Mosquito Control Measures in place at Florida Keys Mosquito Control District

The Florida Keys Mosquito Control District (FKMCD) utilizes integrated mosquito management practices, which involve a variety of methods to control mosquitoes including adulticides, larvicides, source reduction, and biological controls. The primary method of control of the *Aedes aegypti* mosquito is source reduction. Currently, FKMCD has twenty-three domestic inspectors throughout the Florida Keys whose primary responsibility is to find and eliminate domestic breeding habitats. *Gambusia rhizophorae*, also known as the mosquito fish, is also used quite frequently in a variety of habitats. Fish were introduced over 650 times in Key West in 2010; some of the habitats of these introductions include, but are not limited to, abandoned swimming pools and Jacuzzis, cisterns, ponds, fountains, wells, and drains. These fish are an excellent means of biological control for mosquito larvae and are found locally throughout the Keys.

When source reduction or biological control introductions are not feasible in the control of *Aedes aegypti* larvae, FKMCD employs a wide-array of larvicides. These larvicides include *Bacillus thuringiensis israelensis (Bti)*, *Bacillus sphaericus (Bs)*, methoprene, Spinosad, or an oil dispersant such as Bonide or Golden Bear. Larvicides are applied on a daily basis by FKMCD inspectors. The larvicide utilized is largely dependent upon the species, instar, and container size and type in which the mosquito larvae are found. These products are rotated to avoid prolonged exposure of mosquito larvae to a particular larvicide's mode of action. Standard treatment of larval *Aedes aegypti* is *Bti* if the larvae are 1st through 3rd instar. Approximately 2,000 treatments have been made with *Bti* to containers in Key West in 2010. Likewise, standard treatment for *Culex* larvae is *Bs,* again if larvae are in 1st through 3rd stages. Approximately 1,500 treatments with *Bs* were made in 2010. Spinosad was recently introduced into FKMCD's larvicide arsenal in the summer of 2010. This is a new product recently available to rotate into the treatment of any larval species, 1st through 3rd stages. There were approximately 1,000 treatments with Spinosad products in the Key West area in 2010. The main advantage to these treatments is the residual nature of the products, which ranges from 60 to 180 days, depending upon the formulation. There have been approximately 10,000 applications of methoprene in 2010. This product is used as a pretreatment to containers which are not currently holding water but will with an ensuing rainfall. Also, methoprene is generally used in treatment of early instar *Aedes aegypti* larvae as well as a rotational product for standard treatment of all larval stages. It is used in conjunction with oil dispersant for the treatment of pupae as well.

The main delivery method of these larvicides is by hand; however, backpack sprayers and helicopters can also be utilized to treat larger areas. Backpack sprayers are employed in the treatment of tire piles and large groups of breeding containers with temephos. Temephos is an

organophosphate larvicide used for control of *Aedes aegypti* larvae. In 2010, only three such treatments were made on the island of Key West. These backpack sprayers are also used in instances when inspectors do not have access to properties. Under these circumstances, *Bti* is sprayed over fences to treat any possible *Aedes aegypti* larval habitats on the inaccessible property. Treatments such as this occur in situations where mosquito numbers are high and the inspector cannot find larval habitats on the surrounding properties. This method of treatment was used just over 100 times in 2010.

In 2010, FKMCD began the use of aerial larviciding over residential areas for the control of *Aedes aegypti*. This was made possible by the development of a wettable powder form of *Bti* by Valent BioSciences. A total of five treatments have been made in the Key West area, including four trial applications. This method of *Aedes aegypti* control will be used frequently in the upcoming year, with applications occurring at least every two weeks. This delivery of *Bti* via helicopter has increased FKMCD's treatment coverage to include all areas which were previously inaccessible and looks to be a new means which will allow FKMCD to treat large areas in a timelier manner.

Larval control is by far the most efficient means of *Aedes aegypti* control; however, FKMCD also uses adult control methods when population numbers are high and disease is present. Adult control of *Aedes aegypti* is extremely difficult due to the behavior of the species; therefore, adulticide treatments are not regularly employed. The most common and effective treatment for adult *Aedes aegypti* is the use of handheld ULV sprayers. Prior to 2010, permethrin was the insecticide used in these treatments; however, due to the beginning stages of resistance development appearing throughout the local *Aedes aegypti* population, chlorpyrifos has been used. Just over 600 handheld ULV treatments were completed upon residential request and when adult mosquito presence was detected by inspectors, and these treatments can occur between the hours of 0800 and 1700.

When mosquitoes are concentrated in small areas, the District will deploy pick-up trucks with mounted ULV spray systems. Ground treatments are done in the evenings beginning approximately one hour before sunset. Trucks are dispatched to those neighborhoods with high numbers of biting mosquitoes allowing area-specific treatments to control problem populations. Permethrin is the insecticide used in the truck ULV systems. In 2009, a total of 128 truck ULV missions were completed in Key West; in 2010, 51 truck ULV missions were completed. Truck missions are not very effective in the control of *Aedes aegypti* due to the adult mosquito behavior; however, the majority of truck ULV missions on Key West in 2010 were conducted for the control of the salt marsh mosquito, *Aedes taeniorhynchus* and *Culex quinquefasciatus*.

In instances when mosquito populations are high over large areas FKMCD will spray for adult mosquitoes aerially. This is done with either a Bell 206B Jet Ranger helicopter or one of the District's two Briton Norman Islanders. These aircraft are equipped with rotary atomizer type ULV nozzles. FKMCD uses either Naled or permethrin to control adult mosquitoes with our aerial program. When locally-acquired dengue cases began surfacing in September of 2009, FKMCD began aerially treating Key West with permethrin every other morning. In total, 26 missions were completed over the island of Key West in 2009 in an attempt to combat the spread of dengue fever. In 2010, after evaluating the overall effectiveness of these aerial missions, FKMCD has completed 9 aerial missions, all using Naled. Aerial adulticide missions are conducted usually between the hours of 0700 and 0900.

FKMCD has been very diligent in assessing the effectiveness of all materials and methods used in the control of *Aedes aegypti*. Chemical resistance studies are ongoing for a number of insecticides on the local population and will be completed within the next few months. While it is important to continue the use of adulticide products to aid in the control of *Aedes aegypti*, the most effective means of control is source reduction and larviciding which is FKMCD's main emphasis.

Ruj. Kami   JBK(S) 602-1/1/3 (30)
Tarikh   20 Oktober 2010

SERAHAN TANGAN

Pengarah
Institut Penyelidikan Perubatan
Kementerian Kesihatan Malaysia
Jalan Pahang
50588 Kuala Lumpur

Tuan,

PERMOHONAN KELULUSAN UNTUK MENJALANKAN KAJIAN LAPANGAN UNTUK PROJEK "LIMITED-MARK-RELEASE-RECAPTURE OF AEDES AEGYPTI (L.) WILD TYPE AND OX513A(My1) STRAINS"

Dengan segala hormatnya saya diarah merujuk kepada perkara di atas.

2.   Dimaklumkan bahawa permohonan tuan untuk menjalankan kajian lapangan bagi Projek "Limited-Mark-Release-Recapture of Aedes aegypti (L.) Wild Type and OX513A(My1) Strains" telah diberi kelulusan dengan terma dan syarat seperti pada Sijil Kelulusan yang dilampirkan.

3.   Di samping itu, pihak tuan adalah dinasihatkan supaya memberi perhatian sewajarnya terhadap isu-isu yang dikenal pasti di **Lampiran A** sekiranya berhasrat untuk membuat pelepasan secara besar-besaran atau komersial pada masa hadapan.

Sekian, terima kasih.

"BERKHIDMAT UNTUK NEGARA"

Saya yang menurut perintah,

(LETCHUMANAN RAMATHA)
Ketua Pengarah Biokeselamatan
Jabatan Biokeselamatan
Malaysia

s.k.   Ketua Setiausaha
        Kementerian Sumber Asli dan Alam Sekitar

IDENTIFICATION OF ISSUES TO BE ADDRESSED FOR FUTURE RELEASES

Some additional issues have been identified that would be important during the assessment of an application for a larger scale or commercial release of OX513A(My1) mosquitoes. These include:

a) Risk: Release of OX513A(My1) mosquitoes may cause other pests to become more serious

The current proposed release involves a relatively small number of OX513A(My1) mosquitoes and the duration of the field trial is short. However, for a larger release, this risk should be considered more thoroughly.

b) Risk: Increase in the population of another mosquito species due to suppression of the target mosquito

Proper baseline data on, and close monitoring of, any change in the populations of *A. aegypti* and other mosquito species are required as a release of a larger number of mosquitoes is likely to affect these populations.

c) Integrated Pest Management (IPM) programme

For a large scale release to control the population of *A. aegypti,* an IPM programme should be in place to further augment the technology.

d) Stability of the transgenes in the field

Molecular information has to be obtained on the stability of the transgenes through multiple generations in the field. This includes the independent stability of expression of the tTAV gene with or without expression of the DsRed marker gene and the stability of the transgene cassette.

e) Behaviour of OX513A(My1) in the field

The behaviour (e.g. mating, biting, etc.) of OX513A(My1) in the field has to be assessed in comparison to its behavior in containment.

f) Sorting error

The effectiveness in screening and elimination of female mosquitoes should be ensured even when larger numbers are involved, especially since human error could then adversely affect the process.

