor's prior written consent.

15. STUDY PLAN DISTRIBUTION LIST

Study Monitor and Sponsor's Representative	C. BEECH
Study Director	E. YTHIER (original)
Test Facility Lead Quality Assurance	Y. TACIK
Test Facility Management	P. ESCHENBRENNER
Experimental phase Principal Investigator	L. MARTIN

Either a paper copy or an electronic copy (pdf-file) is acceptable.
The Study Director is responsible for forwarding a copy to his QA unit.

16. REFERENCES

- OECD guideline n°204. Fish, Prolonged Toxicity Test: 14-day Study (Adopted 4 April 1984). 9 pp.

- OECD guideline n°203. Fish, Acute Toxicity Test (Adopted 17 July 1992). 9 pp.

- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonization of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

- OECD Principles of Good Laboratory Practice (as revised in 1997). Series on Principles of GLP and Compliance Monitoring, No. 1. ENV/MC/CHEM(98)17 and No. 13. ENV/JM/MONO (2002)9. Organisation for Economic Co-operation and Development, Paris, France.

- Code de l'Environnement. Article Annexe II à l'Article D523-8 du Code de l'Environnement du 16 octobre 2007.

APPENDIX 1. ACKNOWLEDGEMENT OF STUDY PLAN

EXPERIMENTAL PHASE:

SynTech Research

Principal Investigator: Lucie MARTIN 03 OCT 2012 Date

Principal Investigator will be responsible for forwarding a copy to his QA unit.

APPENDIX 2. CERTIFICATE OF ANALYSIS

MERCK

Certificate of Analysis
CertiPUR® Reference Material

Merck Volumetric Standard
1.02403 Potassium dichromate
Batch No. 102403H

Secondary Reference Material for redox titrations
traceable to Standard Reference Material of NIST
(National Institute of Standards and Technology, USA).

Merck volumetric standards are used for the adjustment and control of volumetric solutions. They are manufactured under stringently controlled conditions in order to guarantee the highest quality standards. The general standard corresponds at least to the "GR" grade. It's suitability for use as a volumetric standard is based on a direct comparison with Standard Reference Material obtained from NIST.

The oxidimetric assay of this batch is

99.97%

- Measurement uncertainty: +/-0,05% (coverage factor *k=2 ; confidence level 95%).*
- The content indicated is based on a molecular mass M = 294.184 g/mol dried substance.
- Directly traceable of NIST SRM potassium dichromate batch 136e.
- Volumetric standard for standardisation of volumetric solutions in accordance to the chapter reagents of the Pharmacopoeia (USP).
- Standardisation was carried out using sodium thiosulfate solution as titration solution using a potentiometric procedure.
- Drying: When used as a volumetric standard, the potassium dichromate must be dried at 110°C for 2 hours.
- Storage: The volumetric standard should be stored at room temperature (+15 to +25°C) tightly closed (in the original container) and protected from light and moisture.
- The original unopened container may be used until: 31.07.2015

Date of release: 08.07.2010

Dr. S. Frey

Dr. Stefan Frey
(responsible laboratory manager quality control)

APPENDIX 3. GLP CERTIFICATE

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE
GROUPE INTERMINISTERIEL DES PRODUITS CHIMIQUES

CERTIFICAT DE CONFORMITE AUX BONNES PRATIQUES DE LABORATOIRE SELON LES DIRECTIVES 2004/9/CE ET 2004/10/CE
CERTIFICATE OF COMPLIANCE WITH GOOD LABORATORY PRATICES ACCORDING TO DIRECTIVES 2004/9/CE AND 2004/10/CE

Certifient n°: 2012/14

Société ou organisme : SYNTECH RESEARCH France - Le bois de Loyse
Company : 71570 LA CHAPELLE DE GUINCHAY

Installation d'essais : SYNTECH RESEARCH France - Le bois de Loyse
Test facilities : 71570 LA CHAPELLE DE GUINCHAY

Vu les articles D 523-8 et suivants du code de l'environnement relatifs au groupe interministériel des produits chimiques,
Having regard to the articles D.523-8 and onwards relating to the interministerial group of chemical products (GIPC),

Vu les résultats de l'inspection périodique réalisée par le Comité français d'accréditation (COFRAC) - Section Laboratoires – le : 29 et 30 juin 2011
Having regard to the results of the periodic inspection realised by the French Committee of accreditation (COFRAC) Laboratory Section – on the : *29 et 30 June 2011*

Vu l'avis du GIPC en date du : 29 novembre 2011
Having regard to the GIPC's opinion dated *29 November 2011*

La conformité aux principes des BPL de l'installation précitée est reconnue dans les domaines suivants :
Compliance with the principles of GLP is recognized for the facility above in the following areas:

4 - études écotoxicologiques sur les organismes aquatiques et terrestres
(environmental toxicity studies on aquatic and terrestrial organisms)
5 - études portant sur le comportement dans l'eau, dans le sol et dans l'air : bioaccumulation
(studies on behaviour in water, soil and air; bioaccumulation)
6 - études portant sur les résidus *(residue studies)*
7 - études portant sur les effets, sur les mécososmes et les écosystèmes naturels
(studies on effects on mesocosms and natural ecosystems)

Fait à Paris, le 16 janvier 2012

Le Président,

Jean-Pierre FALQUE-PIERROTIN

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

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

Appendix 2
Individual Data and Statistical Analysis

(4 pages)

Study number : **232SRFR12C1**
Trial number : **SRFR12-001-232XC1**

Assessment timing	Acute and sublethal effects	Mortality ; Abnormal behaviour/appearance		
		C101	T102	R103*
Day 1	Number of dead	0	0	0
	Number of unusual behaviour	0	0	0
	Number of moribund	0	0	0
Day 2	Number of dead	0	0	2
	Number of unusual behaviour	0	0	1
	Number of moribund	0	0	0
Day 3	Number of dead	0	0	4
	Number of unusual behaviour	0	0	0
	Number of moribund	0	0	0
Day 4	Number of dead	0	0	4
	Number of unusual behaviour	0	0	1
	Number of moribund	0	0	0
Day 5	Number of dead	0	0	5
	Number of unusual behaviour	0	0	1
	Number of moribund	0	0	1
Day 6	Number of dead	0	0	6
	Number of unusual behaviour	0	0	1
	Number of moribund	0	0	1
Day 7	Number of dead	0	0	7
	Number of unusual behaviour	0	0	1
	Number of moribund	0	0	0
Day 8	Number of dead	1	0	7
	Number of unusual behaviour	0	0	0
	Number of moribund	0	0	1
Day 9	Number of dead	1	0	8
	Number of unusual behaviour	0	0	0
	Number of moribund	0	0	1
Day 10	Number of dead	1	0	9
	Number of unusual behaviour	0	0	0
	Number of moribund	0	0	0
Day 11	Number of dead	1	0	9
	Number of unusual behaviour	0	0	0
	Number of moribund	0	0	1
Day 12	Number of dead	1	0	10
	Number of unusual behaviour	0	0	NA
	Number of moribund	0	0	NA
Day 13	Number of dead	1	0	10
	Number of unusual behaviour	0	0	NA
	Number of moribund	0	0	NA
Day 14	Number of dead	1	0	10
	Number of unusual behaviour	0	0	NA
	Number of moribund	0	0	NA

* Item groups significantly different from control after 14 days
L = Living; D = Dead; NA = Not Applicable

Items	
C101	Control (70% w/w non-GM mosquitoes)
T102	Test item (70% w/w OX513A mosquitoes)
R103	Potassium dichromate (100 mg a.s./L)

14-day Exposure	
LR_{50}/ER_{50}	> 70% w/w OX513A mosquitoes
NOER	70% w/w OX513A mosquitoes
LOER	> 70% w/w OX513A mosquitoes

	Size and weight of the fish at the beginning of the test									Mean	Loading (mg/L)*
Length (mm)	26	22	25	21	22	24	23	20	19	23	22,50	689,4
Weight (mg)	371,0	105,4	243,4	98,7	114,5	401,4	167,7	95,5	183,1	287,6	206,8	

* Based on 10 organisms randomly sampled the day before the start of the test and on test units of 3L test medium for 10 fish

		Size and weight of the surviving fishes at the end of the test										Mean	Loading (mg/L)*
C101	Length (mm)	25	23	21	22	24	22	20	21	24	/	22,44	595,0
	Weight (mg)	299,8	275,6	101,0	125,1	387,6	108,6	85,4	100,7	301,2	/	198,3	
T102	Length (mm)	24	23	20	21	27	25	22	23	25	22	23,20	709,6
	Weight (mg)	238,7	187,9	124,3	98,6	412,2	398,4	132,1	157,6	257,0	121,9	212,9	
R103	Length (mm)	/	/	/	/	/	/	/	/	/	/	/	/
	Weight (mg)	/	/	/	/	/	/	/	/	/	/	/	

* Based on surviving test organisms at the end of the test and on test units of 3L test medium

One-way ANOVA: 14-day Mortality versus Item

```
Source     DF      SS      MS      F      P
Item   2  6,0667  3,0333  91,00  0,000
Error      27  0,9000  0,0333
Total      29  6,9667
S = 0,1826   R-Sq = 87,08%   R-Sq(adj) = 86,12%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean   StDev  ---+---------+---------+---------+------
C101   10  0,1000  0,3162    (---*--)
R103*  10  1,0000  0,0000                              (---*--)
T102   10  0,0000  0,0000  (--*--)
                          ---+---------+---------+---------+------
                           0,00      0,35      0,70      1,05

Pooled StDev = 0,1826

Dunnett's comparisons with a control

Family error rate = 0,05
Individual error rate = 0,0273
Critical value = 2,33

Control = level (C101) of Item
Intervals for Item mean minus control mean

Level    Lower   Center   Upper  --------+---------+---------+---------+-
R103*   0,7095   0,9000  1,0905                              (-----*----)
T102   -0,2905  -0,1000  0,0905  (----*-----)
                                 --------+---------+---------+---------+-
                                       0,00      0,35      0,70      1,05
```

One-way ANOVA: 14-day Length versus Item

```
Source     DF       SS       MS     F      P
Item   1  0,00084  0,00084  0,70  0,414
Error      17  0,02026  0,00119
Total      18  0,02110
S = 0,03452   R-Sq = 3,97%   R-Sq(adj) = 0,00%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean   StDev    +---------+---------+---------+---------
C101    9  1,3691  0,0309    (---------------*--------------)
T102   10  1,3824  0,0375              (-------------*-------------)
                            +---------+---------+---------+---------
                          1,344     1,360     1,376     1,392

Pooled StDev = 0,0345

Dunnett's comparisons with a control

Family error rate = 0,05
Individual error rate = 0,0500
Critical value = 2,11

Control = level (C101) of Item
Intervals for Item mean minus control mean

Level     Lower   Center    Upper     +---------+---------+---------+---------
T102   -0,02018  0,01329  0,04676     (----------------*---------------)
                                      +---------+---------+---------+---------
                                   -0,020     0,000     0,020     0,040
```

One-way ANOVA: 14-day Weight versus Item

```
Source     DF      SS      MS     F      P
Item   1  0,0110  0,0110  0,19  0,665
Error      17  0,9590  0,0564
Total      18  0,9700
S = 0,2375   R-Sq = 1,13%   R-Sq(adj) = 0,00%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean   StDev  ----+---------+---------+---------+-----
C101    9  2,2314  0,2588  (----------------*----------------)
T102   10  2,2795  0,2168        (---------------*---------------)
                          ----+---------+---------+---------+-----
                            2,10      2,20      2,30      2,40

Pooled StDev = 0,2375

Dunnett's comparisons with a control

Family error rate = 0,05
Individual error rate = 0,0500
Critical value = 2,11

Control = level (C101) of Item
Intervals for Item mean minus control mean

Level    Lower  Center   Upper  -----+---------+---------+---------+----
T102   -0,1821  0,0481  0,2783  (------------------*------------------)
                                -----+---------+---------+---------+----
                                  -0,12      0,00      0,12      0,24
```

Appendix 3
Environmental Conditions Data

(1 page)

Study number : **232SRFR12C1**
Trial number : **SRFR12-001-232XC1**

Assessment timing	Parameters	Fresh Test Medium			Aged Test Medium		
		C 101	T 102	R 103	C 101	T 102	R 103
Day 1	pH	7,57	7,58	6,61	7,31	7,34	6,29
	T°C	21,8	21,8	21,8	21,6	21,4	21,6
	O2 (mg/L)	9,9	9,9	9,8	7,4	7,6	7,9
Day 4	pH	7,61	7,64	/	7,28	7,25	6,27
	T°C	21,6	21,6	/	21,8	21,6	21,6
	O2 (mg/L)	9,9	9,9	/	7,3	7,5	7,9
Day 8	pH	7,55	7,51	/	7,22	7,18	6,21
	T°C	21,8	21,8	/	21,6	21,8	21,6
	O2 (mg/L)	9,8	9,7	/	7,2	7,4	7,5
Day 11	pH	7,57	7,56	/	7,34	7,23	6,18
	T°C	21,8	21,6	/	21,6	21,6	21,6
	O2 (mg/L)	9,9	9,8	/	7,4	7,5	7,3
Day 1 to Day 14		Temperature (°C)			20,5 - 22,4		
		Light intensity (lux)			1120 - 1340		

Appendix 4
Software Verification

(1 page)

MINITAB:

MINITAB ONE-WAY ANOVA VERIFICATION

01/06/2012 17:24:03

```
Welcome to Minitab, press F1 for help.
```

One-way ANOVA: C2 versus C1

```
Source  DF     SS     MS     F      P
C1       3  341,9  114,0  9,01  0,000
Error   36  455,6   12,7
Total   39  797,5

S = 3,557   R-Sq = 42,87%   R-Sq(adj) = 38,11%

                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level       N    Mean  StDev    -+---------+---------+---------+---------
Sample 1   10  83,600  4,033                              (------*------)
Sample 2   10  79,400  2,503                    (------*------)
Sample 3   10  78,600  3,307                  (------*------)
Sample 4   10  75,400  4,142    (------*------)
                                -+---------+---------+---------+---------
                               73,5      77,0      80,5      84,0

Pooled StDev = 3,557
```

Published example of statistical analysis used:
"Procedure for computing one-way ANOVA". In "Fowler, J., Cohen, L., & Jarvis, P. (1998). Practical statistics for field biology. Second edition. John Wiley & Sons Ltd, Chichester, England". From pp181 to pp184.

L. DESLANDES

01 JUN 2012

On computers n° : 01279
01951
01813

The cited example was used to validate MINITAB Software one-way ANOVA.

Appendix 5
Certificate of Analysis

(1 page)

MERCK

Certificate of Analysis
CertiPUR® Reference Material

Merck Volumetric Standard
1.02403 Potassium dichromate
Batch No. 102403H

Secondary Reference Material for redox titrations
traceable to Standard Reference Material of NIST
(National Institute of Standards and Technology, USA).

Merck volumetric standards are used for the adjustment and control of volumetric solutions. They are manufactured under stringently controlled conditions in order to guarantee the highest quality standards. The general standard corresponds at least to the "GR" grade. It's suitability for use as a volumetric standard is based on a direct comparison with Standard Reference Material obtained from NIST.

The oxidimetric assay of this batch is

99.97%

- Measurement uncertainty:+/-0,05% (coverage factor *k=2 ; confidence level 95%).*
- The content indicated is based on a molecular mass M = 294.184 g/mol dried substance.
- Directly traceable of NIST SRM potassium dichromate batch 136e.
- Volumetric standard for standardisation of volumetric solutions in accordance to the chapter reagents of the Pharmacopoeia (USP).
- Standardisation was carried out using sodium thiosulfate solution as titration solution using a potentiometric procedure.
- Drying: When used as a volumetric standard, the potassium dichromate must be dried at 110°C for 2 hours.
- Storage: The volumetric standard should be stored at room temperature (+15 to +25°C) tightly closed (in the original container) and protected from light and moisture.
- The original unopened container may be used until: 31.07.2015

Date of release: 08.07.2010

Dr. S. Frey

Dr. Stefan Frey
(responsible laboratory manager quality control)

99395

Appendix 6
GLP Certificate

(1 page)

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE

GROUPE INTERMINISTERIEL DES PRODUITS CHIMIQUES

CERTIFICAT DE CONFORMITE AUX BONNES PRATIQUES DE LABORATOIRE SELON LES DIRECTIVES 2004/9/CE ET 2004/10/CE

CERTIFICATE OF COMPLIANCE WITH GOOD LABORATORY PRATICES ACCORDING TO DIRECTIVES 2004/9/CE AND 2004/10/CE

Certificat n°: 2012/14

Société ou organisme : SYNTECH RESEARCH France - Le bois de Loyse
Company : 71570 LA CHAPELLE DE GUINCHAY

Installation d'essais : SYNTECH RESEARCH France - Le bois de Loyse
Test facilities : 71570 LA CHAPELLE DE GUINCHAY

Vu les articles D.523-8 et suivants du code de l'environnement relatifs au groupe interministériel des produits chimiques,
Having regard to the articles D.523-8 and onwards relating to the interministerial group of chemical products (GIPC),

Vu les résultats de l'inspection périodique réalisée par le Comité français d'accréditation (COFRAC) - Section Laboratoires – le : 29 et 30 juin 2011
Having regard to the results of the periodic inspection realised by the French Committee of accreditation (COFRAC) – Laboratory Section – on the : 29 et 30 June 2011

Vu l'avis du GIPC en date du : 29 novembre 2011
Having regard to the GIPC's opinion dated : 29 November 2011

La conformité aux principes des BPL de l'installation précitée est reconnue dans les domaines suivants :
Compliance with the principles of GLP is recognized for the facility above in the following areas:

4 - études écotoxicologiques sur les organismes aquatiques et terrestres
(environmental toxicity studies on aquatic and terrestrial organisms)
5 - études portant sur le comportement dans l'eau, dans le sol et dans l'air ; bioaccumulation
(studies on behaviour in water, soil and air; bioaccumulation)
6 - études portant sur les résidus (*residue studies)*
7 - études portant sur les effets, sur les mécososmes et les écosystèmes naturels
(studies on effects on mesocosms and natural ecosystems)

Fait à Paris, le 16 janvier 2012

Le Président,

Jean-Pierre FALQUE-PIERROTIN

Secrétariat général du GIPC - DGCIS, Service de l'industrie, bureau de la chimie - 12, rue Villiot - 75572 Paris cedex 12
Téléphone : 01 53 44 96 10 – Télécopie : 01 53 44 91 72

MINISTÈRE DE L'ÉCONOMIE
DES FINANCES ET DE L'INDUSTRIE

Appendix I

TRANSGENIC PROTEIN tTAV: Assessment of allergenic risk

Background

tTAV is a recombinant tetracycline repressible activator protein.