LEMBAGA BIOKESELAMATAN KEBANGSAAN

SIJIL KELULUSAN

(subseksyen 16(3) Akta Biokeselamatan 2007)

Dengan ini disahkan bahawa permohonan kelulusan

**INSTITUT PENYELIDIKAN PERUBATAN,
KEMENTERIAN KESIHATAN MALAYSIA**

yang beralamat di

JALAN PAHANG, 50588 KUALA LUMPUR

untuk

**PROJEK "LIMITED MARK-RELEASE-RECAPTURE OF
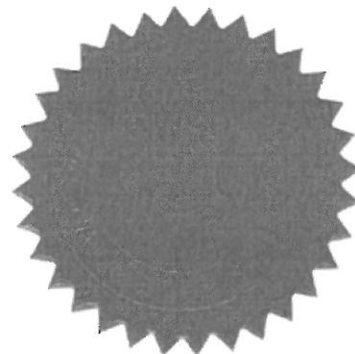AEDES AEGYPTI (L.) WILD TYPE AND OX513A(My1)STRAINS"**

telah diluluskan oleh Lembaga Biokeselamatan Kebangsaan pada

5 OKTOBER 2010

mengikut peruntukan subseksyen 16(3) Akta Biokeselamatan 2007. Sijil kelulusan ini adalah tertakluk kepada terma dan syarat seperti di **LAMPIRAN 1**.

Pengerusi
Lembaga Biokeselamatan Kebangsaan

TERMS AND CONDITIONS FOR CERTIFICATE OF APPROVAL

FOR LIMITED MARK-RELEASE-RECAPTURE OF AEDES AEGYPTI (L.) WILD TYPE AND OX513A STRAINS

Part A

Information and/or documentation that should be submitted to NBB at least two weeks prior to the start of field trials

a)   Documentation from District Council/Majlis Daerah or relevant authorities on the presence or otherwise of aquaculture, poultry and pharmaceutical industries within a vicinity of 500 meters of the release sites, and information on whether any of these industries regularly use tetracycline in their operations.

b)   Confirmation from the relevant health authorities that the sites selected has been free from any dengue outbreak for at least 3 months before the start of the field trial.

c)   Detailed information on the positioning of the ovitraps and BG-Sentinel traps (documentation on setting-up of traps, including GPS information and photographs has been proposed by the applicant). Proper cautionary measures should be taken to ensure that that traps are positioned at suitable locations/heights for effective trappings.

d)   A consent letter should be provided from the Local Council for the district/s where the release sites are located for the proposed MRR field trial.

e)   Public Notification and Consensus - It is mandatory that the applicant through a public forum obtains prior consensus and approval from the inhabitants in the release sites regarding the proposed MRR field trial.

Part B

Actions to be taken and reported to NBB during /after the field trial

a)   All proposed activities and methods submitted in the dossier and agreed upon through other means of communication with the applicant should be appropriately and responsibly adhered to.

b)   Sex sorting must be carried out in compliance with the SOP submitted (SOP for Sex Sorting of Aedes aegypti Mosquitoes). Additionally, all OX513A(My1) mosquitoes for release must be checked and not merely a 'quality control sample'.

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c)   All extra insects/ recaptured insects are to be transported in shatter-proof double-covered containers for subsequent identification, analytical studies or appropriate disposal (according to SOP at IMR.)

d)   At the end of the field trial, fogging for a 400m radius is required according to the Ministry of Health's guidelines and a clean-up operation (*gotong-royong*) should be conducted to eradicate all breeding grounds. A second fogging should be conducted one week after the end-of-field-trial fogging.

e)   At the end of the field trial (first fogging), the applicant is required to continue monitoring for another month to ensure no residual OX513A strains are left behind. The traps should be checked on a daily basis. During this additional one month monitoring period, fogging should be done if any residual OX513A(My1) is detected.

f)   Upon completion of the open field trial, a comprehensive report should be submitted to the National Biosafety Board within two months from the end of the trial.

3

getação e estufa acoplada (332,8 m² de área total) no Centro de Tecnologia Du Pont - Paulínia, localizado em Paulínia/SP, para desenvolver atividades de pesquisa em regime de contenção com plantas geneticamente modificadas pertencentes à classe de risco I. Após análise das medidas de biossegurança descritas na solicitação, a CTNBio entendeu que os OGMs e derivados devem ser utilizados na unidade operativa apenas para os fins propostos. Assim sendo e atendidas as recomendações da CTNBio e as medidas de biossegurança contidas no processo, essa atividade não é potencialmente causadora de significativa degradação do meio ambiente ou prejudicial à saúde humana.

No âmbito das competências do art. 14 da Lei 11.105/05, a CTNBio considerou que as medidas de biossegurança contidas no processo e neste parecer técnico atendem às normas e à legislação pertinente que visam garantir a biossegurança do meio ambiente, agricultura, saúde humana e animal.

A CTNBio esclarece que este extrato não exime a requerente do cumprimento das demais legislações vigentes no país, aplicáveis ao objeto do requerimento.

A íntegra deste Parecer Técnico consta do processo arquivado na CTNBio. Informações complementares deverão ser solicitadas por escrito à Secretaria Executiva da CTNBio.

EDILSON PAIVA

EXTRATO DE PARECER TÉCNICO Nº 2.764/2010

O Presidente da Comissão Técnica Nacional de Biossegurança - CTNBio, no uso de suas atribuições e de acordo com o Artigo 14, inciso XIX, da Lei 11.105/05 e do Art. 5º, inciso XIX do Decreto 5.591/05, torna público que na 139ª Reunião Ordinária, ocorrida em 16 de dezembro de 2010, a CTNBio apreciou e emitiu parecer técnico para o seguinte processo:

    Processo nº: 01200.000987/2010-59
    Requerente: Monsanto do Brasil Ltda
    CNPJ: 64.858.525/0001-45
    Endereço: Av. Nações Unidas, 12901, Torre Norte, 7º e 8º Andares, 04578-910, São Paulo, SP
    Assunto: Liberação Comercial de milho geneticamente modificado resistente a insetos e tolerante ao glifosato MON88017 e suas progênies.

A CTNBio, após apreciação do pedido de Parecer Técnico para a liberação comercial de milho geneticamente modificado resistente a insetos e tolerante ao glifosato MON88017, bem como de quaisquer progênies dele derivados, concluiu pelo seu DEFERIMENTO nos termos deste parecer técnico.

A Monsanto do Brasil Ltda, detentora do Certificado de Qualidade em Biossegurança - CQB 03/97, solicitou à CTNBio Parecer Técnico a biossegurança do milho geneticamente modificado resistente a insetos e tolerante ao glifosato MON 88017, para efeito de sua liberação no meio ambiente, seu uso comercial e quaisquer outras atividades relacionadas a esse OGM e quaisquer progênies dele derivados. O milho MON 88017 (evento 88017) foi produzido por transformação de embriões imaturos de milho A x Hi-II mediada por Agrobacterium. A análise molecular mostrou que o milho MON 88017 contém um inserto único e intacto contendo dois cassetes de expressão, isto é, a sequência codificadora do gene cp4 epsps e a sequência codificadora do gene cry3Bb1, respectivamente. O número de insertos foi determinado mostrando que o milho MON 88017 contém uma cópia do DNA do cassete de expressão (cry3Bb1 e cp4 epsps) introduzido em um único locus de integração no genoma do milho com todos os elementos genéticos intactos. As análises de segregação em 10 gerações confirmaram a herdabilidade e a estabilidade dos genes cp4 epsps e cry3Bb1 no milho MON 88017. Os produtos da expressão dos genes cry3Bb1 e cp4 epsps inseridos no milho MON 88017 são as proteínas Cry3Bb1 e CP4 EPSPS, respectivamente. A proteína CP4 EPSPS é responsável pela característica de tolerância ao herbicida glifosato, enquanto que a proteína Cry3Bb1 é responsável pela característica de resistência a larvas coleópteros praga do gênero Diabrotica. O organismo doador do gene cry3Bb1 é o Bacillus thuringiensis, bactéria gram-positiva, formadora de esporos, enquanto que o doador do gene cp4 epsps é a bactéria Agrobacterium sp. Cepa CP4, que produz uma proteína EPSPS naturalmente tolerante ao glifosato. Os níveis de expressão das proteínas Cry3Bb1 e CP4 EPSPS em vários tecidos do milho MON 88017 foram avaliados a partir do cultivo do milho MON 88017 e do milho convencional plantados nos Estados Unidos e no Brasil. Os dados de expressão das proteínas Cry3Bb1 e CP4 EPSPS no milho MON 88017 no Brasil e nos Estados Unidos mostraram valores que promovem eficácia de controle de larvas de coleópteros praga do gênero Diabrotica e de tolerância ao glifosato neste milho geneticamente modificado. Nenhum efeito pleiotrópico foi observado no milho MON 88017 durante os experimentos de campo realizados em diferentes países e também a partir do seu uso comercial. Até o momento não existem exemplos de efeitos interativos entre as proteínas Cry, como a Cry3Bb1, e a proteína CP4 EPSPS. Do ponto de vista de avaliação da segurança alimentar do milho MON 88017 e das proteínas Cry3Bb1 e CP4 EPSPS nele expressas, estudos na composição química e nutricional, a segurança das proteínas Cry3Bb1 e CP4 EPSPS presentes na dieta, em alimentos e rações derivados do milho MON 88017, o potencial de toxicidade e de alergenicidade das proteínas Cry3Bb1 e CP4 EPSPS foram avaliados quanto aos riscos para humanos e animais. Em termos de segurança alimentar, o milho MON 88017 mostrou-se substancialmente semelhante ao milho convencional, assim como a outros híbridos comerciais. As proteínas Cry3Bb1 e CP4 EPSPS foram avaliadas quanto ao seu potencial de toxicidade a humanos e animais. Nos estudos de toxicidade oral aguda com camundongos foi demonstrado que as proteínas Cry3Bb1 e CP4 EPSPS não apresentam toxicidade aguda e não causam nenhum efeito adverso. As análises de bioinformática demonstraram

que as proteínas Cry3Bb1 e CP4 EPSPS não compartilham de similaridades estruturais e de sequência com toxinas conhecidas ou proteínas biologicamente ativas que causam efeitos adversos à saúde humana e animal. As proteínas Cry3Bb1 e CP4 EPSPS são oriundas de fontes não alergênicas, não possuem similaridade com alérgenos conhecidos e são rapidamente digeridas em fluido gástrico simulado, além de constituírem uma porção muito pequena da proteína presente nos grãos do milho MON 88017. Em conjunto, estes dados permitem concluir que as proteínas Cry3Bb1 e CP4 EPSPS apresentam probabilidade desprezível de causar alergias e que o milho MON 88017 é tão seguro quanto o milho convencional com respeito ao risco de alergenicidade. Do ponto de vista de segurança ambiental foram realizados estudos com o milho MON 88017, respectivamente, nos Estados Unidos e no Brasil. Os estudos incluíram avaliações das características agronômicas fenotípicas no campo, avaliações fenotípicas e ecológicas, interações ecológicas, potencial de fluxo gênico e coexistência e avaliação potencial como planta daninha. As avaliações das características agronômicas fenotípicas no campo em experimentos realizados nos Estados Unidos e no Brasil mostraram que o milho MON 88017 e o milho controle são equivalentes e têm o mesmo comportamento no meio ambiente. A avaliação das interações ecológicas em experimentos realizados nos Estados Unidos e no Brasil com base no monitoramento de insetos específicos, doenças e estresses abióticos não mostrou diferenças na suscetibilidade a pragas ou estresses ambientais. Os dados fenotípicos e ecológicos indicaram que o milho MON 88017 não confere nenhuma vantagem seletiva ao milho, sendo tão seguro quanto o milho controle convencional. Uma avaliação do potencial de fluxo gênico indicou que o milho MON 88017 é similar ao milho convencional. As características agronômicas e fenotípicas do milho MON 88017 foram avaliadas em relação potencial como planta daninha e os dados coletados permitem concluir que o milho MON 88017 não apresenta risco de se tornar uma planta daninha ou de gerar impacto ecológico significativo quando comparado ao milho convencional. Além dos dados fornecidos pela requerente, a CTNBio consultou literatura científica independente para avaliar a segurança alimentar e ambiental e a ocorrência de algum efeito independente este evento de transformação. A CTNBio determina que o monitoramento pós-liberação comercial do milho MON 88017 deve seguir os parâmetros aprovados na 135ª reunião Ordinária ocorrida em 19 de agosto de 2010.

No âmbito das competências que lhe são atribuídas pelo Art. 14 da Lei 11.105/05, a CTNBio considerou que o pedido atende às normas e legislação vigentes que visam garantir a biossegurança do meio ambiente, agricultura, saúde humana e animal e concluiu que o milho MON 88017 é substancialmente equivalente ao milho convencional, sendo seu consumo seguro para a saúde humana e animal. No tocante ao meio ambiente, concluiu a CTNBio que o cultivo do milho MON 88017 não é potencialmente causador de significativa degradação do meio ambiente, guardando com a biota relação idêntica ao milho convencional.

A íntegra deste Parecer Técnico consta do processo arquivado na CTNBio. Informações complementares ou solicitações de maiores informações sobre o processo acima listado deverão ser encaminhadas por escrito à Secretaria Executiva da CTNBio.

EDILSON PAIVA

EXTRATO DE PARECER TÉCNICO Nº 2.765/2010

O Presidente da Comissão Técnica Nacional de Biossegurança - CTNBio, no uso de suas atribuições e de acordo com o artigo 14, inciso XIX, da Lei 11.105/05 e do Art. 5º, inciso XIX do Decreto 5.591/05, torna público que na 139ª Reunião Ordinária, ocorrida em 16 de dezembro de 2010 de , a CTNBio apreciou e emitiu parecer técnico para o seguinte processo:

    Processo nº: 01200.002644/2010-29
    Requerente: Instituto de Ciências Biomédicas da Universidade de São Paulo- USP.
    CQB: 0046/98
    Endereço: Avenida Prof. Lineu Prestes, 2415 - Cidade Universitária. São Paulo - SP. CEP: 05508-900 Fones: (11) 3091-7350 Fax: (11) 3091-7420
    Assunto: Solicitação de parecer para liberação planejada de insetos geneticamente modificados no meio ambiente.
    Extrato Prévio: 2455/2010, Publicado no D.O.U No. 139, 22 de julho de 2010.
    Decisão: DEFERIDO
    RESUMO: A CTNBio, após apreciação da solicitação de Parecer Técnico para liberação planejada de insetos geneticamente modificados no meio ambiente, conclui pelo deferimento nos termos deste parecer técnico. O presidente da CIBio da Instituto de Ciências Biomédicas da Universidade de São Paulo, Prof. Dr. João Gustavo Perssini Amarante Mendes, solicita à CTNBio parecer técnico para condução de experimento de liberação planejada de insetos geneticamente modificados no meio ambiente do classe II de risco biológico. Os insetos serão liberados em cinco áreas distintas, sob responsabilidade da Profa. Dra. Margareth de Lara Capurro-Guimarães. Os insetos a serem liberados são mosquitos da espécie Aedes aegypti, linhagem OX513A com a expressão de uma proteína fluorescente que atua na superfamília GFP, fator ativador de transcrição tetraciclina-repressível (tTA). Os mosquitos transformados terão duas novas atividades biológicas, que são: fluorescência e letalidade repressível. Os insetos são provenientes da empresa Oxitec- Oxford Insect Tecnologies (Oxford, Inglaterra) e mantidos e multiplicados pela Organização Social Biofábrica Moscamed Brasil detentora do CQB 312/10. Estes insetos servirão de modelos biológicos para controle de vetores de doenças que afetem humanos, não apresentando qualquer vantagem seletiva adaptativa em relação aos parentais não geneticamente modificados. O proponente solicita que informações por ele indicadas sejam consideradas sigilosas pela CTNBio. O pesquisador responsável declara que a equipe técnica que conduzirá o experimento dispõe de infraestrutura adequada e qualificação técnica para gerir o risco associado à atividade proposta. No âmbito das competências conferidas pela Lei 11.105/05, e regulamentadas pelo Decreto 5.591/2005, a Comissão considerou que os protocolos experimentais e as demais medidas de biossegurança propostas atendem às normas da CTNBio e à legislação pertinente que visam garantir a biossegurança do meio ambiente, agricultura, saúde humana e animal.

A CTNBio esclarece que este extrato não exime a requerente do cumprimento das demais legislações vigentes no país, aplicáveis ao objeto do requerimento.

A íntegra deste Parecer Técnico consta do processo arquivado na CTNBio. Informações complementares ou solicitações de maiores informações sobre o processo acima listado deverão ser encaminhadas por escrito à Secretaria Executiva da CTNBio.

EDILSON PAIVA

DESPACHO DO PRESIDENTE
Em 16 de dezembro de 2010

O Presidente da Comissão Técnica Nacional de Biossegurança - CTNBio, no uso de suas atribuições e de acordo com o artigo 14, inciso XIX, da Lei 11.105/05 e do Art. 5º, inciso XIX do Decreto 5.591/05, torna público, após decisão ocorrida na 139ª Reunião Ordinária da CTNBio, em 16/12/2010, que ficam APROVADOS, os seguintes relatórios de liberação planejada após sua conclusão. Processos: 01200.003462/2008-51;   01200.000469/2008-69; 01200.000469/2009-00; 01200.003736/2007-21; 01200.001614/2009-61;   01200.000479/2008-56;   01200.003675/2008-82; 01200.005834/2006-11; 01200.006108/2007-05; 01200.003067/2008-78;   01200.000904/2007-26;   01200.00868/2007-09; 01200.001940/2005-45; 01200.001381/2009-05.