Genetically modified, transgene homozygous, mosquitos (*Aedes aegypti)* have been developed to control and limit mosquito population growth and vector transmission.

The transgene codes for a protein (tTAV) that inhibits cellular function. The dominant lethal transgene is carried in genetically modified male mosquitos that are released to breed with wild-type females. The trait prevents the resulting progeny that carry the gene from reaching maturity in the absence of tetracycline.

The tTAV gene is expressed in a number of transgenic insect tissues and it is probable, therefore, that the gene will be transcribed in the salivary glands of transgenic mosquitos.

The concern that has been raised is that if tTAV protein has inherent allergenic properties, and if this protein is indeed in the saliva, then the protein could potentially induce allergic sensitisation in those bitten by female mosquitos (male mosquitos do not bite). There is a case to answer because allergic reactions due to sensitisation to normal mosquito salivary proteins have been described (Kulthanan et al., 2010). Alternatively/additionally, a related concern is that tTAV might have a level of homology with a known protein allergen sufficient to elicit an allergic reaction in those already sensitised to the cross-reactive protein allergen.

The two issues addressed here are: (a) whether there is an inherent allergenic hazard, and (b) whether there are possible human health risks with respect to allergic sensitisation.

Inherent allergenic hazard

The primary approach adopted to evaluate the inherent allergenic potential of tTAV has been to use a suite of bioinformatic tools to examine whether tTAV displays sequence homology with, or structural similarity to, known protein allergens. This approach was developed originally for the purposes of determining whether transgenes introduced into crop plants had the potential to cause allergic sensitisation and food allergy in future consumers. However, it must be appreciated that the factors that confer on proteins allergenic activity are independent of the route through which encounter with/exposure to protein occurs. That is, the properties that confer on proteins an ability to cause food allergy are the same as those that will enable a protein to cause allergic sensitisation of the respiratory tract. Thus, for instance, ovalbumin from hens' eggs can cause food allergy and also respiratory allergy among those working in egg processing plants (James and Crespo, 2007). Moreover, there is now growing evidence that allergic sensitisation to peanut proteins can occur via skin contact in addition to dietary exposure (Kimber et al., 2014). It is therefore legitimate to use this well-established and well-validated bioinformatics approach to evaluate whether proteins have intrinsic allergenic hazard irrespective of the route(s) through which exposure may occur.

In the first series of bioinformatics analyses it was reported, using standard assessment criteria, that tTAV lacked sequence homology with known allergens (or toxins) (Goodman, 2011). This was subsequently confirmed in a second updated analysis in which it was again established that tTAV lacked significant homology with any known allergens. In the same series of investigations it was also reported that a second transgene product, DsRed2, a red fluorescent marker protein derived from coral and sea anemone species, also lacked homology with any known allergens (Goodman, 2013).

The conclusion drawn from that second series of bioinformatics analyses was that tTAV (and DsRed2) lacks allergenic potential and does not display cross-reactivity with any known protein allergens (Goodman, 2013).

On the basis of these data it can be stated that tTAV protein does not have the inherent potential to induce allergic sensitisation. The tTAV protein also lacks cross-reactivity with known human allergens and will therefore fail to elicit allergic reactions in subjects sensitised to other proteins.

The conclusion is that neither tTAV, nor DsRed2, represent an allergenic hazard.

Human allergy health risks

It can be argued that if tTAV (and DsRed2) lack inherent allergenic properties (either the ability to cause the acquisition of sensitisation, or the ability to elicit allergic reactions in subjects sensitised to cross-reactive proteins), then there are no health risks irrespective of the route of exposure.

However, for the purposes of completeness it is important to emphasise that even if there did exist an allergenic hazard then the likelihood that that would translate into a human health risk is very low.

In this instance exposure would be associated solely with bites by female mosquitos resulting in the intradermal delivery of salivary proteins. Although there is a precedent for the acquisition of sensitisation to proteins constitutively borne in mosquito saliva, the amount of transgene product that would be encountered via this route would be exceedingly small, if present at all, and unlikely to elicit an immune response.

Conclusions

- The available evidence indicates that tTAV (and DsRed2) lacks the inherent potential to induce allergic sensitisation.
- In addition, neither tTAV, nor DsRed2, display a level of homology with known human allergens that would be required for the elicitation of cross-reactive allergic reactions.
- Levels of exposure to tTAV (and DeRed2) via mosquito bite will be extremely low, if present at all, and unlikely to initiate an immune response.
- The transgene proteins to not pose human health risks with regard to allergy or allergic sensitisation.

References

Goodman RE (2011) Bioinformatics evaluation of transgenic protein tTAV from mosquito. Report to Oxitec Ltd (unpublished).

Goodman RE (2013) Bioinformatics analysis for risks of allergenicity and toxicity of proteins encoded by the two genes introduced into genetically engineered mosquitos (*Aedes aegypti*), strain OX513A for production of sterile males to reduce vector transmission of important human diseases. Report to Oxitec Ltd (unpublished).

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

Kimber I, Griffiths CEM, Basketter DA, McFadden JP, Dearman RJ (2014) Epicutaneous exposure to proteins and skin immune function. Eur. J. Dermatol. 24, 10-14.

Kulthanan K, Wongkamchai S, Triwongwaranat D (2010) Mosquito allergy: clinical features and natural course. J. Dermatol. 37, 1045-1031.

Ian Kimber, January 2015

Appendix J

Study Title

Bioinformatics analysis for risks of allergenicity and toxicity of proteins encoded by the two genes introduced into genetically engineered mosquitos (*Aedes aegypti*), strain OX513A for production of sterile males to reduce vector transmission of important human diseases

Authors

Richard E. Goodman

Study Completed On

5 September, 2013

Performing Laboratory

Food Allergy Research and Resource Program
Food Science and Technology
University of Nebraska
143 Food Science & Technology
Lincoln, NE 68583-0955

Laboratory Project ID

Study Number: REG Oxitec OX513A

Summary:
Genetically modified (GM) *Aedes aegypti* mosquitoes were developed by Oxitec Limited by the insertion of a single contiguous DNA segment comprising two genes to produce male mosquitoes that carry a lethal dominant trait under control of a promoter that allows successful reproduction only in cultured conditions in the presence of tetracycline. One gene encodes the fluorescent red protein (DsRed2) from an Anthozoan species (corals and sea anemones) that has been used as a visible selection marker in a number of plant transformation events (Jach et al., 2001; Dietrich and Maiss, 2002; Wenck et al., 2003; Mirabella et al., 2004; Stuitje et al., 2003). The second gene regulates the reproductive development of the mosquitoes by production of a protein with a tetracycline-repressible transcriptional activator fused to and controlling a segment of a *Herpes simplex* virus VP16 protein. The second gene is a dominant lethal trait as the large majority of males and females carrying this trait die before functional adulthood. The engineered male mosquitoes are released into the environment to breed with normal females, but production of the next generation of progeny fails to develop to adulthood (Gossen and Bujard, 1992; Gong et al., 2005; Phuc et al, 2007; Kongmee et al., 2010, Fu et al., 2010).

Regulatory agencies in countries where the genetically modified mosquitoes might be released will have to evaluate potential human safety issues that might be presented by the GM mosquitoes. Although the safety assessment process for genetically modified organisms (GMOs) is normally applied in consideration of the safety of the organism for food use, as that is the majority of GMO's currently seen by regulators, in this case regulators may consider risks to humans that may be exposed to mosquito proteins through bites by female GM mosquitoes (males do not bite). Potential exposure routes include: worker exposure to female mosquitoes during mosquito rearing; incomplete sex separation leading to release of some homozygous female OX513A mosquitoes together with the males; incomplete penetrance of the lethal trait leading to production of some functional heterozygous female adult OX513A mosquitoes among the offspring of the released homozygous OX513A males and wild females. The exposure would be expected to occur through bites and saliva, not through dietary exposure (in humans). The primary risk of severe reactions would be assumed to be from the transfer of a protein that causes systemic allergic reactions in allergic individuals rather than sensitization de novo. Thus a bioinformatics evaluation to ensure the newly expressed proteins are not allergens and are not highly identical to allergens as described here, was used as an important evaluation step to minimize potential risks for humans. Finally, an evaluation was performed to consider if the protein has any properties that would be considered toxic in the context of human exposure to a mosquito bite. The bioinformatics searches performed and reported here did not uncover any concerns of potential allergenicity, allergenic cross-reactivity or potential toxicity that would demonstrate a need for further testing regarding safety. The conclusion of the bioinformatics evaluation and the evidence of expression patterns demonstrated that the DsRed2 and tTAV proteins do not present a risk of allergy or toxicity to humans.

1.0 Introduction

Oxitec Limited, UK has developed genetically modified *Aedes aegypti* mosquitoes by inserting DNA encoding two proteins in transgenic strain OX513A, using the LA513 transposon - also known as OX513 (Phuc et al., 2007). One protein DsRed2 (Matz et al., 1999; Jach et al., 2001; Dietrich and Maiss, 2002; Wenck et al., 2003; Stuitje et al., 2003; Mirabella et al., 2004; Phuc et al., 2007) was used as a marker protein for efficient selection of recombinant mosquitoes. The second protein is the recombinant tetracycline repressible transcriptional activator protein (tTAV) described by Phuc et al. (2007). The purpose is to produce homozygous male mosquitoes carrying a dominant lethal gene that prevents the successful development of progeny from wild-type females mating with the GM males into adults Phuc et al , 2007).. Since this species is a vector for Dengue Fever and Yellow Fever as well as other arboviral diseases, there is an urgent need to reduce the reproductive success of this mosquito as one mechanism to reduce human disease. Laboratory as well as field trials are currently in progress to evaluate the effectiveness of this system (Bargielowski et al., 2011; Lacroix et al., 2012, Harris et al, 2011, Harris et al, 2012).

The human safety component of GM organisms (GMOs) normally focuses on the safety of food produced from the GMO (Codex, 2003) and mosquitoes are not consumed by humans. However, the female mosquitoes must feed on the blood of mammal hosts (usually humans in the case of *Aedes aegypti*) in order to provide nutritional requirements for egg production. Proteins in the saliva of mosquitoes are known to cause allergic reactions in humans. In addition, there is a potential for a toxic protein to be introduced through a mosquito bite.

While it would be important to consider whether one or both of the newly introduced proteins (DsRed2 or tTAV) in this GM mosquito might be present in saliva of GM mosquitoes, the primary evaluation should be whether the proteins are known to be allergens or toxins, or whether they are nearly identical to any known allergen or toxic protein. That is why Oxitec Limited requested a bioinformatics analysis of the two expressed proteins. In 2011 a bioinformatics study was performed and reported on tTAV regarding potential similarities to allergens and toxins (Goodman, 2011). The current report provides results from a new updated search that includes the DsRed2 marker protein and tTAV compared to all compiled allergens or toxins in public databases. This report describes the sequences, the datasets, the methods and the results of the bioinformatics evaluation of the DsRed2 and tTAV proteins, using the amino acid sequence information provided by Oxitec as the query sequences.

2.0 Purpose

The purpose of this study is to perform an evaluation of the potential allergenicity and toxicity of the DsRed2 and tTAV proteins that are encoded by the genes introduced in OX513A mosquitoes (*Aedes aegypti*) based on published literature about the source of the genes and bioinformatics (sequence comparisons) of proteins with known allergens and toxins. The intent is to guide decisions regarding whether additional safety tests would be needed for evaluating these proteins as potential sources of allergy or toxicity if there is any human exposure through the bite of the female mosquitoes.

3.0 Methods

3.1 Scientific literature search strategies. The PubMed database (http://www.ncbi.nlm.nih.gov/pubmed) maintained by the U.S. National Library of Medicine was used as the primary data source for scientific literature on allergy and toxicity. The primary question is whether the source of the gene is a common cause of allergy or toxicity. The data (authors, publication, date and abstracts) from searches were saved to files for review. All publication abstracts were manually reviewed and any likely relevant publications suggesting adverse health risks were investigated further by reading the journal articles.

3.1.1 Search for allergenicity. Search terms "gene source" AND "allergen" as well as "gene source" AND "allergy" were used on 10 August, 2013.

3.1.2 Search for toxicity. Search terms "gene source" AND "toxin" as well as "gene source" AND "toxicity" were used on 10 August, 2013.

3.2 Amino acid sequences of query proteins. The mosquito transfection clone construct used to develop OX513A was described in Phuc et al., 2007.

3.2.1 DsRed2. The DsRed2 amino acid sequence from OX513A was supplied by Oxitec Limited (Table 1) and is identical to the protein expressed by the transient expression vector pX-DR, GI:237652127 (Chen et al., 2009) except for an additional three amino acids at the N-terminus that was added in constructing the mosquito insertion transposon (personal communication, C. Beech, 17 May, 2013).

3.2.2 tTAV. The tTAV amino acid sequence from OX513A was supplied from Oxitec Limited (Table 1) and is 100% identical to GI: 60542785, Accession AJ865387, from Gong et al., 2005 and Phuc et al., 2007.

Table 1 Amino acid sequences of the novel OX513A transgenic event mosquito proteins.

Protein (in OX513A) Common name of nearest source organism *Latin name*	**Protein name** Nearest published sequence GI: Protein length (aa) Percent ID to Oxitec [native publication]	**Protein sequence for OX513A proteins (supplied by Oxitec Ltd.)**
DsRed2 Coral *Discosoma sp.*	**DsRed** GI:55976617 225 amino acids 97% (219/225 aa) [Matz et al., 1999]	1 MARMASSENV ITEFMRFKVR MEGTVNGHEF EIEGEGEGRP YEGHNTVKLK 51 VTKGGPLPFA WDILSPQFQY GSKVYVKHPA DIPDYKKLSF PEGFKWERVM 101 NFEDGGVATV TQDSSLQDGC FIYKVKFIGV NFPSDGPVMQ KKTMGWEAST 151 ERLYPRDGVL KGETHKALKL KDGGHYLVEF KSIYMAKKPV QLPGYYYVDA 201 KLDITSHNED YTIVEQYERT EGRHHLFL
tTAV GI: 60542785 Synthetic construct from two proteins Bacterial tetracycline repressor *Escherichia coli* *Herpes simplex* virus 1 (human)	**Tetracycline repressor protein** (3-208 aa) GI:486188873 **Transactivating tegument protein** (211-338 aa)	1 MGSRLDKSKV INSALELLNE VGIEGLTTRK LAQKLGVEQP TLYWHVKNKR 51 ALLDALAIEM LDRHHTHFCP LEGESWQDFL RNNAKSFRCA LLSHRDGAKV 101 HLGTRPTEKQ YETLENQLAF LCQQGFSLEN ALYALSAVGH FTLGCVLEDQ 151 EHQVAKEERE TPTTDSMPPL LRQAIELFDH QGAEPAFLFG LELIICGLEK 201 QLKCESGSGP AYSRARTKNN YGSTIEGLLD LPDDDAPEEA GLAAPRLSFL 251 PAGHTRRLST APPTDVSLGD ELHLDGEDVA MAHADALDDF DLDMLGDGDS 301 PGPGFTPHDS APYGALDMAD FEFEQMFTDA LGIDEYGG

3.3 Sequence database search strategies.

The AllergenOnline version 13 (http://www.allergenonline.org/) and the NCBI Entrez Protein (http://www.ncbi.nlm.nih.gov/BLAST/) databases were used as the protein amino acid data sources for the sequence comparisons for allergens and toxins (31,601,460 sequences on 14 August, 2013). The AllergenOnline database was updated in 12 February 2013 and is maintained by the Food Allergy Research and Resource Program of the University of Nebraska. Protein entries in the Entrez search and retrieval system is compiled and maintained by the NCBI of the

National Institutes of Health (U.S.A.). The database is potentially updated or modified daily, and therefore the date of sequence searches by BLASTP is relevant to the dataset used in the BLASTP searches. BLASTP and FASTA3 are unique computer algorithms that provide similar local alignments and results if the appropriate scoring matrices and criteria are used.

3.3.1 FASTA3 overall search of AllergenOnline. The potential sequential and inferred structural similarities of the DsRed2 and tTAV proteins were evaluated using version 13 of AllergenOnline.org.

3.3.2 FASTA3 of AllergenOnline by 80 aa segments. This short segment search is based on the recommendation of Codex (2003). The rationale is that this might help in identifying structural motifs, much shorter than the intact protein, which might contain a conformational IgE binding epitope. It should also help to identify potentially cross-reactive proteins that are not true homologues of an allergen that have significant local identities that might provide an immunological target for IgE antibodies in those with allergies to the matched allergen. A match of >35% with a known allergen will suggest further testing for possible cross-reactivity.