EDILSON PAIVA

CONSELHO NACIONAL DE DESENVOLVIMENTO CIENTÍFICO E TECNOLÓGICO
DIRETORIA DE ADMINISTRAÇÃO

DESPACHOS DO DIRETOR
Em 15 de dezembro de 2010

17ª RELAÇÃO DE DISTRIBUIÇÃO DE COTA PARA IMPORTAÇÃO - LEI 8.010/90

PROCESSO	ENTIDADE	VALOR US$
0016/1990	Universidade Federal do Rio Grande do Sul	100,00
0066/1990	Fundação da UFPR para o Desenvolvimento da Ciência, Tecnologia e Cultura	949,00
0083/1990	Fundação de Desenvolvimento da UNICAMP	123.483,64
0144/1990	Universidade Federal do Rio Grande do Norte	10.333,71
0160/1990	Fundação Arthur Bernardes	2.415,83
0207/1991	Fundação de Ciências Aplicadas e Tecnologia Espaciais	7.614,75
0355/1992	Associação das Pioneiras Sociais	29.892,86
0534/1993	Fund. Coordenação de Projetos, Pesquisas e Estudos Tecnológicos	1.885,45
0633/1995	Escola de Engenharia de São Carlos	14.751,72
0653/1995	Universidade Federal do Espírito Santo	1.773,41
0740/1998	Fund.Centro de Pesquisa e Desenvolvimento em Telecomunicações	122.679,00
0750/1998	Pontifícia Universidade Católica do Rio de Janeiro	2.400,00
0814/2001	Centro Brasileiro de Pesquisas Físicas	944,96
0846/2002	Centro de Tecnologia da Informação Renato Archer	419,98
0948/2005	Fund. de Apoio ao Desenvolvimento de Ensino Superior do Norte de Minas	1.299,01
1012/2007	Fundação de Desenvolvimento Científico e Cultural	5.769,00

4ª RELAÇÃO DE CANCELAMENTO DE COTA PARA IMPORTAÇÃO - LEI 8.010/90

PROCESSO	ENTIDADE	VALOR US$
0003/1990	Fundação de Desenvolvimento da Pesquisa	-300.000,00
0243/1991	Instituto Ludwig de Pesquisa sobre o Câncer	-2.364,50
0248/1991	Fundação de Apoio à Física e à Química	-270,00

Failure to supply all applicable information can delay the processing of this application   **PLEASE TYPE OR PRINT <u>CLEARLY</u>**

No controlled material, organisms or vectors may be imported or moved interstate unless the data requested on this form is furnished and certified (9 CFR 94, 95, and 122).	According to the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information collection is 0579-0015. The time required to complete this information collection is estimated to average between 1.6 and 3 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information.	FORM APPROVED OMB NO. 0579-0015

U.S. DEPARTMENT OF AGRICULTURE ANIMAL AND PLANT HEALTH INSPECTION SERVICE VETERINARY SERVICES National Center for Import-Export, Products Program 4700 River Road, Unit 40 Riverdale, MD 20737-1231 **APPLICATION FOR PERMIT TO:** IMPORT OR TRANSPORT CONTROLLED MATERIAL OR ORGANISMS OR VECTORS	1. MODE OF TRANSPORTATION *(Please 'X')*: [X] AIR [ ] SEA [ ] LAND [ ] ANY 2. U.S. Ports of Entry BALTIMORE, MD; DULLES AIRPORT; FT LAUDERDALE; JFK INTERNATIONAL AIRPORT; LAGUARDIA INTERNATIONAL AIRPORT; MIAMI, FL; NEWARK, NJ; PORT EVERGLADES, FL

3. IMPORTER *(Name, organization, complete address, telephone and fax number of individual who will receive and be responsible for the imported material)*	4. SHIPPER(s): *(Name and Address of producer/shipper)* Dr. Luke Alphey / Oxitec Limited 71 Milton Park, Oxford, Oxfordshire, United Kingdom, OX14 4RX Telephone: +44 (0) 1235 532393 Fax: +44 (0) 1235 861138

Name/Org.: Ms. Vickie A. Forster / Agent for Oxitec, Ltd

Address: c/o Dr. Gary Clark - USDA/ARS, 1600 S.W. 23rd Drive, Gainesville, Florida, 32608

Mail: Forster & Associates Consulting, LLC, 230 Steeplechase Circle, Wilmington, Delaware, 19808

Tel/alt: 302-239-6576 / 302-383-3313

Email: vaforster@comcast.net

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5. DESCRIBE THE MATERIAL TO BE IMPORTED *(Provide the following information, as applicable: Animal species and tissue of origin of animal product, country of origin of the animals from which raw animal product was sourced, processing country, recombinant system and genetic inserts, antibody immunogens, stabilizers, nutritive factors of animal origin in media.)* *(COMPLETE VS FORM 16-7 for cell cultures and their products)*

Material	Country of Origin	Species	Cell Culture
Mosquitoes - Aedes albopictus (eggs)and Aedes aegypti (eggs). Mosquitoes have been transformed by direct microinjection with genetic inserts comprising of a fluorescent marker gene from the EGFP family, DsRed or CFP family, and the RIDL genetic system.	Malaysia Mexico United Kingdom	Aedes aegypti, Aedes albopticus Aedes aegypti, Aedes albopticus Aedes aegypti, Aedes albopticus	No

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6. QUANTITY, FREQUENCY OF IMPORTATION, AND EXPECTED COMPLETION DATE *(estimate)*

Material	Quantity	Frequency of Importation	Expected Completion Date
Mosquitoes - Aedes albopictus (eggs)and Aedes aegypti (eggs). Mosquitoes have been transformed by direct microinjection with genetic inserts comprising of a fluorescent marker gene from the EGFP family, DsRed or CFP family, and the RIDL genetic system.	up to 500g total	up to 1 shipment per week through 12/31/2012	12/31/2012

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7. PROPOSED USE OF MATERIAL AND DERIVATIVES *(Also, for animal pathogens or vectors describe facilities/biosafety procedures)*

Material	Proposed Use	Facilities / Biosafety Procedures
Mosquitoes - Aedes albopictus (eggs)and Aedes aegypti (eggs). Mosquitoes have been transformed by direct microinjection with genetic inserts comprising of a fluorescent marker gene from the EGFP family, DsRed or CFP family, and the RIDL genetic system.	The imported eggs shipment will be opened and maintained inside the BSL2 insectary prior to field release. The proposed field release will occur in one or more of the following Florida counties: Bay, Clay, Indian River, Manatee and/or Monroe. The release of RIDL mosquitoes will have no impact on TES listed in these counties. Please see attached Suppression Trial protocol for detailed information regarding the proposed use.	Containment Protocol: The eggs are shipped in shatter-resistant plastic containers within additional packaging, closed and sealed with tape. The box will be labeled as to its contents, origin, destination, and contact telephone numbers. Upon arrival at the trial site, the shipment will be opened and maintained inside the BSL2 insectary prior to field release. Aedes mosquitoes in the insectary: Guidelines published by the American Mosquito Control Association for propagation and maintenance of mosquitoes as a minimum standard for mosquito containment. They will be reared at 80% relative humidity at 80 degrees Fahrenheit with a photoperiod of 12 hours light:12 hours dark. Eggs are kept in sealed plastic bags inside covered containers in the appropriate cages. Larvae are reared in containers with lids and pupae are collected at appropriate times so that no adults emerge in larval pans. All adult mosquitoes are kept in sturdy, screened, tightly closed cages within the insectary. All surplus eggs, larvae, pupae, or adults are killed by one of the following methods:1) freezing at minus 20 degrees Centigrade for 48 hours; 2) autoclaving for 30 minutes at 250 degrees F (121 degrees C); or 3) incineration. All excess larvae or pupae will be placed in a 50% bleach solution for 24 hours after which the liquid waste will be diluted with tap water and drained into the insectary sink. Any remaining solid waste will be placed into a plastic biohazard bag and disposed of using standard biohazard waste disposal procedures.

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8. IF FOR USE IN ANIMALS, <u>SPECIFY</u> THE ANIMAL SPECIES

Material	Species

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9. TREATMENT OF MATERIAL <u>PRIOR</u> TO IMPORTATION INTO THE U.S *(Processing/purification methods, including time at specific temperatures, pH, other treatments, disease safeguard etc.)*

Material	Treatment

Mosquitoes - Aedes albopictus (eggs)and Aedes aegypti (eggs). Mosquitoes have been transformed by direct microinjection with genetic inserts comprising of a fluorescent marker gene from the EGFP family, DsRed or CFP family, and the RIDL genetic system.	Aedes mosquitoes are kept in a well maintained insectary according to ACL-2 conditions. SOPs determine the care and maintenance of the colonies and the genetic transformation procedures. Wild caught insects are not introduced directly into the laboratories and insects are sourced from well respected government laboratories or other institutions. Insects that are known to have resistance to pesticides used in current control measures are prohibited from the insectaries (with the exception of DDT, which is no longer used for controlling insects). Staff are health screened prior to start of employment and then monitored at regular intervals. Personnel from or those that have visited disease endemic countries are prohibited from entering the insectaries until disease incubation periods are completed. Bite records are maintained within the insectaries. The Aedes mosquito eggs are laid onto seed germination paper under insectary conditions (27 C and 80% RH) and matured for 3 days. The eggs are then air dried and packaged in a sealed plastic bag with a damp paper towel for transportation.

10. METHOD OF FINAL DISPOSITION OF IMPORTED MATERIAL AND DERIVATIVES

Material	**Method of Final Disposition**
Mosquitoes - Aedes albopictus (eggs)and Aedes aegypti (eggs). Mosquitoes have been transformed by direct microinjection with genetic inserts comprising of a fluorescent marker gene from the EGFP family, DsRed or CFP family, and the RIDL genetic system.	All adult mosquitoes are kept in sturdy, BSL-2 facilities within the insectary. All surplus eggs, larvae, pupae, or adults are killed by one of the following methods:1) freezing at minus 20 degrees Centigrade for 48 hours; 2) autoclaving for 30 minutes at 250 degrees F (121 degrees C); or 3) incineration. All excess larvae or pupae will be placed in a 50% bleach solution for 24 hours after which the liquid waste will be diluted with tap water and drained into the insectary sink. Any remaining solid waste will be placed into a plastic biohazard bag and disposed of using standard biohazard waste disposal procedures.

I CERTIFY AS AUTHORIZED BY THE COMPANY/INSTITUTION THAT I REPRESENT, THAT THIS MATERIAL WILL BE USED IN ACCORDANCE WITH ALL RESTRICTIONS AND PRECAUTIONS AS MAY BE SPECIFIED IN THE PERMIT.

11. SIGNATURE OF APPLICANT Customer Copy of Application 09211044	12. TYPED NAME AND TITLE Ms. Vickie A. Forster / Agent for Oxitec, Ltd
13. DATE 03/15/2010	14. APHIS USER FEE CREDIT ACCOUNT NO. OR METHOD OF USER FEE PAYMENT *(for VISA or Mastercard include number and expiration date)*. Payment Type: On-Line Credit Card

VS FORM 16-3 (NOV 99)

Federally Listed & Candidate Species in Monroe County, Florida
Updated February 22, 2008

	Common Name	Scientific Name	Federal Status	Habitat
Mammals	Florida panther	*Puma (= Felis) concolor coryi*	E	High pine, Tropical hardwood hammock, Scrub, Maritime hammock, Mesic temperate hammock, Pine rockland, Scrubby flatwoods, Mesic pine flatwoods, Hydric pine flatwoods, Dry prairie, Wet prairie, Freshwater marsh, Seepage swamp, Pond swamp, Mangrove
	Puma (=mountain lion)	*Puma (= Felis) concolor (all subsp. except coryi)*	T/SA	Same as above
	Key deer	*Odocoileus virginianus clavium*	E	Tropical hardwood hammock, Mesic temperate hammock, Pine rockland, Mesic pine flatwoods, Hydric pine flatwoods, Freshwater marsh, Mangrove, Saltmarsh. Keys only
	Key Largo cotton mouse	*Peromyscus gossypinus allapaticola*	E	Tropical hardwood hammock. Key Largo only
	Key Largo woodrat	*Neotoma floridana smalli*	E	Tropical hardwood hammock. Key Largo only
	Lower Keys marsh rabbit	*Sylvilagus palustris hefneri*	E	Beach dune/Coastal strand, Freshwater marsh, Mangrove, Saltmarsh. Keys only
	Rice rat	*Oryzomys palustris natator*	E, CH	Freshwater marsh, Mangrove, Saltmarsh. Keys only
	West Indian manatee	*Trichechus manatus*	E, CH	Fresh and saltwater habitats, Mangroves
Birds	Audubon's crested caracara	*Polyborus plancus audubonii*	T	Improved pastures, Mesic temperate hammock, Mesic pine flatwoods, Hydric pine flatwoods, Dry prairie, Wet prairie. Last documented in 1993
	Bachman's warbler	*Vermivora bachmanii*	E	Migrant 1880s?
	Cape Sable seaside sparrow	*Ammodramus maritimus mirabilis*	E, CH	Wet prairie, Freshwater marsh
	Everglade snail kite	*Rostrhamus sociabilis plumbeus*	E	Hydric pine flatwoods, Freshwater marsh, Pond swamp

	Common name	Scientific name	Status	Habitat
	Ivory-billed woodpecker	Campephilus principalis	E	Last documented in 1919
	Piping plover	Charadrius melodus	T, CH	Sandy beaches, mudflats, sandflats, spoils islands, areas adjacent to inlets and passes.
	Red knot	Calidris canutus rufa	C	
	Roseate tern	Sterna dougallii dougallii	T	Beach dune/Coastal strand, Saltmarsh, Seagrass, Nearshore reef. Keys only
	Wood stork	Mycteria americana	E	
Reptiles	American crocodile	Crocodylus acutus	T, CH	Mangrove, Seagrass
	American alligator	Alligator mississippiensis	T/SA	
	Eastern indigo snake	Drymarchon corais couperi	T	High pine, Tropical hardwood hammock, Scrubby high pine, Beach dune/Coastal strand, Maritime hammock, Mesic temperate hammock, Pine rockland, Scrubby flatwoods, Mesic pine flatwoods, Hydric pine flatwoods, Dry prairie, Cutthroat grass, Freshwater marsh, Seepage swamp, Flowing water swamp, Pond swamp, Mangrove
	Green sea turtle [1]	Chelonia mydas	E	Beach dune/Coastal strand, Seagrass, Nearshore reef
	Hawksbill sea turtle [1]	Eretmochelys imbricata	E	Beach dune/Coastal strand, Seagrass, Nearshore reef
	Leatherback sea turtle [1]	Dermochelys coriacea	E	Beach dune/Coastal strand, Seagrass, Nearshore reef
	Loggerhead sea turtle [1]	Caretta caretta	T	Beach dune/Coastal strand, Seagrass, Nearshore reef
Fish	Gulf sturgeon [2]	Acipenser oxyrinchus desotoi	T	
	Smalltooth sawfish [2]	Pristis pectinata	E	
Invertebrates	Bartram's hairstreak butterfly	Strymon acis bartrami	C	
	Elkhorn coral [2]	Acropora palmata	PT	
	Florida leafwing butterfly	Anaea troglodyta floridalis	C	

	Common name	Scientific name	Status	Notes
	Miami blue butterfly	*Cyclargus (=Hemiargus) thomasi bethunebakeri*	C	
	Schaus swallowtail butterfly	*Heraclides aristodemus ponceanus*	E	
	Staghorn coral [2]	*Acropora cervicornis*	PT	
	Stock Island tree snail	*Orthalicus reses (not incl. nesodryas)*	T	
Plants	Big Pine partridge pea	*Chamaecrista lineata keyensis*	C	Keys only
	Blodgett's silverbush	*Argythamnia blodgettii*	C	Keys only
	Cape Sable thoroughwort	*Chromolaena frustrata*	C	Excluding Keys
	Everglades bully	*Sideroxylon reclinatum austrofloridense*	C	
	Florida indigo	*Indigofera mucronata keyensis*	C	Keys only
	Florida prairie clover	*Dalea carthagenensis floridana*	C	Last documented in 1966 on Key Biscayne
	Florida semaphore cactus	*Consolea corallicola*	C	Keys only
	Garber's spurge	*Chamaesyce garberi*	T	
	Key tree-cactus	*Pilosocereus robinii*	E	Keys only
	Sand flax	*Linum arenicola*	C	Keys only
	Wedge spurge	*Chamaesyce deltoidea serpyllum*	C	Keys only

E=Endangered; T=Threatened; PE=Proposed Endangered; PT=Proposed Threatened; C=Candidate; SA=Similarity of Appearance to a listed taxon; XN=Experimental Population, Non-Essential; CH=Critical Habitat; PCH=Proposed Critical Habitat; [1]=National Marine Fisheries Service has lead for this species in the water, please contact National Marine Fisheries Service for more information and/or consultation for aquatic projects; [2]=National Marine Fisheries Service has lead for this species, please contact National Marine Fisheries Service for more information and/or consultation.