3.3.3 BLASTP of NCBI Entrez with "allergen" as keyword limit. The BLASTP is available on the NCBI Entrez website (http://www.ncbi.nlm.nih.gov/BLAST/). The current version is BLASTP 2.2.28+ (28 July, 2013). A BLASTP search was used comparing the DsRed2 and tTAV query sequences against the entire Entrez Protein database, with a limit option selected to query entries for "allergen", to align only with proteins identified as allergens. The purpose of this BLASTP search is to ensure that a significant match with a newly discovered allergenic sequence that has not yet been entered into AllergenOnline is not overlooked. Evaluation of the *E* value, the length of the alignment and the percent identity of any identified match is necessary to judge the significance of any alignment using BLASTP.

3.3.4 BLASTP of NCBI Entrez without keyword limit. The purpose of this BLASTP search is to compare the DsRed2 and tTAV proteins to all known protein sequences to evaluate whether there are other similar proteins from other organisms that might provide information of safe exposure to homologues of this protein.

3.3.5 BLASTP of NCBI Entrez with "toxin" as keyword limit. The purpose of this BLASTP search is to identify matches to known toxic proteins (toxins) and if alignments share significant identities, to determine potential risks that would require further testing. The sequences of the DsRed2 and tTAV proteins were compared to the NCBI Entrez database using "toxin" as a keyword search limit.

4.0 Results and Discussion. The summary results for the PubMed search using the various protein sources and search terms, and the amino acid sequences of the DsRed2 and tTAV proteins, are presented here.

4.1 PubMed Searches. The PubMed scientific literature database was searched for evidence that the DsRed2 and tTAV proteins are linked to allergy or toxicity. The search demonstrated that there is no published evidence that the two proteins are allergens or toxins for mammals and no evidence that implies there might be an association with allergenicity or toxicity. An important consideration of the safety assessment related to potential toxicity, is an understanding of the mechanism of action of the newly expressed protein. If the protein is an enzyme, potential biological impacts of any new metabolites should be considered. If the inserted DNA or new protein is a transcription or translational regulator, potential targets or measured effects in source organisms or other host organisms should be considered. Identical or nearly identical genes introduced in OX513A mosquitoes have been have been inserted in transgenic animals, plants or arthropods by many scientists and the proteins have been expressed within cells of various tissues of the hosts, without adverse impacts (e.g., for DsRed2 see Ryu et al., 2013; for tetracycline repressible transactivator protein see Gong et al., 2005). Since these proteins have been produced directly within the cells of diverse eukaryotic species without obvious toxic effects, it is highly unlikely that incidental exogenous exposure by any route (ingestion, inhalation or injection) would have adverse biological impacts on human or mammalian health.

4.1.1 Allergenicity.

The terms "*Discosoma*" AND "allergen" as well as "*Discosoma*" AND "allergy" were used to search PubMed for evidence of allergy from the source organism of the DsRed2 protein, *Discosoma sp.* No references were listed when "allergen" was used. Two references were listed when "allergy" was used (Teterina et al., 2010; Tawfik et al., 2008), but in both cases the studies used the DsRed protein as a fluorescent label to study disease processes and there was no causal relationship with allergy.

Literature searches to evaluate the potential allergenicity (and toxicity) of the source for the TAV protein are somewhat complex. The source of the gene/protein for the tetracycline repressor protein (amino acids 3-208 of TAV) part of the protein is the Tn10 plasmid in *E. coli* (Gossen and Bujard, 1992; Altschmeid et al., 1988), which is produced as a fusion protein with the *Herpes simplex* virus protein 16 C by the design of the gene construct (Gong et al., 2005, Phuc et al., 2007).

The *E. coli* bacterium has used as a cloning and expression host for many allergens and toxins. In addition, some strains of *E. coli* are known to produce toxins. Therefore it was expected that simple searches of "*E. coli*"

AND "allergen" or "toxin" would find many irrelevant publications. A search of the terms "*Escherichia coli*" AND "allergen" in PubMed returned 665 publication references. Since it is not possible to efficiently read 655 publications for evidence of allergenicity, an additional term, "tetracycline" was used to refine the source since tTAV is a tetracycline repressible transactivator. Only one publication was found then, Nishihara et al., 1998, describing the design of a transgenic bacterium that included regulatory expression of a cedar pollen allergen, Cry j 2 in *E. coli*. The results suggest that there are no natural allergens in *E. coli*. In order to ensure that the addition of the third term was not too restrictive, an additional strategy was used. Since, the sequence of the tTAV is identical to, or nearly identical to tetracycline repressible transactivator proteins that have been cloned from a few species of bacteria including *Salmonella sp.*, *Shigella sp.*, *Acinetobacter sp.*, and of the taxonomic family Enterobacteriaceae, a search was performed using "*Acinetobacter*" AND "allergen" as alternative search terms. Five publications were identified. Jadhav et al. (2013) found *Acinetobacter sp.* associated with isolates of nosocomial infections in a number of patients in hospitals, along with many other microbes, without any connection to allergy. Qiu et al. (2011) published data from a study demonstrating that an intentional lung infection with *Acinetobacter baumannii* reversed the Th2 response of eosinophilia and "allergic response" to ovalbumin in a mouse model. Skorska et al. (2007) tested skin prick tests and IgE responses against a number of gram negative and gram positive microbes commonly found in organic dust, using subjects who work in a poultry hatchery in Poland. The brief report did not detail proteins or specific data, only a trend that more workers showed positive precipitin reactions extracts (typically an IgG antibody complex reaction, indicating Th1 response) to *Escherichia coli* and *Acinetobacter baumannii* along with other fungi and bacteria than control subjects. Valerio et al. (2005) demonstrated by PCR of 16S ribosomal DNA, that a few bacterial species were present in cultures and culture medium of the allergenic house dust mite (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), including *Acinetobacter sp.*, however the prevalence was low and no proteins were identified or relationship to allergenicity in looking for the source of endotoxin in the allergenic mite cultures. Dutkiewicz et al. (2002) measured IgE levels to extracts of a number of bacterial species using sera from workers in a potato processing facility to evaluate potential causes of work-related asthma. Although *Acinetobacter calcoaceticus* was mentioned, there was no reference to it as an important target for IgE, whereas some other bacteria were implicated. Interestingly there is little evidence in the literature for microbial proteins causing allergic reactions, so the importance of the few reports on bacterial precipitin antibody binding reactions to bacterial proteins is not obvious (RE Goodman, personal observations). A search with "Acinetobacter" AND "allergy" listed 48 references. An attempt to focus on the protein was performed by inclusion of a third term "tetracycline repressor protein", and did not identify any publications. An evaluation of the entire list of 48 publications demonstrated that most reported identification of *Acinetobacter spp* .and other microbes in a study group, often from an institute of allergy, or in some cases (e.g. Renz et al., 2011), an animal

model was used to demonstrate that an infection with *Acinetobacter spp.* may suppress allergic responses. Other research points to the conclusion that exposure to *Acinetobacter spp.* protects individuals against allergy, possibly due to the lipopolysaccharide content that skews reactions toward a Th1 response (Debarry et al., 2010).

The terms "Herpes" AND "VP16" AND "Allergy" were used to evaluate the source of the transactivating portion of tTAV, with nine articles found. However all nine were only listed as they are from investigators at the National Institute of Allergy and Infectious Diseases. They did not demonstrate a relationship between the Herpes simplex VP16 and allergy. A search with "Herpes" AND "VP16" AND "allergen" yielded no publications.

Thus, a search of the literature for publications linking the source organisms, *Discosoma sp.*, *Escherichi coli, the* surrogate source (*Acinetobacter sp.*), and *Herpes simplex* did not uncover evidence that would implicate the sources or the proteins used in OX513A as likely allergens.

4.1.2 Toxicity. A search of PubMed using the taxonomic organism names for the taxonomic sources of the DsRed2 and tTAV along with the terms "toxin" or "toxicity" AND the protein ("DsRed2" or "tTAV") were reviewed.

For "Discosoma" AND "toxin", no references were found. For "Discosoma" AND "toxicity" two publications were found. One by Long et al. (2005) presented information on a new, mutated form of the red fluorescent protein from *Discosoma sp.*, and suggested that the DsRed2 protein might be toxic if expressed in transgenic mice, referring to an earlier paper by Hadjantonakis and Papaioannou (2004). However, after a careful read of the 2004 paper by Hadjantonakis and Papaioannou, I believe that their study did not demonstrate toxicity to DsRed2, rather it showed a failure to maintain highly expressing red-fluorescent stem cell lines, which might be due to position effects or a number of other technical issues. The second paper found in this search was by Murata et al. (2011), in which they indicate using DsRed2 as a marker in transgenic mice did not demonstrate any toxicity problems in the transgenic mice. A further search with "DsRed2" found 217 publications and rapid review did not indicate toxicity. Instead the publications demonstrate that the DsRed2 protein can be expressed in various cells of transgenic mice and transfected mouse cells that have been used for various physiological or toxicological studies without apparent toxicity. For example, a study by Ryu et al. (2013) reported successful transformation and use of transgenic mice with the DsRed2 reporter in a chimeric situation with maintenance of polyclonal tissues having cells that are from EGFP transformed or DsRed2 transformed mice, with no reported toxicity. These were generated by mating two lines of transgenic mice, one with DsRed2 and one with EGFP. The mice were surviving and healthy. In addition, Nordin et al. (2013) demonstrated that the OX513A mosquito larvae, which do express both the DsRed2 and tTAV

proteins can be consumed by two species of fly larvae (*Toxorhynchites spp.*) without apparent toxicity. From this literature search, there does not appear to be published evidence that the DsRed2 protein is toxic to microbes, fungi, insects, plants or mammals.

The tTAV protein is a fusion of two proteins. The tetracycline repressible transactivator protein is from *E. coli.* The second of the fusion protein is from the *Herpes* simplex virus. The first search with "Escherichia coli" AND "toxin" returned over 26,890 publications. Adding the term "tetracycline" reduced that to 229 publications, still a large number. Scanning titles of the 229 reveals that most are related to specific virulence factors or outbreaks of disease in livestock. Addition of the term "transactivator" removed all but one of the 229 publications. Baur et al., (1997) described tetracycline regulated expression of the diphtheria toxin A gene in human glioma cells using a rTA element. The toxicity was related to the toxicity of the toxin A gene. These results demonstrate that while the specific bacterium is associated with toxic responses, there is no obvious indication that the tetracycline activator protein is associated with toxicity.

As in the search for allergens, an additional search was performed using the *Acinetobacter sp.* as a search term since the proteins are 99% identical and the species are from the same family of bacteria. The intent was again to verify whether other researchers who study this species might have uncovered toxicity associated with the protein. A search with "Acinetobacter" AND "toxin" found 210 publications. There is ample evidence that the organism, *Acinetobacter sp.* is an opportunistic pathogen and that it does produce toxins such as lipopolysaccharide. However, limiting the search by including AND "tetracycline" reduced the publication list to two papers related to antibiotic resistance (Wieczorek et al., 2008 and Loeffelholz et al., 1987). Addition of "transactivator" reduced the list to zero. Finally, "Acinetobacter" AND "tetracycline repressor protein" identified only one publication, Thompson et al. (2007), which did not identify toxicity associated with the protein. This search did not identify evidence of any toxicity associated with the tetracycline repressor protein of *Acinetobacter sp.*

The VP16 protein of *Herpes simplex* virus is a transcriptional regulator that functions by binding to specific DNA sequences (TAATGARAT consensus sequence) present in virus genes that are up-regulated in the early cycle of viral infection (Simmen et al. 1997). It functions within the cell and is not expected to be taken up by eukaryotic cells or be active in cells that do not express the protein. In order to consider possible toxicity the terms "Herpes" AND "toxicity" as well as "Herpes" AND "toxin" were search in PubMed, returning 1381 references and 326 references respectively. Adding "VP16" as a search term reduced the number to 7 and 6 references respectively. The publications were searched for evidence of toxicity related to VP16 and no direct evidence was found. The

studies generally relate to attempts to use *Herpes simplex* virus as a transfection vector for efficient gene transfer to mice for mechanistic studies or possibly to humans to treat disease. The vectors have caused toxicity, thus a small number of publications are identified in this search. The conclusion of reading those publications that seemed most relevant was negative, that is, no direct toxicity associated with the VP16 protein.

The combined search information failed to identify any evidence that the proteins fused to provide the complete tTAV protein or the DsRed2 proteins have any known toxicity.

4.2 Sequence comparison of the DsRed2 and tTAV proteins in OX513A to allergens. The amino acid sequences of the DsRed2 protein and the tTAV protein (Table 1) were compared to known allergens using both a full-length FASTA alignment search and a sliding window of 80 comparisons against AllergenOnline.org, version 13. Additionally, a BLASTP search was performed against the NCBI database using keyword search limits of "allergen" and "toxin".

4.2.1 Full length FASTA3 vs. AllergenOnline. Results of the full length FASTA3 searches of the DsRed2 protein against AllergenOnline version 13 did not identify any significant alignment with an allergen. Scoring results for the DsRed2 protein showing alignments with *E* scores less than 1 are shown in Table 3 and demonstrate no significant matches with any allergen. The low-level alignment with various sequences of the same carrot PR-10 protein are insignificant matches. Their identities (%) are markedly below the level that is likely to indicate cross-reactivity (< 50% identity, Aalberse, 2000) and it is also below the 35% identity level suggested by Codex (2003) as a match that may possibly be cross-reactive. Thus, there is no scientific basis for assuming the DsRed2 protein is sufficiently similar to any allergen to suspect cross-reactivity and there is no rationale for performing serum IgE tests based on overall alignment, the most predictive bioinformatics comparison.

Table 2. Overall FASTA3 search of AllergenOnline.org database with the DsRed2 protein (225 amino acids). Only proteins identified with matches to allergens or putative allergens in AllergenOnline.org version 13, having an *E* score smaller than 1 are shown. None of the results were significant using the criteria of >35% identity over alignments of at least 80 amino acids.

Sequence GI #	Organism	Description	Length aa	*E* score	% Identity	aa Alignment length
302379155	*Daucus carota* carrot	Pathogenesis Related protein (Bet v 1 like)	154	0.23	23.2	125
302379157	*Daucus carota* carrot	Pathogenesis Related protein (Bet v 1 like)	154	0.36	23.2	125
302379159	*Daucus carota* carrot	Pathogenesis Related protein (Bet v 1 like)	154	0.36	23.2	125
19912791	*Daucus carota* carrot	Pathogenesis Related protein (Bet v 1 like)	154	0.42	23.2	125
302379151	*Daucus carota* carrot	Pathogenesis Related protein (Bet v 1 like)	154	0.42	23.2	125
302379153	*Daucus carota* carrot	Pathogenesis Related protein (Bet v 1 like)	154	0.58	23.2	125

Similarly, the tTAV protein amino acid sequence does not show any significant FASTA alignment to any known allergen in the AllergenOnline.org database (Table 3). The only alignment was to tropomyosin of the sea snail or whelk (Neptunea polycostata) and at only 22.1% identity with an E score of 0.053, which is considered irrelevant for potential cross-reactivity.

Table 3. Overall FASTA3 search of AllergenOnline.org database with the tTAV protein (338 amino acids). Only proteins identified with matches to allergens or putative allergens in AllergenOnline.org version 13, having an *E* score smaller than 1 are shown. None of the results were significant using the criteria of >35% identity over alignments of at least 80 amino acids. In addition to testing the complete 338 aa tTAV protein, the 208 aa tetracycline repressor protein and the VP16 127 aa proteins were tested independently and only the N-terminal 208 aa segment of the intact tTAV aligned with tropomyosin of sea snail. The complete tTAV alignment is shown here.

Sequence GI #	Organism	Description	Length aa	*E* score	% Identity	aa Alignment length
219806590	*Neptunea polycostata* Sea snail (whelk)	Tropomyosin	284	0.053	22.1	181

4.2.2 Sliding 80-amino acid window FASTA3 vs. AllergenOnline.org database. Results of the comparisons of the DsRed2 and tTAV protein sequences were tested against all of the sequences in Allergenonline.org version 13. The comparisons did not identify any possible match of > 35% identity with any known allergen in the database. Thus the risk of cross-reactions for allergic individuals is very low, and the data indicate there is no reason to perform serum IgE testing as there is not a target allergen to suspect cross-reactivity. Tables 5 and 6 give an indication of the results obtained for DsRed2 and tTAV proteins respectively.

Table 4. Scanning 80-mer Sliding Window Search Results for DsRed2 protein

80mer Sliding Window Search Results

Database	AllergenOnline Database v13 (February 12, 2013)
Input Query	>DsRed2 MARMASSENVIIEFMRFKVRMEGTVNGHEFEIEGEGEGRPYEGHNTVKLKVTKGGPLPFA WDILSPQFQYGSKVYVKHPADIPDYKKLSFPEGFKWERVMNFEDGGVATVTQDSSLQDGC FIYKVKFIGVNFPSDGPVMQKKTMGWEASTERLYPRDGVLKGEIHKALKLKDGGHYLVEF KSIYMAKKPVQLPGYYYVDAKLDITSHNEDYTIVEQYERTEGRHHLFL
Length	228
Number of 80 mers	149
Number of Sequences with hits	0

No Matches of Greater than 35% Identity Found

AllergenOnline Database v13 (February 12, 2013)

Table 5. Scanning 80-mer Sliding Window Search Results for tTAV protein of OX513A

80mer Sliding Window Search Results

Database	AllergenOnline Database v13 (February 12, 2013)
Input Query	>query MGSRLDKSKVINSALELLNEVGIEGLTTRKLAQKLGVEQPTLYWHVKNKRALLDALAIEM LDRHHTHFCPLEGESWQDFLRNNAKSFRCALLSHRDGAKVHLGTRPTEKQYETLENQLAF LCQQGFSLENALYALSAVGHFTLGCVLEDQEHQVAKEERETPTTDSMPPLLRQAIELFDH QGAEPAFLFGLELIICGLEKQLKCESGSGPAVSRARTKNNYGSTIEGLLDLPDDDAPEEA GLAAPRLSFLPAGHTRRLSTAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDS PGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDEYGG
Length	338
Number of 80 mers	259
Number of Sequences with hits	0

No Matches of Greater than 35% Identity Found

AllergenOnline Database v13 (February 12, 2013)

4.2.3 Eight amino acid match. Because some countries still require a search for any exact match of 8 or more contiguous amino acids between the GM protein and any known allergen, that comparison was performed using AllergenOnline.org database, version 13. Both the DsRed2 and the tTAV full length sequences were copied into the AllergenOnline.org search query box and tested. The results of these searches were negative.