Development of genetically engineered *Aedes aegypti*

Transformation system

The inserts were constructed using standard laboratory technique, including purification of the plasmid DNA to ensure that no bacterial protein or chromosomal material remains associated with the plasmid. *Aedes aegypti* mosquitoes of Rockefeller strain were reared in an insectary maintained at 28°C and 75-80% humidity with a 12-hour light/dark cycle. Mosquitoes were transformed by standard micro-injection methods according to the methods of Jasinskiene et al. (1998), using the plasmid LA513 at a concentration of 500 ng/µl co-injected with a 400 ng/µl concentration of the *piggyBac* helper plasmid, phsp-pBac as described by Handler et al. (1998) as a source of transposase. *piggyBac* was used as a non-autonomous transposable element, or transposon, co-injected with a non-integrating source of *piggyBac* transposase. The *piggyBac* element was discovered in a baculovirus infecting tissue culture cells derived from *Trichoplusia ni*, the cabbage looper moth (Cary et al., 1989; Elick et al., 1996; Fraser et al., 1995; Fraser et al., 1996; Fraser et al., 1983; Wang and Fraser, 1993). A non-autonomous transposon is prevented from moving within or outside the genome of its host because it does not produce the transposase enzyme that is necessary for such movement (Handler and James, 2000). It has been reported that the non-autonomous *piggyBac* vector is very stable in the *Aedes* genome even when exposed to exogenous transposase under a wide variety of conditions (O'Brochta et al., 2003; Sethuraman et al., 2007; Thibault et al., 1999).

Inserted genes

Two traits have been inserted in the strain, conditional lethality and a fluorescent marker gene.

The *conditional or repressible lethality trait*, where the lethality is repressed in the laboratory or mass-rearing by applying artificial conditions (in the case of RIDL, dietary supplementation with tetracycline and /or analogues) and it is not repressed, therefore active, under conditions that do not include the dietary supplement, such as the environment. This has the beneficial consequence that the RIDL system is activated in insects that are released into the environment and their progeny. The basis of the conditional lethality in RIDL system developed by Oxitec uses the "tet-off" gene expression system (Gossen and Bujard, 1992). The "tet-off" system is based on a synthetic fusion protein called the tetracycline-repressible transactivator or "tTA". tTA is produced by the fusion of a sequence specific binding protein from *E. coli* to a eukaryotic transcriptional enhancer from herpes simplex virus (VP16). The fusion protein binds to a short specific DNA sequence known as the tet operator (tetO). When bound to tetO, tTA acts as a transcriptional activator. tTA has been further optimised for expression in insects, tTAV for example (Gong et al., 2005). These modifications made minor changes to the nucleic acid sequence and do not affect the properties of the encoded protein. tTA and tTAV bind tetracycline and tetracycline like chemicals with high affinity, and in the bound form will not bind DNA, therefore in the presence of small concentrations of the chemical, tTA does not bind to DNA and does not act as a transcriptional activator.
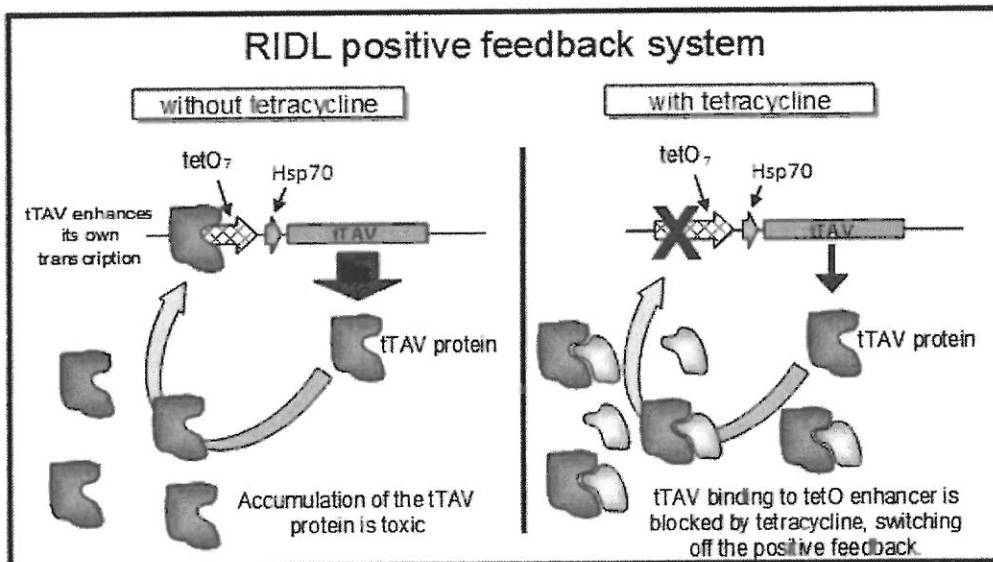
Figure 1: Schematic representation of the RIDL system. In the absence of tetracycline (left panel), small amounts of tTAV protein generated from basal expression of the Hsp70 promoter (Hsp70) can bind to the tetO binding sites (tetO$_7$), creating a positive feedback loop that enhances expression of tTAV. When the tTAV protein accumulates in sufficient quantities it affects cellular function, resulting in lethality. In the presence of tetracycline (right panel), tTAV is prevented from binding to the tetO sites and can therefore not enhance the expression from hsp70 minimal promoter. This prevents the build-up of tTAV, hence avoiding its lethal affects to cells.

tTA (and tTAV etc) are intracellular proteins. The effect of expression or over-expression of the tTAV appears to be cell-autonomous, which allows expression in one cell and does not affect those in adjacent cells, as it can be expressed at high levels under the control of tissue-specific promoters without affecting other tissues. tTA protein is only mildly deleterious to mammalian tissue culture cells when produced intracellularly at high levels, evidenced by their loss of expression over time. This also confirms that it is a relatively unstable protein, probably due to the presence of a ubiquitin degron. A degron is a specific amino acid sequence in a protein that directs the starting place of degradation. The lethal mode of action is thought to be by transcriptional squelching (Gill and Ptashe, 1988) and/or interference with ubiquitin-dependent proteolysis (Gillespie et al., 1997; Salghetti et al., 2001) and not because of a toxic or insecticidal action. A further review of autocidal or lethal genetic systems is available in the Final Environmental Impact Statement, Appendix C (USDA, 2008).

The *fluorescent marker gene* is introduced to act as a marker gene for the transformation, showing insects that have successfully received the genetic construct. The fluorescent marker gene is from the family of Cnidarian proteins, Discosoma coral (Ip and Wan, 2004; Matz et al., 1999; Shagin et al., 2004) and fluoresces orange-red with a known and specific excitation and emission wavelength profile. Fluorescent proteins are widely used in medicine, molecular biology, biotechnology and agriculture and have already been used in commercial transgenic organisms (GloFish®) and field releases in the USA and other countries including New Zealand, and Malaysia[1]. A review of the database on field releases to the environment in the USA[2] revealed over 60 field releases with

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[1] http://bch.cbd.int [accessed 10 Feb 2011]
[2] http://www.isb.vt.edu/search-release-data.aspx, [accessed 10 Feb 2011]

fluorescent proteins have been conducted since 1997 with no adverse incidents reported. Fluorescent protein markers should not confer any selective advantage that could increase the invasiveness of species that could acquire the gene, however remotely through horizontal gene transfer.

There are no antibiotic resistance genes or plasmid backbone sequences in the strain to be released.

The strain has described in Phuc et al. (2007)[3] and has been further introgressed into Asian and Latin American background strains at Oxitec Ltd in the United Kingdom. The Latin American strain will be used for the trial.

Table 1 shows the genetic elements and their sources, inserted into OX513A *Ae. aegypti* mosquitoes.

Table 1: Genetic elements inserted into OX513A *Aedes aegypti* mosquitoes

| Genetic Element	Donor Organism and Common name	Reference	Function	Comments
piggyBac 3'	*Trichoplusia ni* (cabbage looper moth)	(Cary et al., 1989; Thibault et al., 1999)	DNA transposable element with sequence deletions to prevent mobility.	.
Act5C	*Drosophila melanogaster* (vinegar fly)		Promoter element driving the expression of the marker gene	
DsRed2	*Discosoma* (coral)	(Ip and Wan, 2004)	Red fluorescent protein marker gene	The fluorescent marker has been used in a wide range of vertebrate and invertebrate species as marker genes, confer no competitive advantage of disadvantage to the recipient and no adverse ecological or other consequences resulting from their incorporation into the mosquito.
Drosomycin 3' UTR	*Drosophila melanogaster* (vinegar fly)		Terminator region (polyadenylation signal)	
TetO$_7$	*Escherichia coli* (bacteria)		Non-coding binding site for tTAV element	
Hsp70	*Drosophila sp.* (vinegar fly)		Promoter element driving tTAV element	

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[3] As LA513A, the original, alternative name for OX513A

adh intron	*Drosophila sp.* (vinegar fly)		Enhances gene expression	
tTAV	Synthetic DNA based on a fusion of sequences from *Escherichia coli* and herpes simplex virus (VP16 transcriptional activator)	(Gong et al., 2005; Gossen and Bujard, 1992)	Tetracycline repressible transcriptional activator	tTA protein binds to and activates the expression from the tetracycline response element (tRE) which includes the specific DNA sequence to which tTA binds (tetO). tTA also binds tetracycline with a high affinity, which then cannot bind DNA. In effect tTA acts as tetracycline regulated switch. High-level expression of tTA is deleterious to cells as it can repress their normal transcription. tTA has been used in fungi, mice, plants, mammalian cultures with no known adverse effects on the environment or human health.
3'UTR	*Drosophila sp.* (vinegar fly)		Terminator region	
piggyBac 5'	*Trichoplusia ni* (cabbage looper moth)	(Cary et al., 1989)	DNA transposable element with sequence deletions to prevent mobility	

Molecular characterisation

A single copy of the insert is present in OX513A and the strain is homozygous for the introduced traits. The graphic below shows the chronology of the generation of the strain proposed for use in the trial.