4.2.4 BLASTP of NCBI Entrez using "allergen". The full-length amino acid sequences of the DsRed2 and tTAV proteins were compared to sequences in NCBI-Entrez, which were designated as "allergen" in the NCBI database on 14 August, 2013.

The top two aligned matches to DsRed2 have (Tables 6) have significantly small *E* scores, suggesting some evolutionary homology. However, the identity matches are low (25% in a 212 amino acid alignment to a recombinant pollen allergen Cry j 1 fused to a green fluorescent protein and 24% in a 212 amino acid alignment to cockroach allergen Bla g 1 fused to a green fluorescent protein). The low identity match is not considered a likely indication of allergic cross-reactivity (Aalberse, 2000). But importantly, the two matched sequences are synthetic constructs that include a green fluorescent marker protein that was originally derived from *Aequorea victoria* (GI:634009) described by Tsien (1998). The alignments of DsRed2 to those two synthetic constructs were only in the region of the green fluorescent protein, which is not known to cause allergies. The other alignments of DsRed2 were not significant as judged by the very large *E* score values (>0.001) and low identity matches (25% to 57%) with very short-partial protein alignments. The aligned proteins would not be considered homologues of the DsRed2.

Table 6. BLASTP of NCBI Entrez with DsRed2 using the keyword "allergen". The scoring alignments with *E* scores below 10 are shown for this DsRed2 protein vs. all proteins labeled with the keyword "allergen" in the NCBI Entrez database on 14 August, 2013, using BLASTP. The sequence identities are low and / or the length of alignments are very short, indicting unlikely homology and that the overall structure is unlikely to be similar. Thus even if the NCBI sequence is a proven allergen, there is very little likelihood of cross-reactivity by the DsRed2 protein.

Sequence GI#	Organism	Description	Length aa	*E* score	% identity	aa Alignment length
223005744	*Synthetic construct* Cryptomera japonica AND GFP origin *Aequorea victoria*	Synthetic construct of T cell epitopes of the Cry j 1 *Cryptomera japonica)* and green fluorescent protein	412	1e-16	25	212
529482053	*Synthetic construct* *Blatella germanica* AND GFP origin *Aequorea*	Synthetic construct of cockroach allergen Bla g 1 and green fluorescent protein	416	1e-14	24	212

	victoria					
116333554	*Lactobacillus brevis* bacteria	Hypothetical protein LVIS_0955	321	0.34	36	50
156370878	*Nematostella vectensis* Sea anemone	Predicted protein sea anemone MD-2 like protein	299	3.1	32	38
493609361	*Oscillochloris trichoides* bacteria	Allergen V5/Tpx-1 family protein	495	4.0	29	77
403416894	*Fibroporia radiculosa* Brown rot fungus	Predicted protein MD-2 like protein	524	4.1	57	30

The tTAV protein only showed one very minor alignment by BLASTP limited by "allergen" (Table 7). This alignment is insignificant and does not represent an indication of possible cross-reactivity as described by Aalberse (2000) and Goodman et al. (2008).

Table 7. BLASTP of NCBI Entrez with tTAVusing the keyword "allergen". The only identified scoring alignments with *E* scores below 10 is shown for this tTAV. The sequence identities are low and / or the length of alignments are very short, indicting unlikely homology and that the overall structure is unlikely to be similar. Thus even if the NCBI sequence is a proven allergen, there is very little likelihood of cross-reactivity by the tTAV protein.

Sequence GI#	Organism	Description	Length aa	E score	% identity	aa Alignment length
493199135	*Treponema vincentii* Spirochette	Hypothetical protein, SCP like protein	221	1.7	55	20

4.2.5 BLASTP of NCBI without keyword limit. The full-length of the DsRed2 and tTAV proteins were compared to all sequences in NCBI-Entrez database on 14 August, 2013. The DsRed2 protein has 100 alignments of over 80% identity, mostly with synthetic constructs of transfection vectors as it is a marker gene/protein. Similarly, tTAV has many alignments of near-100% identity with synthetic constructs with partial alignments to tetracycline repressor proteins and with *Herpes simplex* 1 VP16. Because so many entries in NCBI show synthetic constructs that researchers are using for transfecting various organisms, it is difficult to trace out the origin of the proteins. The original literature on constructs is necessary to evaluate the origins (see the Introduction and Section 4.1 for references).

4.3 BLASTP of NCBI Entrez with "toxin". The full-length sequences of the DsRed2 and tTAV proteins were compared to sequences in NCBI-Entrez, which were designated as "toxin" in the NCBI database on 14 August, 2013. The top aligned proteins with E scores smaller than 10 are shown for each of the two proteins (Tables 8– 9).

DsRed2. The best scoring alignment with DsRed2 was to synthetic constructs. For the best aligned protein, the primary alignment of 98% identity is to a 223 amino acid portion of the synthetic protein construct that is from a red fluorescent protein (DsRed1) that is in the Green Fluorescent protein family, with a secondary poor alignment of 24% identity over 210 amino acids to the green fluorescent protein (Liu et al. 2011). The second best aligned sequence was a similar construct by the same authors (Liu et al., 2011), using slightly different order and sequences. The construct was used to transform cells for tests of physiological function. There is no evidence that the red fluorescent portion of the protein (with nearly 100% identity to DsRed2) is toxic. The third sequence with the next best alignments is a similar synthetic construct for testing cell physiology using a botulinum toxin substrate, by a different group, but with two green fluorescent proteins rather than one green and one red (Itoh et al., 2002). The fourth protein sequence with an alignment is another cloning construct with a green fluorescent protein and with a botulinum toxin in the same construct (Band et al. 2010). The fifth protein, phytoene dehydrogenase from the toxic bacterium Corynebacterium ulcerans is the highest scoring protein that is not a fluorescent marker protein (Sekizuka et al., 2012). However, the alignment is poor and the sequence is merely one of the sequences discovered by whole genome sequencing of the bacterium. The protein sequence (GI:397655072) was then compared to all of NCBI by BLASTP and it turns out to be one of the highly conserved enzymes related to phytoene desaturases, that are rather ubiquitous. No published evidence was found that this protein is toxic and furthermore, the sequence alignment to DsRed2 is weak. The final aligned protein is a transcription regulator from *Closteridium botulinum*, a toxic organism. A search of PubMed did not identify any publications that describe toxicity associated with the transcriptional regulators of *C. botulinum*. In general transcriptional regulators are only functional if they are expressed inside the cell of the organism containing the gene that is being regulated. I found no evidence that this protein could be taken in by the cells of other, non-bacterial organisms and cause gene expression changes.

Table 8. BLASTP of NCBI Entrez "toxin" with DsRed2 from OX513A. The best scoring alignments to putative toxins shown in the NCBI Entrez database on 14 August, 2013, were identified by BLASTP with the full-length sequence of the DsRed2 protein from OX513A.

Sequence GI#	Organism	Description	Length aa	*E* score	% identity	aa Alignment length
172054575	Synthetic construct Primary alignment	EGFP-Pak1-Rac1-dsRed1-CAAX fusion with Rac related to a botulinum toxin substrate Alignment only to red fluorescnt protein peptide	798	2e-145	98	223
172054575	Synthetic construct Secondary alignment	EGFP-Pak1-Rac1-dsRed1-CAAX fusion with Rac related to a botulinum toxin substrate Alignment only to green fluorescnt protein peptide	798	1e-14	24	210
16796513	Synthetic construct Primary alignment	dsRed1/Pak1/Rac1/ECFP fusion protein Alignment to red fluorescent protein	775	1e-144	98	222
16796513	Synthetic construct Secondary alignment	dsRed1/Pak1/Rac1/ECFP fusion protein Alignment to green fluorescent protein	775	4e-14	30	145
23095931	Synthetic construct Primary alignment	Raichu-1011x, rac and cdc42 Alignment to green fluorescent protein	763	1e-15	26	203
23095931	Synthetic construct Secondary alignment	dsRed1/Pak1/Rac1/ECFP fusion protein Alignment to green fluorescent protein	763	2e-14	26	209
259490938	Synthetic construct	deltaLC-GFP-BoNT/A rev	1230	2e-15	25	212
397655072	Corynebacterium ulcerans bacteria	Phytoene dehydrogenase	544	0.17	21	95
182674319	Corynebacterium botulinum bacteria	Transcriptional regulator AraC family	395	8.4	45	33

tTAV. The best scoring alignment with tTAV was to a tetracycline repressor protein TetR from *Escherichia fergussonii* (Table 9). The alignment is very significant, however the bacteria is considered a source of toxicity. But the TetR protein has very high identity with the TetR proteins of many species of bacteria. It is also a regulatory protein expressed in the bacteria, which acts on the DNA of the bacteria to suppress or promote expression of bacterial proteins. It is not known to be taken up by cells of other organisms and cause any toxic effects. The next alignment is almost identical to the first and from another species of the genus. In fact it is 100% identical to a shorter (196 aa) segment of the protein of tTAV. Running a BLAST comparison of these two proteins demonstrates very high identity matches with many cloned gene/proteins as the activity is useful for tetracycline dependent gene regulation, as used in OX513A. There were quite a few alignments of much lower length and identity, all beginning near the N-terminus of the tTAV protein. However, there was also a very poor alignment to a segment of tTAV beginning at amino acid 224, but that was also a transcriptional regulator. No evidence was found of homology of the tTAV protein to a true toxin.

Table 9. BLASTP of NCBI Entrez "toxin" with tTAV protein from OX513A. The best scoring alignments to putative toxins shown in the NCBI Entrez database on 14 August, 2013, were identified by BLASTP with the full-length sequence of the tTAV protein from OX513A. There were 93 alignments with *E* scores less than 10 using this sequence and the term "toxin" by BLASTP. However, in many cases it is clear that the sequence is "identified" as a toxin due to the toxicity of the source organism (from a number of toxic bacteria). Importantly, only a few sequences had high identity matches over any extensive length of sequence. Those were evaluated further here.

Sequence GI#	Organism	Description	Length aa	*E* score	% identity	aa Alignment length
218561676	*Escherichia fergusonii* bacteria	Tetracycline repressor protein TetR	208	3e-150	99	208
388377844	*Escherichia coli* bacteria	Tetracycline repressor protein TetR	197	4e-142	100	196
394430501	*Escherichia coli* bacteria	Tetracycline repressor protein TetR	141	1e-81	90	136
190903672	*Escherichia coli* bacteria	Tetracycline repressor protein class A from transposon 1721	219	2e-65	53	202
388363196	*Escherichia coli* bacteria	Tetracycline repressor protein from E. coli strain o111:H8, TetR C	225	2e-64	51	210

310286451	*Escherichia coli* bacteria	Tetracycline repressor protein Class A from E. coli	217	1e-63	50	209
397654352	*Corynebacterium ulcerans* bacteria	TetR family transcriptional regulator	203	1e-12	33	147
300850578	*Enterococcus faecalis* bacteria	TetR family transcriptional regulator	220	2e-11	31	159
292642929	*Enterococcus faecalis* bacteria	TetR family transcriptional regulator	222	2e-08	26	155
429514288	*Enterococcus faecalis* bacteria	TetR/AcrR family transcriptional regulator	189	1e-5	44	62

4.4 Bioinformatics summary for the DsRed2 and tTAV proteins of OX513A. Although the results of literature searches to the sources of the genes transferred into OX513A were challenging due to the some extensive annotations that suggest allergy or toxicity associated with the source organisms, careful evaluation of the abstracts and publications as well as refined searches did not identify publications with sufficient evidence to suspect the DsRed2 or tTAV proteins represent risks of allergy or toxicity.

None of the results from the bioinformatics searches of the DsRed2 or tTAV protein amino acid sequences indicate that these proteins represent a risk of allergy or toxicity that is greater than a typical dietary protein. There were no matches of either protein to known allergens with more than 50% identity over the full-length. There were no matches of >35% identity over 80 or more amino acid segments compared to known or putative allergens. There were no identical matches of 8 or more contiguous amino acid segments. These highly conservative comparisons did not identify sequence similarities that would suggest the proteins are allergens or are sufficiently similar to an allergen to cause cross-reactions. They did not identify matches to toxins to suggest they may be toxic.

5.0 Conclusions

No convincing evidence was found to suggest that the DsRed2 protein or the tTAV protein expressed in the OX513A mosquitos represent risks of allergy or toxicity to humans (or other mammals). Based on the guidelines of the Codex Alimentarius Commission (2003 and 2009), and on common practices for evaluation of potential risks of allergy or toxicity from GMO (plants, animals or microbes), there is no reason to perform additional tests to evaluate potential risks of allergy or toxicity for these proteins. Although the guidelines are intended primarily evaluating potential food safety concerns regarding potential risks from genetically engineered organisms, the same safety evaluation process is scientifically sound as an approach for evaluating other potential routes of exposure, namely via airway (inhalation of insect body parts) or through insect bites (e.g. mosquito saliva). There is no evidence that these proteins pose any risk of eliciting allergic or toxic reactions.

6.0 References

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

Appendix K

OXITEC

Study Report: Western Blot Analysis of *Aedes aegypti* OX513A Saliva for the Detection of tTAV and DsRed2 Proteins

[Confidential business information (CBI) deleted]

SR-00004 Edition 2.b

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

Table of Contents

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

ACKNOWLEDGEMENTS

Oxitec would like to extend their gratitude to Dr Eric Calvo of the National Institutes of Health (NIH), Bethesda, USA for training Oxitec staff in mosquito saliva collection techniques, providing purified recombinant Aegyptin and providing rabbit anti-sera against recombinant Aegyptin.

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

1 STATEMENT OF DATA CONFIDENTIALITY

Confidential business information (CBI) has been deleted from this report.

2 STATEMENT CONCERNING GOOD LABORATORY PRACTICES

This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

3 ASSOCIATED PERSONNEL

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

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

4 TEST FACILITY

This research was performed at the laboratory of Oxitec Limited located at:

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5 OBJECTIVES

The objective of this study was to investigate whether there is a detectable presence of the tetracycline-controlled transactivator protein (tTAV) or the marker protein DsRed2 in the saliva of adult female *Aedes aegypti* homozygous for the OX513 rDNA construct, known as OX513A.

6 SUMMARY

To test whether tTAV and/or DsRed2 protein is present in the saliva, which is a secretion of the salivary glands, of homozygous adult female *Aedes aegypti* expressing the OX513 construct, OX513A *Aedes aegypti* were reared in the presence of doxycycline hyclate to adulthood. Saliva was collected from bloodfed adult females between 10 and 15 days post-eclosion. Saliva was collected from these insects as well as from wild type *Aedes aegypti* females and two pools (OX513A and WT) created that were used for the entire study. Western blot analysis using a polyclonal tTAV antibody (anti VP16 tag antibody) and a polyclonal DsRed2 antibody was carried out, using an Enhanced Chemiluminescence (ECL) based detection method. Sample integrity was confirmed using an antibody detecting a secreted salivary protein in mosquitoes, Aegyptin. Aegyptin detection was also used as a basis to determine that equivalent amounts of saliva were loaded in control and sample lanes between the test saliva samples of OX513A and the WT control saliva samples.

The Limit of Detection (LOD) for tTAV and DsRed2 on the western blots was determined using recombinant tTAV and recombinant DsRed2. Purified tTAV and DsRed2 proteins from OX513A could not be used as sufficient quantity cannot be extracted from the insects for this study.

Results from western blot analyses were captured using the ChemiDoc-IT 500 Imaging System (UVP), and signals were quantified by relative densitometry, using the VisionWorks LS Acquisition and Analysis Software (UVP).

The Limit of Detection (LOD) for recombinant tTAV was determined to be 0.8 ng and the LOD for recombinant DsRed2 was determined to be between 5.0 and 2.5 ng.

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

The introduced proteins, tTAV and DsRed2 were not detected in OX513A *Aedes aegypti* saliva at and above these LODs in the 5 µl of saliva analysed. 5 µl of OX513A saliva equates to the quantity of saliva collected from approximately 5.5 female adult mosquitoes based on this study (270 µl of pooled saliva collected from approximately 300 *Aedes aegypti* adult females homozygous for OX513A rDNA construct).

7 INTRODUCTION

Genetically engineered *Aedes aegypti* strain OX513A carrying the rDNA construct OX513 was developed by Oxitec Ltd in 2002. This strain carries a genome integrated tetracycline transcriptional activator gene (tTAV), whereby the tTAV is produced in the absence of tetracycline or analogues and accumulated to levels lethal to those mosquitoes[1]. The OX513 rDNA construct also carries a gene coding for the DsRed2 protein as a marker.

In this study, pooled saliva samples collected from homozygous OX513A adult females (OX513A saliva) were investigated for the presence of tTAV and DsRed2 proteins. Pooled saliva samples from non-genetically engineered wild type *Ae.aegypti* adult females (WT saliva) were used as negative controls.