Figure 2: Chronology of strain of the generation of the OX513A strain.
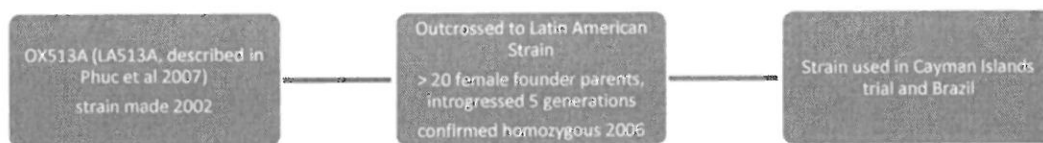
The flanking region around the insertion of the gene product has been sequenced showing that the insertion was mediated by *piggyBac* into a TTAA site in the genome as expected for this integration method. Molecular analysis (PCR) for the presence of plasmid backbone sequences, which include origins of replication and an antibiotic resistance gene, showed that these sequences were not

present. PCR techniques are very sensitive and can be tailored to detect unique genetic fragments within the modified insects, based on the genomic regions flanking the insertion site. Such a methodology has been developed and validated for OX513A insects and is available on request.

Genotypic and phenotypic stability

The modified OX513A strain has been maintained in the laboratory for over 60 generations with no signs of instability of the genetic trait observed. The fitness of the *Ae. aegypti* OX513A mosquitoes have been observed in the laboratory and under semi- field conditions in comparison to the wild type strain of *Ae. aegypti*. There were no significant differences between the OX513A strain and with wild type in the numbers of eggs laid, number of larvae hatched and the number of pupae in F1 generations, nor the number of days in each stage of life (Lee et al., 2009).

Method of detection

The OX513A strain can easily be identified as larvae and pupae in the laboratory and field conditions due to the presence of a fluorescent marker gene. The genetic marker can reliably be seen in larval stages of the mosquito if a fluorescent microscope is used with excitation at 558nm wavelength and emission at 583nm. Some degree of staff training is required to achieve the best results, but the fluorescence is strong and robust under a variety of conditions for other insects (Simmons et al., submitted) and used successfully with field samples in Grand Cayman.

The marker gene is heritable allowing progeny to be identified using the same techniques. Further confirmation of the presence of the transgene can be gained by the use of molecular biology techniques, namely polymerase chain reaction (PCR) techniques can be used to identify the transgenic mosquitoes.

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Draft Environmental Assessment

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Field study of Genetically Modified *Aedes aegypti* (Diptera: Culicidae)

Summary
On March 15, 2010, the Animal and Plant Health Inspection Service (APHIS) received an application for an authorization of release into the environment (field study) under 7 CFR 371.4 of Genetically Engineered mosquitoes, species *Aedes aegypti*.

Oxitec Limited has developed a genetically sterile *Ae. aegypti* to control local populations of the same mosquito. *Ae. aegypti* is a peri-domestic species closely associated with human habitations. Breeding is tied to artificial water containers, such as pot plant holders, water tanks, tyres, discarded plastic and metal containers such as soda cans, drains and roof guttering as well as ephemeral containers, such as puddles. *Ae. aegypti* is responsible for the transmission of arboviral diseases, particularly being the primary vector of dengue fever, but also having the potential to transmit chikungunya and yellow fever. Dengue is increasing worldwide, is endemic in Puerto Rico and local transmission has recently been reported in Florida. There is no vaccine or therapeutic treatment for dengue disease, and control is by population reduction of *Ae. aegypti*. Current control methods for the vector are increasingly ineffective and new solutions are required. The need for new mosquito control solutions in Key West is emphasized by the recent reporting of local transmission of dengue fever (CDC, 2010).

The *Ae. aegypti* have been engineered to express two genetic elements: a repressible lethality trait and a fluorescent marker protein, described by Phuc et al. (2007). The use of repressible lethality traits for making insects genetically sterile is also known as RIDL® technology. The males of the strain (when released) are designed to mate with local *Ae. aegypti* mosquitoes; their progeny, however, will not survive to adulthood, in the absence of a specific supplement. The specific supplement is only used in the laboratory to allow rearing of the insects and will not be present in the environment in sufficient quantity to allow the insects to survive. The technique is based on the Sterile Insect Technique (SIT) that has been used successfully in the USA since the 1950s for the control of agricultural pest insects. Attempts to use conventional SIT have not succeeded with mosquito species, in the past as the radiation doses required for sterilization of the mosquitoes have compromised the fitness of the mosquito species (Helinski et al., 2009), although recent attempts have been more successful (Helinski et al., 2008).

The field study, which includes the release of the genetically sterile male *Ae. aegypti*, is requested for the

specific location of Key West, Monroe County, Florida in conjunction with the Florida Keys Mosquito Control District. Male mosquitoes do not bite or transmit disease. The engineered *Ae. aegypti* for the proposed field release in Key West have previously been released in the Cayman Islands and Malaysia and has been granted an approval for field release in Brazil.

APHIS has prepared a draft environmental assessment (EA) for the genetically engineered *Ae. aegypti* which are considered regulated articles under the regulations in 7 CFR Part 371.4.

This draft EA analyzes the alternatives available to APHIS for its decision regarding this request for authorization of release into the environment (field study) of male-sterile mosquitoes subject to carefully tailored measures proposed by APHIS. Based on the scope of the draft EA, the specific decisions to be made are:

• Should APHIS grant the request for a release into the environment (field study) of genetically engineered sterile male *Ae. aegypti*?

• Would the preferred alternative, if selected (see alternative 2 below), have significant impacts on the quality of the human and/or animal health requiring preparation of an EIS?

• What conditions should be imposed to prevent any potential human or animal health risk from genetically engineered male *Ae. aegypti* until APHIS can determine if an EIS is required and it can be completed.

The draft EA has been prepared to analyze the alternatives available to APHIS for responding to this request and to provide the public with documentation of APHIS' review and analysis of any potential individual and cumulative environmental impacts associated with the authorization of environmental release of male-sterile mosquitoes subject to carefully tailored conditions proposed by APHIS. The draft EA considers and evaluates two (2) reasonable alternatives. The alternatives analyzed in the draft EA include:

1. No Action
2. Grant Request – Issue Permit

Table of Contents

I. Purpose and Need

A. Introduction and spread in USA

Introduction

USDA Veterinary Services has received an application from Oxitec Ltd to move and field test genetically engineered sterile male *Aedes aegypti* mosquitoes in Key West, Monroe County, Florida. The *Ae. aegypti* have been engineered to express two genetic elements: a repressible lethality trait and a fluorescent marker protein. The field test is planned in conjunction with the Florida Keys Mosquito Control District (FKMCD) to evaluate the use of genetically sterile male *Ae. aegypti*, to reduce the population of local *Ae. aegypti*, the mosquito vector of dengue and other viral diseases. The technique is based on the Sterile Insect Technique that has been used successfully in the USA since the 1950s for the control of agricultural pest insects (Dyck et al., 2005). The need for new mosquito control solutions in Key West is important due to the recent reporting of local transmission of dengue fever (CDC, 2010).

Spread of Aedes aegypti

Worldwide, *Ae. aegypti* is a tropical species with a cosmopolitan range extending from 40° N to 40° S latitude. The species originated in Africa but achieved pan-tropical distribution in the 1930s and is now found throughout most tropical to subtropical world regions. Global distribution has been closely associated with human mass migration and passive transport (Gubler, 2006; Lounibos, 2002).

Ae. aegypti is a peri-domestic species closely associated with human habitations. Breeding is tied to artificial water containers, such as pot plant holders, water tanks, tyres, discarded plastic and metal containers such as soda cans, drains and roof guttering as well as ephemeral containers, such as puddles.

In the United States, *Ae. aegypti* occurs in Mississippi, Alabama, Georgia, Florida, Tennessee, Kentucky, South Carolina, North Carolina, Virginia, New York, New Jersey, the District of Columbia, Arkansas, Illinois, Indiana, Kansas, Louisiana, Missouri, Oklahoma, Texas and Arizona (reviewed by Darsie and Ward, 2005). Densities are greatest in the Gulf Coastal states[1].

B. Economic and Disease Importance

Ae. aegypti is the primary vector of dengue fever, although it is also capable of transmitting other viral diseases such as chikungunya and yellow fever. There is no therapeutic treatment or vaccine for dengue currently available (although several candidates are at various stages of development, none are yet approved for use). Globally the number of dengue cases annually exceed 50 million (Guzman et al., 2010; WHO-TDR, 2006). Dengue fever is a febrile illness caused by a virus (Arbovirus) with a wide range of symptoms, ranging from headache and fever to extreme joint pain and in some countries it is known as "breakbone fever". Some cases can be so mild that they go unreported. Dengue viruses consist of four related serotypes, and several serotypes can be present in a region at once. Exposure to one serotype provides immunity, but not cross-immunity to the other serotypes, which in turn is thought to increase the risk of contracting the more severe form of dengue, dengue hemorrhagic fever (DHF).