Western blotting detection[2] was carried out to determine the absence or presence of these proteins in OX513A saliva samples using an Enhanced Chemiluminescence (ECL) approach. Purified tTAV and DsRed2 proteins from OX513A are unavailable as sufficient quantity cannot be extracted from the insects to conduct the study. Therefore recombinant tTAV (rtTAV) and DsRed2 (rDsRed2) proteins were used to determine the limit of detection (LOD) of the western blot analysis in detecting these proteins in OX513A saliva.

For the purpose of this study, the LOD was defined as "the lowest quantity of a protein that an operator can visibly discern on a developed western blot image and create a user defined-region (UDR) and meets the acceptance criteria such that D_{SLC} is less than 10% of D_S" (Figure1). To be valid, all pixels on the complete western blot image must be less than a pixel intensity of 65,536 for a 16-bit grey scale image (as measured by the VisionWorks LS Acquisition and Analysis Software (UVP) used). 65,536 is the maximum pixel intensity of a 16-bit grey-scale image.

An endogenous 30 kDa saliva protein (Aegyptin)[3] was used as positive control in both OX513A and WT saliva to ensure their integrity and the equivalence of the saliva samples used throughout this study. Aegyptin protein is found in the saliva of females of a number of mosquito species including Aedes, Anopheline and Culicine species[4]. The detection signal obtained from the specific binding of the anti-Aegyptin antibody to the Aegyptin protein in the saliva was used to ensure that comparable amounts of total protein were loaded between the OX513A and WT saliva samples. A recombinant version of Aegyptin (rAegyptin[3]) was used as a positive control for the anti-Aegyptin antibody.

The LOD for tTAV and DsRed2 was determined on replicate blots using quantified amounts of recombinant proteins (rtTAV and rDsRed2). To ensure that the LODs obtained using the recombinant proteins were applicable for the detection of tTAV and DsRed2 in OX513A saliva, LODs were determined in the presence of WT saliva.

8 MATERIALS AND EQUIPMENT

8.1 Saliva

8.1.1 Saliva from approximately 300 *Ae. aegypti* adult females homozygous for OX513A rDNA construct (OX513A saliva) pooled in PBS to give a total volume of 270 µl saliva [therefore 5 µl OX513A saliva equals approximately 5.5 adult mosquitoes].

8.1.2 Saliva from approximately 1000 wild type *Ae. aegypti* (Latin) adult females (WT saliva) pooled in PBS to give a total volume of 890 µl [therefore 5 µl WT saliva equals approximately 5.5 adult mosquitoes]

8.2 Equipment

8.2.1 Microcapillary tube (Sigma-Aldrich, Cat # P1299)
8.2.2 Trans-Blot® Turbo™ Transfer System (BioRad Cat# 170-4155)
8.2.3 ChemiDoc-IT 500 Imaging System and VisionWorks LS Acquisition and Analysis Software (UVP)[5].

8.3 Recombinant proteins

8.3.1 Recombinant Aegyptin (rAegyptin) obtained from Eric Calvo National Institutes of Health (NIH), Bethesda, USA [expressed and purified in the Laboratory of Malaria and Vector Research (NIAID/DIR) by affinity size exclusion chromatography column as described by Calvo[3]]
8.3.2 Recombinant DsRed2 (rDsRed2) protein (Clontech, Cat # 632436)
8.3.3 Recombinant tTAV (rtTAV) protein (produced in *Escherichia coli* by Oxitec, according to Study Report SR-00003)[6]

8.4 Reagents and materials

8.4.1 4x Laemmli Sample Buffer (BioRad, Cat #161-0747)
8.4.2 Defibrinated horse blood (TCS Biosciences, UK, Cat #HB035)
8.4.3 Precision Plus Protein™ WesternC™ Pack (Mwt Marker, BioRad, Cat #161-0385)
8.4.4 Mini-PROTEAN® TGX gel (4-15%, BioRad, Cat #456-1086)
8.4.5 Nitrocellulose membrane (BioRad, Cat #170-4270)
8.4.6 Clarity™ Western ECL Substrate (BioRad, Cat #170-5060)
8.4.7 Tris/Glycine/SDS buffer (BioRad, Cat #161-0732)
8.4.8 Pierce™ Clear Milk Blocking Buffer (Life Technologies, Cat #37587)
8.4.9 Restore™ Western Blot Stripping Buffer (Life Technologies Cat #21059)
8.4.10 Tween 20 (Pierce, Cat # 28320)
8.4.11 PBS (Phosphate buffered saline, 0.01 M phosphate, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4) tablets (SIGMA, Cat # P4417)
8.4.12 TBS-T (Tris-buffered Saline Tween 20, 20 mM Tris, 150 mM NaCl, pH 7.5, 0.1% Tween 20)

8.5 Antibodies

8.5.1 Rabbit Anti-Aegyptin Antibody provided by Eric Calvo - Laboratory of Malaria and Vector Research (NIAID/DIR) (Chagas *et al*[7])
8.5.2 Rabbit Anti-VP16 tag polyclonal antibody (Abcam, Cat #ab4808)
8.5.3 Goat Anti-Rabbit IgG (whole molecule)-Horse Radish Peroxidase Polyclonal Antibody (Abcam, Cat # ab97051)
8.5.4 Living Colors®DsRed Polyclonal Antibody (Clontech, Cat # 632496)

9 METHODS

9.1 Strains

The following *Aedes aegypti* strains were used for this experiment:

OX513A: In the absence of doxycycline hyclate, this strain expresses the tTAV protein during immature stages through a positive feedback loop, resulting in high mortality (>95%). However, when reared in the presence of doxycycline hyclate, expression of the tTAV in this strain is repressed, allowing the OX513A mosquitos to

complete all parts of their lifecycle. Individuals of this strain can be identified during immature stages by the expression of the fluorescent protein DsRed2 in their bodies in a characteristic punctate pattern driven by the Actin5C promoter[1, 8].

Wild Type (WT): The non-genetically engineered background strain of OX513A, originating from Chiapas, Mexico in 2007.

9.2 Insect Rearing

All strains were reared under standard insectary conditions: 26°C [± 2°C], 75% [± 15%] relative humidity, 12h: 12h light: dark cycle. Adults were provided with 10% sucrose solution *ad libitum*. Larvae were reared with the addition of doxycycline hyclate to the water at a final concentration of 1 µg/ml, and fed finely ground Tetramin© fish flakes (Tetra, GmbH, Germany).

9.3 Saliva Collection

The WT and OX513A strains are homozygous for their respective genotypes and so female pupae were collected and directly placed into cages. One week post eclosion, females were offered a blood meal of warmed, defibrinated horse blood (TCS Biosciences, UK). Saliva samples were collected between 10 and 15 days post-eclosion as described below.

Females were cold-anesthetised in the freezer at ≤ -15°C for 15-20 seconds. Legs were removed before mounting them on a microscope slide using double sided tape. Each female's proboscis was inserted into a microcapillary (0.5 µl) containing mineral oil. A 15 minute interval was allowed for salivation at which point females were discarded and pools of 10 samples were collected into 1.5 mL microcentrifuge tubes containing 10 µL of phosphate buffered saline (PBS) pH 7.5 and frozen at ≤ -15°C. Samples were defrosted at room temperature and pooled before being used in this study. [DEV02: Saliva samples were frozen at ≤ -15°C prior to pooling. Saliva samples were thawed at room temperature and pooled. Protocol SP00002 stated that the saliva samples were to be pooled prior to freezing. This does not affect the analysis or results and so no further action was required].

9.4 Production of Recombinant tTAV (rtTAV)

Recombinant tTAV was produced by Oxitec using pET Express and Purify Kit – HisTALON, according to Study Protocol SP_00001[9] and Study Report SR-00003[6].

9.5 Densitometry Methods

Images of the western blot membrane were captured using the ChemiDoc-IT 500 Imaging System (UVP), and signals were quantified by relative densitometry, using the VisionWorks LS Acquisition and Analysis Software (UVP)[5]. To achieve this, the operator defined a rectangular box around a 'Band' of the expected size for the protein being analysed. This selection is referred to as the user defined region ('UDR', Figure 1a). The pixel density within the 'UDR' is referred to as 'Total Density', and is automatically normalised for background by the software, which subtracts the sum of the pixel intensity values of all pixels in an area within three pixels outside of the 'UDR' ('Total Background'), from the sum of the pixel intensity values for all pixels within the 'UDR' ('Total Raw Density').

The 'Total Density' of the 'UDR' containing a 'Band' representing specific binding of the antibody to the target protein is referred to as the 'Specific Density (D_S)'. The 'Total Density' of a sample lane control (D_{SLC}) was measured similarly by defining a rectangular box of identical dimensions to the 'UDR' within the same lane, in an area without specific antibody binding, either above or below the specific signal. 'Total Density' for a 'Blank Lane

Control (D_{BLC})' was defined by creating rectangular box of the same dimensions as the 'UDR' in a nearby blank lane on the same gel (i.e. no protein sample, Figure 1b). 'D_{BLC}' was expected to be low compared to 'D_S' and served as a control for the level of non-specific binding of antibodies to the blocked membrane.

The 'predictable range of the detection method' was accommodated to ensure that the signals from the recombinant proteins and saliva proteins were falling within the range of signal intensity that changed in a predictable way with concentration.

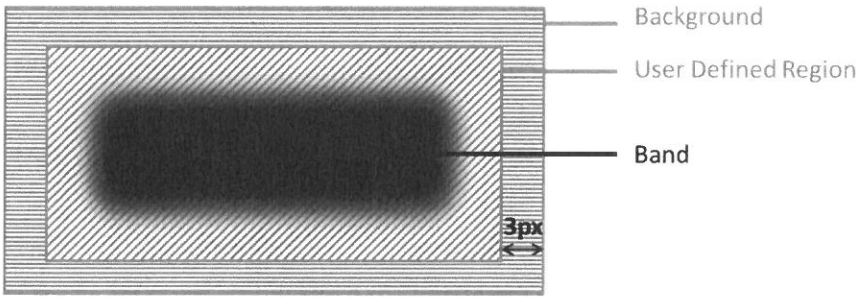

Figure 1a: Schematic representation of a 'UDR' (diagonal stripes), the 'Background' which is a perimeter 3 pixels wide surrounding the 'UDR' (horizontal stripes) and a 'Band' within the 'UDR'. 3px abbreviation denotes the three pixel perimeter.

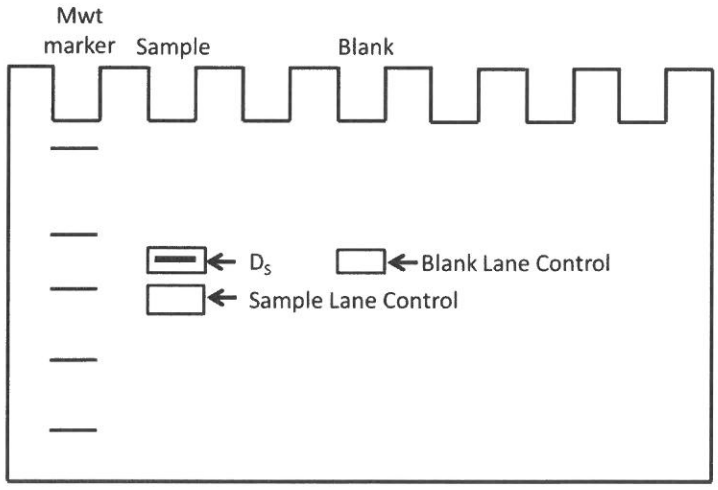

Figure 1b: Diagram of a gel showing how a user defined the UDR's which will give the Total Densities for the Band (D_S), a Sample Lane Control (D_{SLC}) and the Blank Lane Control (D_{BLC}).

9.6 Determination of Endogenous Aegyptin Signals in OX513A and WT Saliva

Two replicate gels (4-15% Mini-PROTEAN® TGX) were run each containing 2-fold serial volumes of both OX513A saliva and WT saliva (8.0, 4.0, 2.0 and 1.0 µl) and a positive control of rAegyptin (2.5 ng), each in 1x Laemmli buffer (diluted in PBS from 4X Laemmli buffer) with 5.5 % β-mercaptoethanol and made up to 12 µl in PBS. All gels were separated at 200V in 1X Tris/Glycine/SDS buffer for approximately 30 minutes. On each gel Molecular weight Marker (Mwt Marker) was loaded onto the outside wells and blank lanes were loaded with PBS in 1X Laemmli Sample Buffer (Table 1). The gel loading plan differed from that detailed in SP_00002[10], see section 12 for deviation (DEV01) details.

Gels were blotted onto nitrocellulose membrane. All gels were transferred using the TransBlot Turbo transfer system using the '1 MiniTGX mixed MW' setting. Blots were probed using polyclonal Rabbit Anti-Aegyptin as the

primary antibody at a 1/2,500 dilution in 10ml TBS-T. All antibody stains were performed at room temperature, shaking, for 1 hour. Polyclonal Goat Anti-Rabbit IgG (whole molecule)-Horse Radish Peroxidase Antibody (HRP) and StrepTactin-HRP (a component of the Precision Plus Protein™ WesternC™ Pack) were used as a secondary detection markers at a 1/30,000 and 1/10,000 dilution in 15 ml TBS-T respectively. HRP-conjugated secondary detection markers were detected using Clarity™ Western ECL Substrate according to the manufacturer's instructions, using 2 ml of substrate per membrane. Following incubation with the primary antibody, all blots were washed 4 times, for 5 minutes at room temperature, in TBS-T. Following incubation with the secondary markers, all blots were washed 3 times, for 5 minutes at room temperature, in TBS-T followed by a 10 minute wash under the same conditions Blots were digitally captured as a 16-bit greyscale image using ChemiDoc-IT 500 Imaging System (UVP) to assess the intensity of signals in the different lanes using VisionWorks LS Analysis Software (UVP). All blots were imaged in increasing exposures, starting at 10 seconds and doubling with each capture, until the image was over exposed (i.e. contained pixels with an intensity of 65,536 - see LOD definition in section 13). The longest exposure, which was not overexposed, was then used for analysis.

A standard curve was plotted for the D_S values of the endogenous Aegyptin signal in both OX513A and WT saliva for each blot to determine equivalence of the total protein levels of the two saliva samples.

Table 1: Gel loading plan for determination of endogenous Aegyptin in OX513A and WT saliva

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Mwt Marker/µl	5.0														5.0
rAegyptin/ng								2.5							
WT Saliva/µl			1.0	2.0	4.0	8.0									
OX513A Saliva/µl										1.0	2.0	4.0	8.0		

Lanes 2, 7, 9 and 14 are blank lanes and loaded with PBS in 1X Laemmli Sample Buffer

9.7 Determination of Limit of Detection (LOD) for rtTAV and Detection of tTAV in OX513A Saliva

Two replicate gels were run containing 5 µl of OX513A saliva and 5 µl of WT saliva and the following amounts of rtTAV; 12.5, 6.3, 3.1, 1.6, 0.8 and 0.4 ng (this range was determined empirically in range-finding studies prior to this study. rtTAV was quantitated using the Bicinchoninic acid assay (BCA) assay in SR_00001[6]) mixed with 5 µl of WT saliva with 1x Laemmli buffer with 5.5 % β-mercaptoethanol and made up to 12 µl in PBS. On each gel Mwt. Marker was loaded in the outside wells and blank lanes were loaded with PBS in 1X Laemmli Sample Buffer (Table 2). Gels were blotted onto nitrocellulose membrane. Blots were probed using polyclonal anti-VP16 tag antibody as the primary antibody at a dilution of 1/500 in 10 ml TBS-T, and the same secondary markers and substrate described in section 9.6.

To ensure equivalence of the saliva samples loaded, the membrane was then stripped of all bound antibodies using Restore™ Western Blot Stripping Buffer. Membranes were incubated with 20 ml stripping buffer at room temperature, shaking, for 15 minutes. Membranes were then re-probed using the anti-Aegyptin antibody as described in section 9.6.

Table 2: Gel loading plan for determination of LOD of rtTAV and detection of tTAV in OX513A saliva

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Mwt Marker/µl	5.0														5.0
rtTAV/ng			12.5		6.3	3.1	1.6	0.8	0.4						
WT Saliva/µl			5.0		5.0	5.0	5.0	5.0	5.0		5.0				
OX513A Saliva/µl													5.0		

Lanes 2, 4, 10, 12 and 14 are Blank Lanes and are loaded with PBS in 1X Laemmli Sample Buffer

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

9.8 Determination of Limit of Detection (LOD) for rDsRed2 and Detection of DsRed2 in OX513A Saliva

Two replicate gels were run containing 5 µl of OX513A saliva and 5 µl of WT saliva and the following amounts of rDsRed2; 40.0, 20.0, 10.0, 5.0, 2.5 and 1.3 ng (this range determined empirically in range-finding studies prior to this study) mixed with 5µl of WT saliva with 1x Laemmli buffer with 5.5 % β-mercaptoethanol and made up to 12 µl in PBS. On each gel Mwt. Marker was loaded in the outside wells and blank lanes were loaded with PBS in 1X Laemmli Sample Buffer (Table 3). Immunoblotting was carried out using Living Colors® DsRed Polyclonal Antibody as a primary antibody at a 1/1,000 dilution in 10 ml TBS-T, and the same secondary markers and substrate described in section 9.6.

To ensure equivalence of the saliva samples loaded, the membrane was then stripped of all bound antibodies using Restore™ Western Blot Stripping Buffer (as described in section 9.7) and re-probed using the anti-Aegyptin antibody as described in section 9.6.

Table 3: Gel loading plan for determination of LOD of rDsRed2 and detection of DsRed2 in OX513A saliva

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Mwt Marker/µl	5.0														5.0
rDsRed2/ng			40.0		20.0	10.0	5.0	2.5	1.3						
WT Saliva/µl			5.0		5.0	5.0	5.0	5.0	5.0		5.0				
OX513A Saliva/µl													5.0		

Lanes 2, 4, 10, 12 and 14 are Blank Lanes and are loaded with PBS in 1X Laemmli Sample Buffer

10 RESULTS

10.1 Determination of Endogenous Aegyptin Signals in OX513A and WT Saliva

OX513A and WT saliva samples were analysed by western blot with an anti-Aegyptin antibody to assess the comparative levels of endogenous Aegyptin in the saliva pools. The outermost lanes in these figures appear bowed, as the lower part of the TGX gel expands prior to the transfer, therefore the lane numberings are aligned to the lane boundaries close to the signals of interest on each blot.

Visually, the signals increased in intensity in a linear fashion with increasing saliva volume for both saliva types, and the strengths of the endogenous Aegyptin signals are equivalent (Figures 2A and 3A). The lower-molecular weight (MW) band of the endogenous Aegyptin signal (the dominant band) was further analysed by densitometry, and the D_S values plotted against saliva volume (Figures 2B and 3B). This analysis also revealed a linear relationship between D_S and saliva volume for both OX513A and WT saliva, both best fit lines are co-linear, with R^2 values of between 0.96 and 0.99, suggesting that the signals from the endogenous Aegyptin from these blots fall within the range of signal intensity that change in a predictable way with amount of protein loaded. Although not identical, the curves were considered similar by the operator, considering the typical intra-blot variations observed with western blots of this nature in range finding studies.

The rAegyptin protein displayed a distinct migration pattern compared to secreted endogenous Aegyptin found in the saliva. rAegyptin migrated slower than the secreted endogenous Aegyptin protein, and produced a single band at approximately 37 kDa, whereas two bands of similar MW (approximately 27 and 30 kDa) were detected by the anti-Aegyptin antibody in the saliva samples. One of the two bands may be a result of cross reactivity of the anti-Aegyptin antibody with another saliva protein, or more likely, differentially processed forms of Aegyptin. The observation that the rAegyptin construct migrates slower than the endogenous Aegyptin is likely due to differences in either protein folding or post-translational modifications, as a result of protein processing pathways in the mammalian 293-F cell expression system in which the rAegyptin construct was expressed[3], compared to the secretory system of expression in the saliva glands of *Ae. aegypti*. Specifically, the difference in

molecular weight between the recombinant Aegyptin and endogenous Aegyptin, is most likely explained as differences in the secretion pathway for Aegyptin, as in a previous study, intracellular (non-secreted) Aegyptin from the excised salivary glands of *Ae. aegypti* appears as a single band on western blot, and migrates at approximately 37kDa, similar to the recombinant construct[3].

Collectively, these data suggest that the endogenous Aegyptin levels (and therefore total protein levels) are equivalent in both OX513A and WT saliva, and therefore equal volumes (5 µl) of OX513A and WT saliva were loaded on the gels for the detection of tTAV and DsRed2 in OX513A saliva in sections 9.7 and 9.8.

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

Figure 2

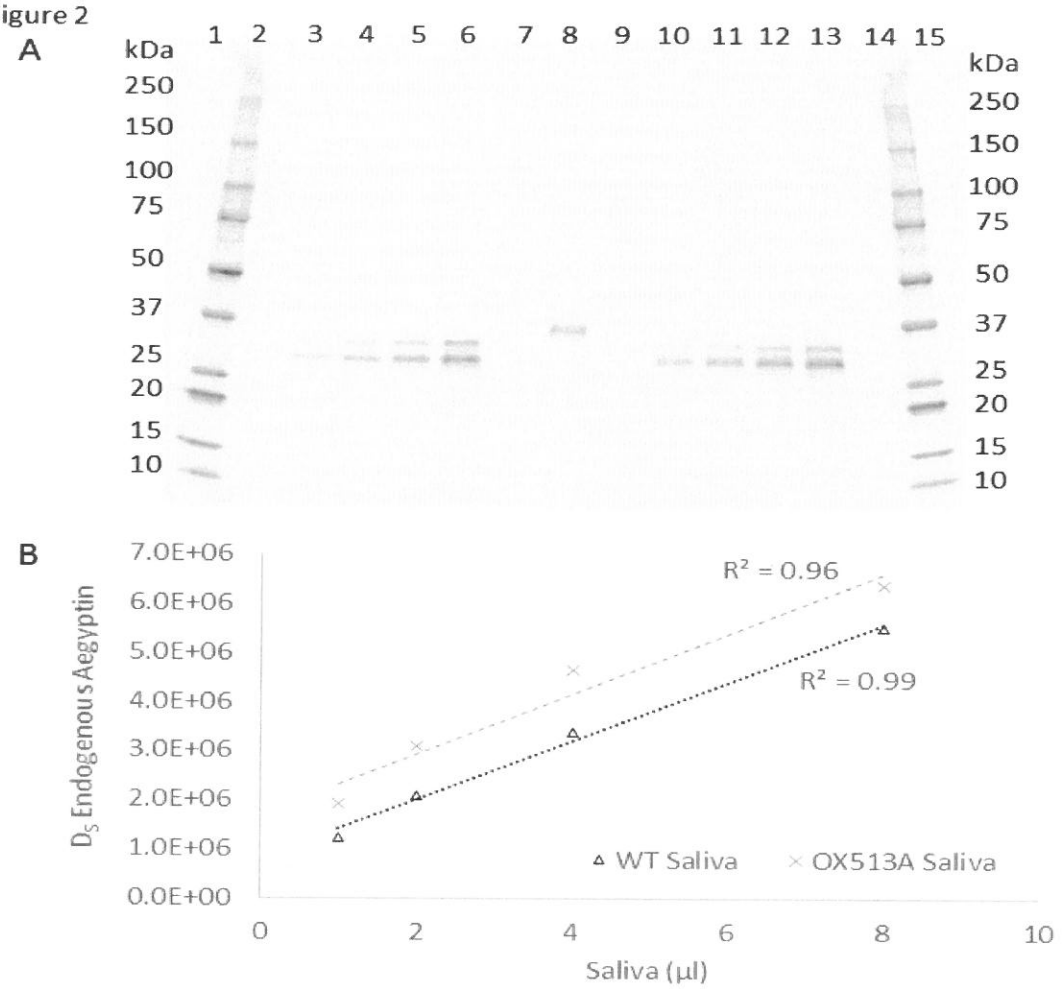

Figure 2: Western blot analysis demonstrating equivalence of endogenous Aegyptin signals in OX513A and WT Saliva

The endogenous Aegyptin in OX513A and WT saliva samples was analysed by western blot. (A) 2-fold serial dilutions of OX513A and WT saliva and 2.5ng of rAegyptin were separated on replicate gels by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with a rabbit anti-Aegyptin antibody to detect the presence of Aegyptin protein. (B) D_s values for the lower-MW band were calculated using VisionWorks LS Acquisition and Analysis Software (UVP). A linear trend line was plotted, and R^2 values calculated, using Microsoft Excel. Δ represents WT Saliva, X represents OX513A saliva. Lane designations are as follows (Blank lanes contain PBS in 1X Laemmli buffer):

1: 5µl Mwt marker
2: Blank lane
3: 1µl WT Saliva
4: 2µl WT Saliva
5: 4µl WT Saliva
6: 8µl WT Saliva
7: Blank lane
8: 2.5ng rAegyptin
9: Blank lane
10: 1µl OX513A Saliva
11: 2µl OX513A Saliva
12: 4µl OX513A Saliva
13: 8µl OX513A Saliva
14: Blank lane
15: 5µl Mwt Marker

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

Figure 3

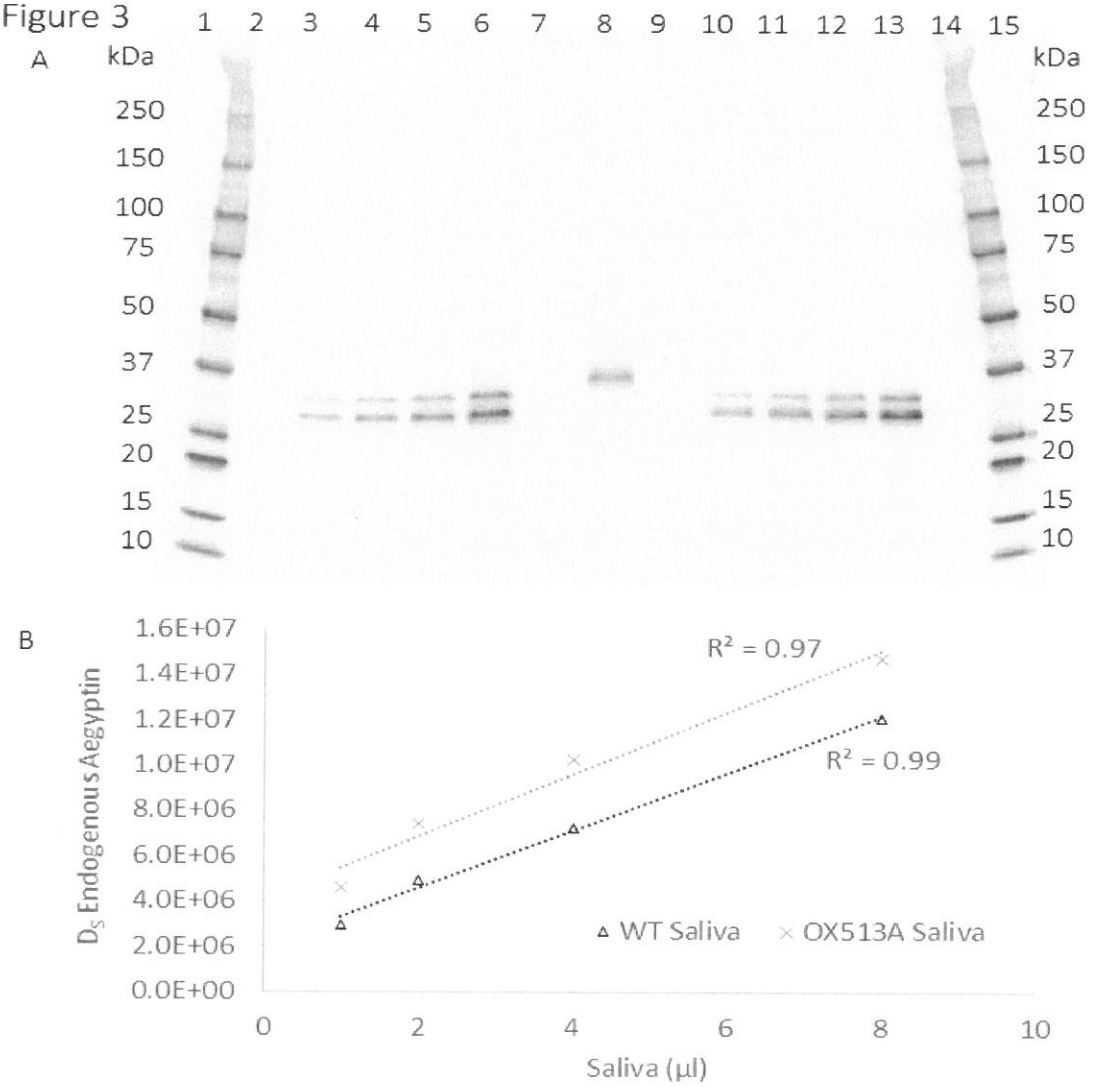

Figure 3: Western blot analysis demonstrating equivalence of endogenous Aegyptin signals in OX513A and WT Saliva (Replicate)

The endogenous Aegyptin in OX513A and WT saliva samples was analysed by western blot. (A) 2-fold serial dilutions of OX513A and WT saliva and 2.5ng of rAegyptin were separated on replicate gels by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with a rabbit anti-Aegyptin antibody to detect the presence of Aegyptin protein. (B) D_s values for the lower-MW band were calculated using VisionWorks LS Acquisition and Analysis Software (UVP). A linear trend line was plotted, and R^2 values calculated, using Microsoft Excel. Δ represents WT Saliva, X represents OX513A saliva. Lane designations are as follows (Blank lanes contain PBS in 1X Laemmli buffer):

1: 5µl Mwt marker
2: Blank lane
3: 1µl WT Saliva
4: 2µl WT Saliva
5: 4µl WT Saliva
6: 8µl WT Saliva
7: Blank lane
8: 2.5ng rAegyptin
9: Blank lane
10: 1µl OX513A Saliva
11: 2µl OX513A Saliva
12: 4µl OX513A Saliva
13: 8µl OX513A Saliva
14: Blank lane
15: 5µl Mwt Marker

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

10.2 LOD for Recombinant tTAV and Recombinant DsRed2 and Detection of tTAV and DsRed2 in OX513A Saliva

LODs for rtTAV and rDsRed2 were determined by western blot analysis where recombinant proteins were loaded with 5µl of WT saliva as the background matrix to be consistent with the test material (OX513A saliva) and control material (WT Saliva). rtTAV has a predicted MW of 39.9kDa and migrates at approximately 46-50kDa when separated by SDS-PAGE, likely due to the helical nature of this protein. This is discussed in more detail in SR_00003[6].

The western blots shown in figure 6A and 7A show rDsRed2 supplied by Clontech gives two bands when analysed by western blot (Figures 6A and 7A). The dominant band, representing monomeric DsRed2, migrates at approximately 30kDa, slightly slower than it's predicted MW (25.7kDa) would suggest. This migration pattern is also documented in the manufacturer's Certificate of Analysis for this protein [ref: http://www.clontech.com/xxclt_ibcGetAttachment.jsp?cItemId=27589]. A higher MW band is also apparent, migrating at approximately 60kDa, approximately twice that of the monomer. DsRed2 natively forms homodimeric and homotetrameric complexes[11], and so this band likely represents dimeric DsRed2. Although samples are denatured prior to SDS-PAGE analysis, complete disruption of all subunit interactions of multimeric complexes is not always achieved. For this study the dominant monomeric band was used for all subsequent analysis.

According to the acceptance criteria detailed in section 7, both replicate blots had the same LOD for rtTAV, 0.8 ng (Figures 4A and 5A). The replicate blots for rDsRed2 had different LODs this protein, 5.0 ng and 2.5 ng for each blot (Figures 6A and 7A). The endogenous Aegyptin signal visually appears to slightly increase in strength from left to right across the blot. This pattern is not apparent in any of the other blots, and is likely due to typical intra-blot variations observed with western blots of this nature, as seen in range finding studies.

D_S values were determined for the signals from the recombinant proteins, and when the D_S values for 'visible' bands are plotted against protein amount loaded reveal a linear relationship, with R^2 values between of 0.96 and 1.00 (Figure 8), suggesting that the signals from the recombinant proteins from these blots fall within the range of signal intensity that change in a predictable way with amount of protein loaded.

Specific bands representing antibody binding to DsRed2 and tTAV proteins, equivalent in size to their recombinant counterparts or otherwise, were not detected in OX513A saliva in either of the replicate blots. The rabbit anti-VP16 tag polyclonal antibody cross-reacts with a 30 kDa protein in WT and OX513A saliva, producing a very slight, but consistent, signal which is difficult to visualise on the western blot images (Fig 4A and Fig 5A). As this protein is also present at a consistent level in WT saliva, it does not represent anti-VP16 antibody binding to tTAV. This cross-reactivity is likely caused by shared epitope between VP16 and an endogenous saliva protein which is being recognised by the polyclonal anti-VP16 antibody. Polyclonal antibodies are by definition a mixture of more than one antibody molecule so multiple epitopes will be recognised by the anti-VP16 polyclonal antibody. The Living Colors®DsRed rabbit polyclonal antibody does not appear to cross react with any saliva proteins.

To ensure equivalence of saliva samples loaded, membranes were stripped of all antibodies using stripping buffer, and re-probed with rabbit anti-Aegyptin polyclonal antiserum to detect endogenous Aegyptin protein in the saliva samples (Figures 4B, 5B, 6B and 7B). Although stripping of the anti-VP16 and anti-DsRed2 antibodies was not complete and faint bands remained, the stripping was sufficient so that these remaining signals did not interfere with the endogenous Aegyptin signal. This analysis revealed that the endogenous Aegyptin signals were visually equivalent across each blot, and therefore demonstrates that equivalent volumes of saliva were loaded in each sample lane.

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

Figure 4

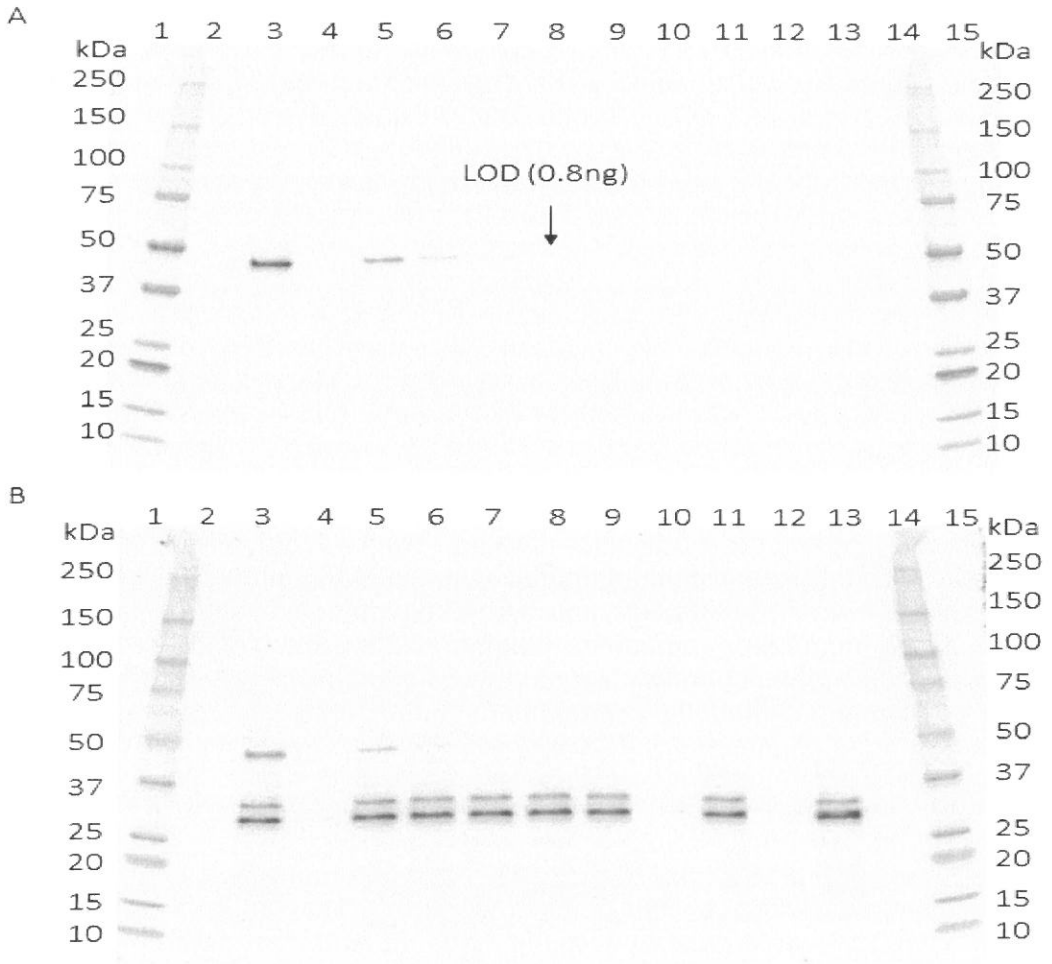

Figure 4: tTAV protein cannot be detected in OX513A saliva using western blot analysis

A 2-fold serial dilution series of rtTAV (mixed with 5µl WT saliva), 5µl of WT saliva and 5µl of OX513A saliva was separated by SDS-PAGE and transferred to a nitrocellulose membrane. (A) The membrane was initially probed with the rabbit anti-VP16 tag polyclonal antibody to detect tTAV protein. (B) The membrane was then stripped of all antibodies and re-probed with rabbit anti-Aegyptin polyclonal antiserum to detect the presence of endogenous Aegyptin protein in the saliva. Lane designations are as follows (Blank lanes contain PBS in 1X Laemmli buffer):

1:	5 µl Mwt marker
2:	Blank lane
3:	12.5ng rtTAV + 5µl WT Saliva
4:	Blank lane
5:	6.2ng rtTAV + 5µl WT Saliva
6:	3.1ng rtTAV + 5µl WT Saliva
7:	1.6ng rtTAV + 5µl WT Saliva
8:	0.8ng rtTAV + 5µl WT Saliva
9:	0.4ng rtTAV + 5µl WT Saliva
10:	Blank lane
11:	5µl WT Saliva
12:	Blank lane
13:	5µl OX513A Saliva
14:	Blank lane
15:	5 µl Mwt Marker

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

Figure 5

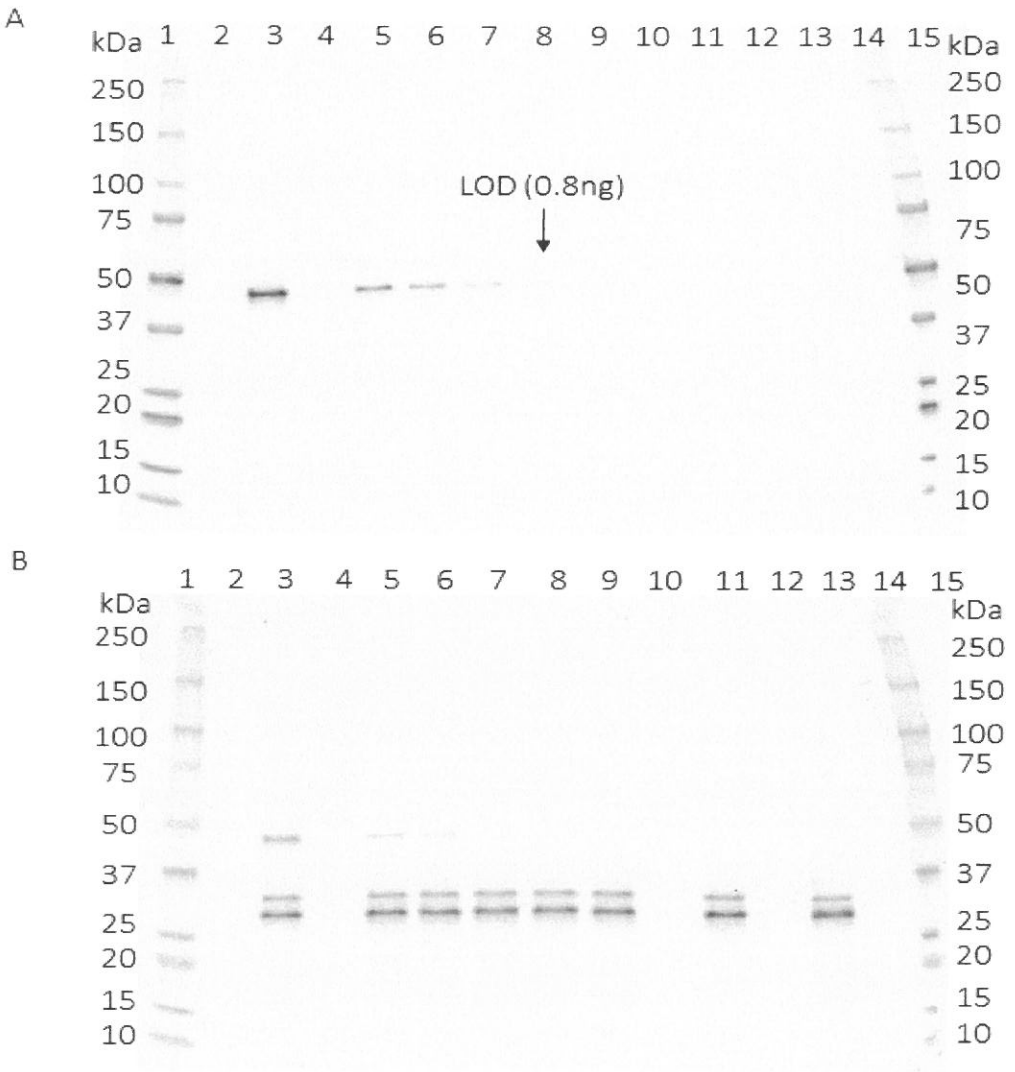

Figure 5: tTAV protein cannot be detected in OX513A saliva using western blot analysis (Replicate)

A 2-fold serial dilution series of rtTAV (mixed with 5µl WT saliva), 5µl of WT saliva and 5µl of OX513A saliva was separated by SDS-PAGE and transferred to a nitrocellulose membrane. (A) The membrane was initially probed with the rabbit anti-VP16 tag polyclonal antibody to detect tTAV protein. (B) The membrane was then stripped of all antibodies and re-probed with rabbit anti-Aegyptin polyclonal antiserum to detect the presence of endogenous Aegyptin protein in the saliva.

1: 5 µl Mwt marker
2: Blank lane
3: 12.5ng rtTAV + 5µl WT Saliva
4: Blank lane
5: 6.2ng rtTAV + 5µl WT Saliva
6: 3.1ng rtTAV + 5µl WT Saliva
7: 1.6ng rtTAV + 5µl WT Saliva
8: 0.8ng rtTAV + 5µl WT Saliva
9: 0.4ng rtTAV + 5µl WT Saliva
10: Blank lane
11: 5µl WT Saliva
12: Blank lane
13: 5µl OX513A Saliva
14: Blank lane
15: 5 µl Mwt Marker

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

Figure 6

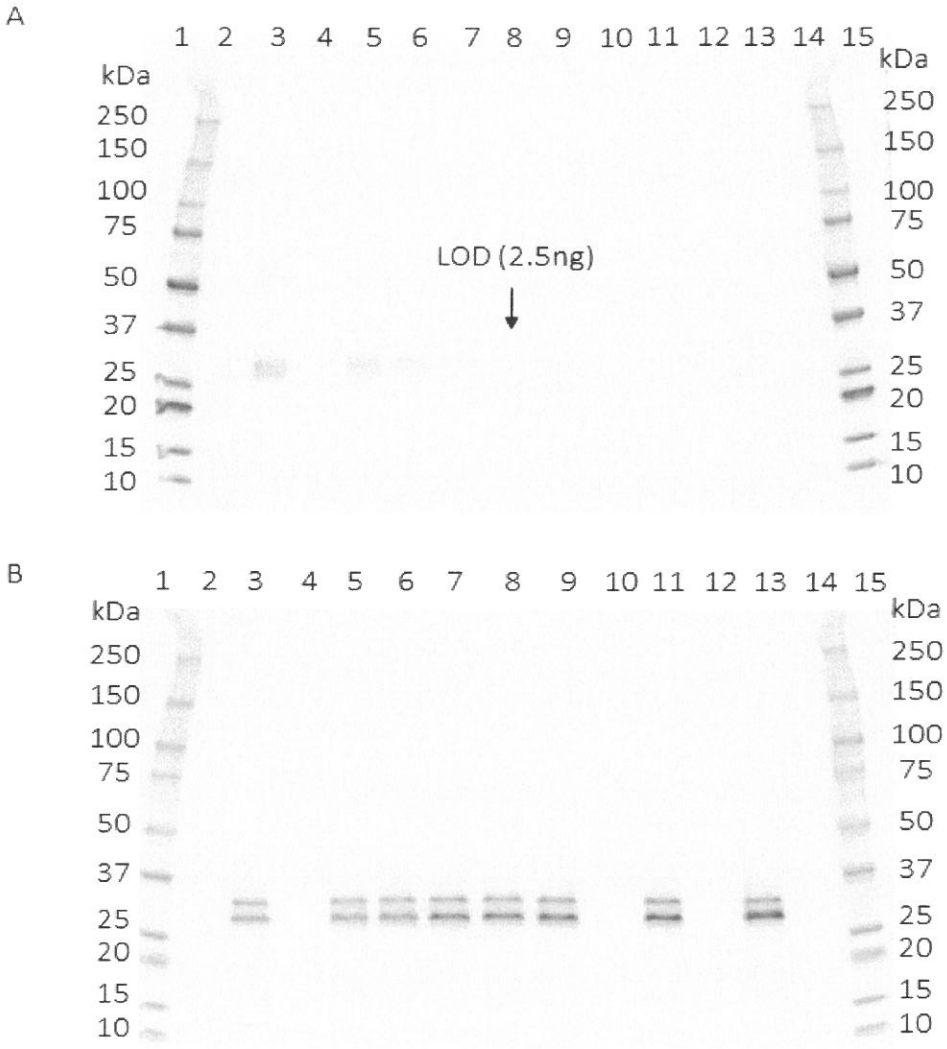

Figure 6: DsRed2 protein cannot be detected in OX513A saliva using western blot analysis

A 2-fold serial dilution series of rDsRed2 (mixed with 5µl WT saliva), 5µl of WT saliva and 5µl of OX513A saliva was separated by SDS-PAGE and transferred to a nitrocellulose membrane. (A) The membrane was initially probed with the Living Colors® DsRed rabbit polyclonal antibody to detect DsRed2 protein. (B) The membrane was then stripped of all antibodies and re-probed with rabbit anti-Aegyptin polyclonal antiserum to detect the presence of endogenous Aegyptin protein in the saliva. Lane designations are as follows (Blank lanes contain PBS in 1X Laemmli buffer):

1:	5 µl Mwt marker
2:	Blank lane
3:	12.5ng rtTAV + 5µl WT Saliva
4:	Blank lane
5:	6.2ng rtTAV + 5µl WT Saliva
6:	3.1ng rtTAV + 5µl WT Saliva
7:	1.6ng rtTAV + 5µl WT Saliva
8:	0.8ng rtTAV + 5µl WT Saliva
9:	0.4ng rtTAV + 5µl WT Saliva
10:	Blank lane
11:	5µl WT Saliva
12:	Blank lane
13:	5µl OX513A Saliva
14:	Blank lane
15:	5 µl Mwt Marker

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

Figure 7

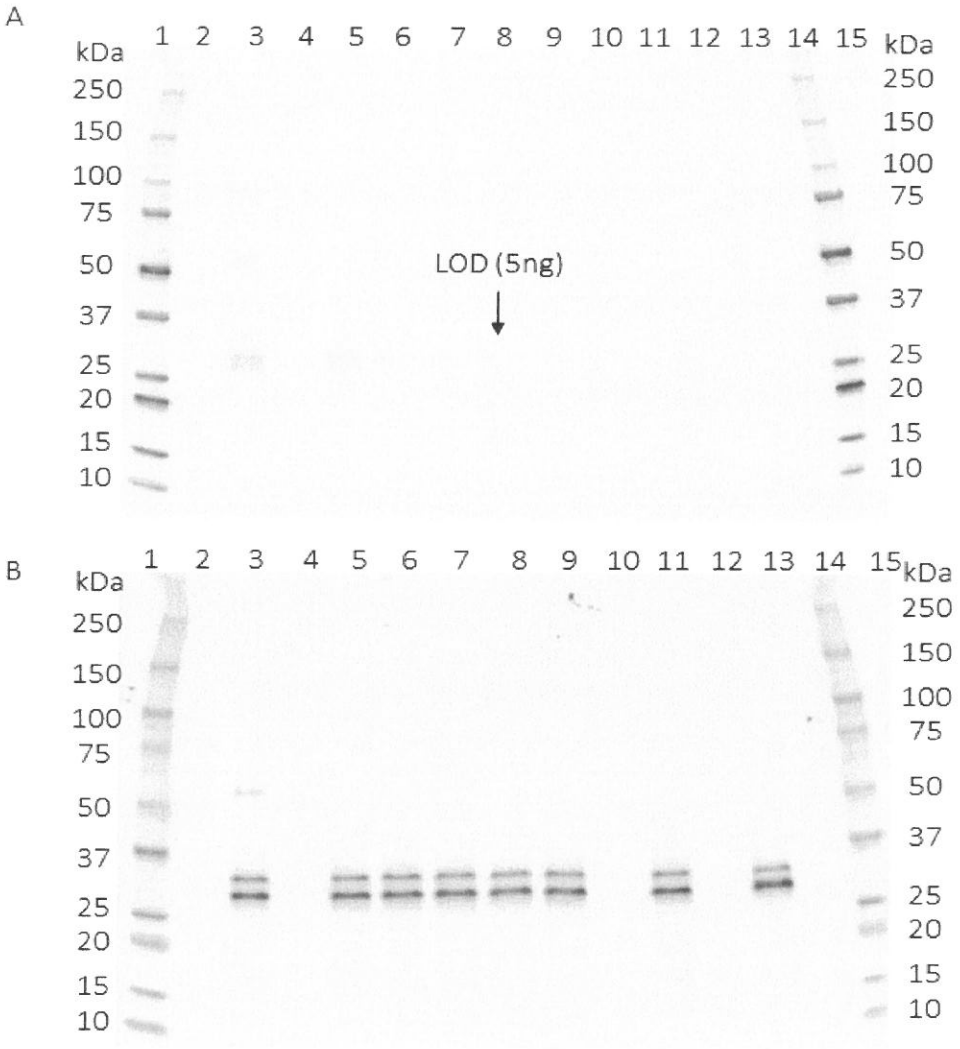

Figure 7: DsRed2 protein cannot be detected in OX513A saliva using western blot analysis (Replicate)

A 2-fold serial dilution series of rDsRed2 (mixed with 5µl WT saliva), 5µl of WT saliva and 5µl of OX513A saliva was separated by SDS-PAGE and transferred to a nitrocellulose membrane. (A) The membrane was initially probed with the Living Colors® DsRed rabbit polyclonal antibody to detect DsRed2 protein. (B) The membrane was then stripped of all antibodies and re-probed with rabbit anti-Aegyptin polyclonal antiserum to detect the presence of endogenous Aegyptin protein in the saliva. Lane designations are as follows (Blank lanes contain PBS in 1X Laemmli buffer):

1:	5 µl Mwt marker
2:	Blank lane
3:	12.5ng rtTAV + 5µl WT Saliva
4:	Blank lane
5:	6.2ng rtTAV + 5µl WT Saliva
6:	3.1ng rtTAV + 5µl WT Saliva
7:	1.6ng rtTAV + 5µl WT Saliva
8:	0.8ng rtTAV + 5µl WT Saliva
9:	0.4ng rtTAV + 5µl WT Saliva
10:	Blank lane
11:	5µl WT Saliva
12:	Blank lane
13:	5µl OX513A Saliva
14:	Blank lane
15:	5 µl Mwt Marker

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

Figure 8

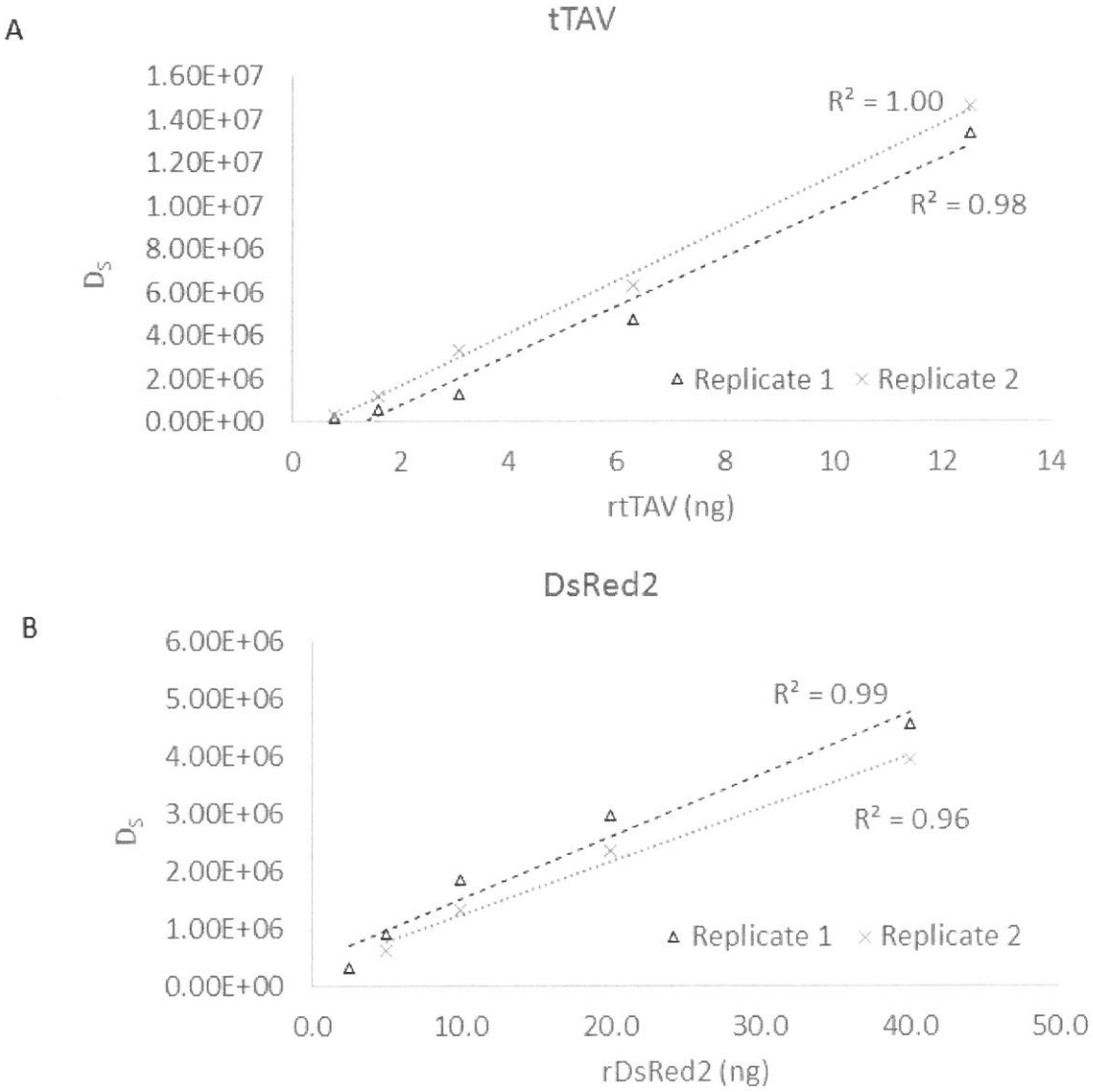

Figure 8: rtTAV and rDsRed2 signals fall within the predictable, linear range for the detection method
D_S values for rtTAV and rDsRed signals from replicate blots for the detection of (A) tTAV and (B) DsRed2 proteins in OX513A saliva were calculated using VisionWorks LS Acquisition and Analysis Software (UVP) and plotted against amount of protein loaded (ng). A linear trend line was plotted, and R^2 values calculated, using Microsoft Excel. Δ represents WT Saliva, X represents OX513A saliva.

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

11 REFERENCE TO LOCATION OF RAW DATA

Raw data for this study will be stored for 5 years and can be found in the following locations;

- Experimental write-up, deviation details and print outs; Laboratory Notebook OX230 pages 30-41
- Raw 16-bit grey-scale images (TIFF files); Oxitec server location S:\G. Regulatory\1.0 USA\FDA\saliva study 2015\Raw data

12 DEVIATIONS

DEV01 Saliva volumes in section 9.6 were incorrectly loaded in ascending order (left to right) instead of descending order as detailed in SP_00002[10]. No action required as this deviation has no impact on the results or analysis.

DEV02 Saliva samples were frozen at ≤ -15°C prior to pooling. Saliva samples were thawed at room temperature and pooled. Protocol SP00002 stated that the saliva samples were to be pooled prior to freezing. This does not affect the analysis or results and so no further action was required.

13 DISCUSSION AND CONCLUSIONS

The data presented in this report shows that tTAV and DsRed2 proteins are not detectable in the saliva of OX513A females by western blot analysis where the limits of detection were determined as 0.8ng for rtTAV and 5.0 - 2.5ng for rDsRed2 in WT saliva. 5 µl of saliva was analysed which equates to the quantity of saliva collected from approximately 5.5 female adult mosquitoes based on this study (270 µl of pooled saliva collected from approximately 300 *Aedes aegypti* adult females homozygous for OX513A rDNA construct).

The equivalence of Aegyptin signal between OX513A and WT saliva was confirmed both visually and by densitometry. Given the subjective nature of the analysis performed, it could be argued that the endogenous Aegyptin signal is marginally stronger in the OX513A saliva compared to WT. If this was the case, OX513A saliva was the test material, and so if there were higher levels of total protein in these samples, it would make it more likely that tTAV and DsRed2 could be detected relative to the WT negative control, and therefore would be unlikely to impact the analysis performed, or the conclusions, drawn from this study.

14 LIST OF ACRONYMS, ABBREVIATIONS AND TECHNICAL TERMS (ALPHABETICAL)

Area	The total number of pixels within the user-defined region 'UDR'.
Background	The area surrounding the perimeter of the user defined-region 'UDR', three pixels wide.
Band	A region of antibody staining clearly visible and discernible by the human eye.
BCA	Bicinchoninic acid.
Blank lane	A lane loaded with PBS in 1X Laemmli loading buffer + 5.5% β-Mercaptoethanol and no protein sample.
D_{BLC}	Density (Blank Lane Control) defined as 'Total Density' for an area of equal size to the 'UDR', in a similar position, in a nearby blank lane representing non-specific binding of antibody to an area where no protein is present.
D_S	Density (Specific) defined as 'Total Density' for the 'UDR' containing a 'band' representing specific antibody binding to the protein of interest
D_{SLC}	Density (Sample Lane Control) defined as 'Total Density' of a region, for an area of equal size to the 'UDR' and situated immediately above or below a 'band' representing antibody

	binding to an area where proteins are likely to be present other than the specific protein of interest.
DsRed2	Fluorescent marker gene from *Discosoma* species.
ECL	Enhanced Chemiluminescense.
kDa	KiloDalton.
LOD	Limit of detection: the lowest quantity of a protein that an operator can visibly discern on a developed western blot image and create a 'UDR' that meets the acceptance criteria such that D_{SLC} is less than 10% of D_S. For the image analysis to be valid, all pixels on the complete western blot image must fall within the dynamic range of the ChemiDoc-IT 500 Imaging System (UVP). This is a pixel intensity within 0 – 65,536 for a 16-bit grey scale image. If this is not the case this image is rejected and an image of a lower exposure time is selected.
MW	Molecular Weight
Mwt Marker	Molecular weight marker.
OX513A saliva	Pooled saliva from homozygous OX513A females.
PBS	Phosphate-buffered saline.
Pixel Intensity	An integer between 0 and 65,536 (for a 16bit grey-scale image) representing the intensity for each pixel
Predictable range	The predictable range of the detection method was be determined by using a linear best fit equation in Microsoft Excel program.
rDsRed2	Recombinant DsRed2.
rtTAV	Recombinant tTAV.
TBS-T	Tris-buffered Saline Tween 20
Total Background	Sum of the pixel intensity values for all pixels within the 'Background'.
Total Density	'Total Raw Density' minus the 'Total Background'.
Total Raw Density	Sum of the pixel intensity values for all pixels within the 'UDR'.
tTAV	Tetracycline-controlled transactivator.
UDR	User defined-region: A rectangle drawn around an antibody specific 'band' or equivalent area by the user, large enough to enclose the entire 'band' or equivalent area.
VP16	*Herpes simplex* Protein 16.
WT saliva	Pooled saliva from wild-type *Aedes aegypti* females.

15 REFERENCES

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

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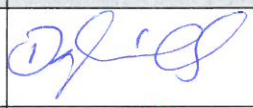
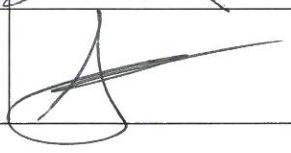
16 APPROVALS

Approval below indicates agreement with information presented in this study report. Raw data has been checked against summary information presented within this report.

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

Appendix L

Appendix L. Larval mosquito surveillance at the Key Haven sewage treatment plant, Monroe County, FL during the 2004-2015 period.

LARVAL MOSQUITO SURVEILLANCE AT THE KEY HAVEN SEWAGE TREATMENT PLANT

MONROE COUNTY, FLORIDA, 2004 – 2015

Florida Keys Mosquito Control District

10th June 2015

Key Haven is a residential development in Monroe County, Florida, near the city of Key West but outside the city limits. Key Haven is located on an island officially known as Raccoon Key, however, everyone refers to the island as "Key Haven". An aerial view of the area is provided below. Key Haven lies to the north of US Route 1 (Overseas Highway). The sewage treatment plant on Key Haven is demarkated by the red circle in the photograph below.

Location of the sewage treatment plant on Key Haven (Raccoon Key).

A closer view of the sewage treatment plant on Key Haven.

Mosquito inspections at Key Haven sewage treatment plant:

According to records maintained by the Florida Keys Mosquito Control District (FKMCD) in the Vector Control Management System (VCMS) database, the first larval inspection at Key Haven sewage treatment plant (STP) was done on 19th February 2004. The latest inspection as of this writing was conducted on 26th May 2015. During that period of 11 years and 3 months, 289 larval inspections were done. Of those inspections only 71 (24.6%) were positive for mosquito larvae. A total of 1,154 larvae was collected; all but one collection were identified as *Culex* sp. or *Culex quinquefasciatus*. The sole exception was a collection made on 7th August 2007 when 60 larvae of *Aedes taeniorhynchus*, the black salt marsh mosquito, were found in a puddle of water on the property of the STP. No *Ae. taeniorhynchus* larvae were collected within the STP itself.

RAW DATA

Survey Date	Dips Taken	No. Larvae	Species	Positive Dips
12/27/2006 15:50	8	1	Cx species	1
8/1/2007 8:50	1	1	Mosquitoes	1
12/26/2007 10:06	5	1	Cx species	1
1/7/2008 8:31	6	1	Cx species	1
2/1/2008 9:53	3	1	Cx species	1
3/18/2008 9:40	5	1	Cx species	1
10/2/2007 11:19	4	2	Cx species	2
12/19/2007 11:39	2	2	Cx species	1
2/11/2008 14:33	6	2	Cx species	1
2/13/2008 8:57	2	2	Cx species	1
3/25/2008 7:30	4	2	Cx species	1
4/1/2008 10:27	4	2	Cx species	1
4/29/2008 7:57	8	2	Cx species	1
4/29/2008 13:53	3	2	Cx species	1
8/21/2007 14:04	2	3	Cx species	2
9/25/2007 12:15	1	3	Cx species	1
2/26/2008 11:29	8	3	Cx species	2
10/16/2007 10:26	1	4	Cx species	1
10/22/2007 9:18	3	4	Cx species	2
2/22/2008 10:39	2	4	Cx species	2
12/20/2006 14:14	6	5	Cx species	1
8/14/2007 10:21	3	5	Cx species	1
3/17/2008 11:18	8	5	Cx species	2
5/13/2008 15:24	6	5	Cx species	4
8/30/2006 14:48	4	6	Cx species	3
10/23/2007 11:48	5	6	Cx species	2
12/26/2007 10:25	2	6	Cx species	1
2/19/2008 11:28	4	6	Cx species	3
4/15/2008 9:31	8	7	Cx species	2
4/14/2005 13:40	1	8	Cx species	1
8/28/2007 11:46	3	8	Cx species	1
10/30/2007 9:33	5	8	Cx species	3
1/22/2008 11:22	3	8	Cx species	1
3/17/2005 10:09	3	10	Cx quinquefasciatus	2
3/20/2007 15:18	3	10	Cx species	1
3/20/2007 15:19	3	10	Cx species	1
7/23/2007 16:05	2	10	Cx species	2
8/7/2007 11:48	1	10	Cx species	1
9/25/2007 11:57	2	10	Cx species	2
3/25/2008 14:39	2	10	Cx species	1
2/19/2004 10:00	1	12	Cx quinquefasciatus	1
10/16/2007 10:51	1	12	Cx species	1
1/18/2008 11:43	15	12	Cx species	2

7/10/2007 15:43	1	15	Cx species	1
7/17/2007 15:04	10	15	Cx species	5
1/29/2008 11:45	6	15	Cx species	3
2/5/2008 13:58	4	15	Cx species	3
2/11/2008 15:09	2	15	Cx species	1
9/18/2007 9:42	3	16	Cx species	2
3/27/2012 10:35	2	18	Cx quinquefasciatus	2
2/16/2006 14:37	2	20	Cx quinquefasciatus	2
3/2/2006 13:43	1	20	Cx species	1
10/19/2006 10:22	1	20	Cx species	1
8/7/2007 11:44	2	20	Cx species	2
8/14/2007 10:04	2	20	Cx species	2
11/13/2007 9:47	3	20	Cx species	3
2/5/2008 10:09	4	20	Cx species	4
3/11/2008 11:36	3	20	Cx species	2
2/4/2015 10:30	1	20	Cx species	1
9/24/2004 10:05	1	30	Cx quinquefasciatus	1
12/10/2007 8:44	1	33	Cx species	1
12/4/2007 11:41	4	35	Cx species	3
1/15/2008 8:02	2	35	Cx species	2
3/24/2005 9:19	3	50	Cx quinquefasciatus	2
5/12/2005 11:33	1	50	Cx quinquefasciatus	1
12/5/2006 11:37	1	50	Cx species	1
4/29/2015 11:23	2	50	Cx species	2
8/7/2007 11:49	5	60	Oc taeniorhynchus	5
1/15/2008 8:33	2	60	Cx species	2
1/7/2008 7:54	4	80	Cx species	3
10/3/2006 11:32	3	100	Cx species	1
3/27/2012 10:35	2	18	Cx quinquefasciatus	2
2/4/2015 10:30	1	20	Cx species	1
4/29/2015 11:23	2	50	Cx species	2

Appendix M

Appendix M. Expert opinion on composition of mosquito saliva and introduction into human host during biting.

What is the total volume of saliva in the salivary gland of a mature adult Ae. aegypti female?

The volume of injected mosquito saliva is problematic to calculate, as the salivation is processed by an active flow of water through the cells stimulated during feeding by serotonin [1]. Perhaps more relevant is to aim at the total amount of protein in the salivary gland before and after the blood meal.

Saliva of mosquitoes have a very high activity of the apyrase enzyme which hydrolyses ATP and ADP to AMP and orthophosphate, thus inactivating the platelet and neutrophil-aggregating properties of these nucleotides, which are released upon tissue injury [2-4]. Apyrase activity thus helps mosquitoes to feed and is a good marker of mosquito saliva. Oil-induced mosquito saliva leads to less than one microliter collections per mosquito. However, on average, saliva collected from 5 mosquitoes contain the same apyrase amount as one pair of adult female salivary glands [5].

Aedes adult female mosquito has near 3 ug of salivary protein, and about half this amount is lost during the blood meal [6]. However, mosquitoes reingest saliva while feeding, and about 25% of the salivary apyrase activity is recovered in the mosquito gut after a blood meal. In short, mosquitoes lose ~1.5 ug of salivary protein during the blood meal, ~0.75 is reingested, and ~0.75, or on the order of 1 ug stays in the host.

What is the volume of saliva injected into a human per bite?

See above.

What is the protein composition of saliva in reference to what proportion of salivary proteins could be estimated to be represented by these two engineered proteins IF they were secreted into saliva?

Aedes saliva contains near 100 polypeptides in several protein families [7] [4, 8]. Some of the most abundantly secreted include the aegyptin and members of the D7 family, in addition of apyrase and antigen-5 members [9]. If all proteins were expressed equally, they would have the same amount in the injected saliva of one mosquito, and thus would be represented at ~10 ng. Of course there is a large variation in the degree of expression, the most expressed being no more than 30% of the total protein in saliva, or 300 ng, with less than one ng for the least expressed.

What is the total protein concentration per unit volume of saliva and the fraction of each major group of proteins as a reference to estimate the fraction that the engineered proteins may represent given the LOD available from Western blotting.

As indicated above, there is a large variation in the degree of expression, the most expressed being no more than 30% of the total protein in saliva (aegyptin, also known as the 30 KDa antigen), or 300 ng, with less than one ng for the least expressed.

Considering the WB protocol that was submitted, the authors used oil induced saliva of 5 mosquitoes as their unit of detection. This corresponds to about one pair of mosquito salivary glands, and about 4 times the amount injected into a host during probing and feeding of the mosquito. Their limit of detection for the recombinant proteins (TAV ~ 0.8 ng and DsRed2 ~ 2.5-5 ng) are in line with the lowest amounts of salivary proteins injected by a mosquito. We can conclude by the report that if secreted in

saliva, the recombinant proteins are in at least equal, or less abundance than the smallest fractions of mosquito saliva. Notice that the known mosquito allergens [10-17] are represented by the abundantly expressed proteins, that would be on the range of dozens or hundreds of nanograms injected per mosquito bite.

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