Dengue fever has emerged as a worldwide problem only since the 1950s. It is estimated that nearly 2.5 billion people live in areas of risk for epidemic transmission. The Pan American Health Organization (PAHO) ran a yellow fever eradication campaign in the 1950s and 1960s, targeting the *Ae. aegypti* mosquito vector (Schliessmann, 1967), which was officially discontinued in the United States in 1970. Since then the mosquito vector has re-infested and has a wider distribution than before the original eradication program (Lloyd, 2003).

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[1] http://entnemdept.ufl.edu/creatures/aquatic/aedes_aegypti02.htm [Accessed 8 Feb 2011]

Although dengue fever rarely occurs in the continental United States, it is endemic in Puerto Rico, and in many popular tourist destinations in Latin America, Southeast Asia and the Caribbean.

The dengue epidemics in Florida, in the Tampa and Miami areas in 1934-35 (Griffitts, 1934; MacDonnell, 1935) affected an estimated 15,000 of the 135,000 population of Miami. The last recorded epidemic in the southeastern states was in Louisiana in 1945 (Ehrenkranz et al., 1971). Most cases of dengue reported in the United States since the 1940s have been imported; however, indigenous transmission of dengue occurred in Texas in 1986 and 1995. Thirteen imported dengue cases (0-4 cases per year) were reported in Florida from 1985 to 1995. Recently, in 2009 and 2010, local transmission of the disease has been reported in the Florida Keys (CDC, 2010), with 22 people diagnosed in 2009 and a further 63 people in 2010 and single cases in Dade-Miami and Broward counties. Case counts for locally acquired dengue and those imported from other countries can be found in the weekly surveillance report of the Florida Department of Health[2]. 2009 saw the first occurrence of locally acquired dengue in the Keys since the 1930s. Dengue outbreaks have a tendency to spread quickly because of the movement of infected humans and mosquitoes. The predicted resumption of travel between the USA and Cuba represents a significant threat for the movement of mosquito-borne diseases like dengue, yellow fever and chikungunya into Florida.

Figure 1 shows a screenshot of the interactive dengue map, available on the CDC website, showing reported dengue cases in 2010.

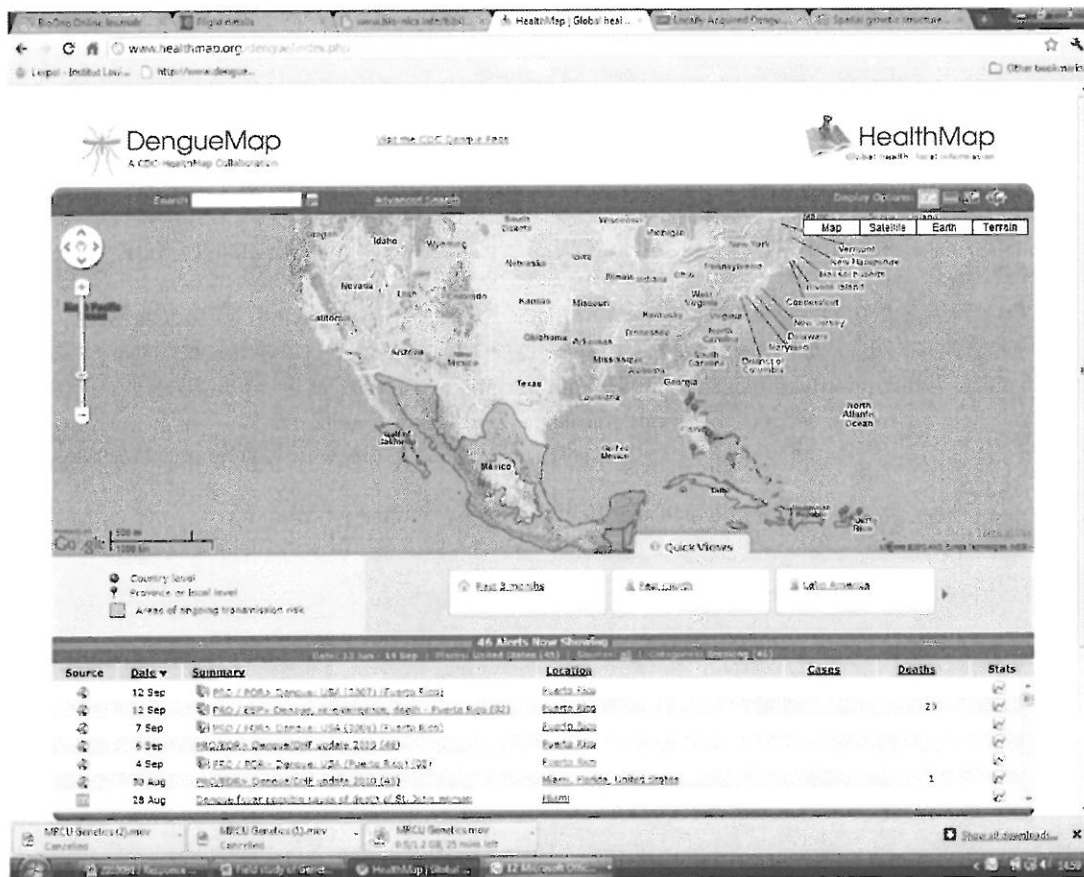**Figure 1:** Distribution of dengue in USA in 2010 (Source: Centers for Disease Control and Prevention http://www.healthmap.org/dengue/index.php).

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[2] http://www.doh.state.fl.us/Environment/medicine/arboviral/surveillance.htm (accessed Jan 24, 2011)

The economic impact of dengue is large and includes a variety of impacts; loss of life, medical expenditure, loss of productivity of the workforce, emergency vector control actions, demand on health care services during an epidemic, and potential loss of tourism revenues as a result of negative publicity.

C. Control of *Aedes aegypti*

Control of *Ae. aegypti* takes a variety of forms, usually as an integrated approach known in the USA as Integrated Mosquito Management (Rose, 2001), which involves some or all of the approaches below.

Use of insecticides for both adults and larvae

Larviciding utilizes the application of insecticides targeted at the immature mosquitoes - the larvae or pupae. These are applied to bodies of water harboring the larvae. However, since larvae do not usually occupy the entire body of water, larvicides are applied where the larvae are, usually the areas near the shoreline of the lake, stream or ditch. Larvicides differ from adulticides in that they are directed at a limited targeted area, i.e. the body of water and often only that area where the larvae grow and mature. Larvicides are classed as stomach toxins, contact larvicides, surface agents, natural agents, bio-pesticides (such as *Bacillus thuringiensis israelensis*) and insect growth regulators (IGRs).

The use of adulticides is primarily by space spraying (also known as fogging). Inside houses, household aerosol space sprays containing synergized pyrethrum or synthetic pyrethroids (allethrin, resmethrin, etc.) are available now. Best results are obtained if doors and windows are kept closed during spraying and for 5-10 minutes after spraying. The major advantage of space treatment is immediate knockdown, quick application, and relatively small amounts of materials required for treatment. Space sprays are most effective indoors.

Outdoors, homeowners can use ULV foggers, portable or fogging attachments for tractors or lawn mowers for temporary relief from flying mosquitoes. Pyrethrins or 5% malathion can be fogged outdoors. The insecticide particles disperse rapidly and may not kill many mosquitoes. The major disadvantage of space spraying is that it will not manage insects for long periods of time. Organized mosquito control uses aerial adult mosquito control using fixed-wing aircraft or helicopters and/or ground adult mosquito control using truck- or boat-mounted equipment.

Source reduction by draining artificial and natural larval breeding sites

Source reduction, incorporates physical control (digging ditches and ponds in the target marsh) and biological control [placing live mosquito fish (*Gambusia*) in the ditches and ponds to eat mosquito larvae]. Other non-chemical control methods include the use of mosquito predators, parasites and diseases to control mosquito larvae. Information about the role of other predators on the development, survival and abundance of *Aedes* mosquitoes is limited and they have not achieved widespread use (Essam Abdel and Deon, 2009), although have been successful in some disease-endemic countries (Diabate et al., 2008; Marten, 1990; Mottram and Kettle, 1997; Stav et al., 2005; Vu et al., 1998).

Use of mosquito traps

Mosquito traps and insect electrocutors are primarily used by homeowners, although some trapping is conducted by mosquito control districts to monitor the population of mosquitoes rather than acting as a control measure. The scientific data on the effectiveness of these traps is sparse and they are not regarded as an overall control measure.

Even a well-organized mosquito control program, using integrated mosquito management measures, cannot always be effective against the mosquitoes as there can be just too many breeding sites to cover. Therefore new tools are required to be integrated into the mosquito management programs.

The current control methods used in the FKMCD are included in **Appendix I**.

D. Biology of *Aedes aegypti*

The genus *Aedes* contains about 700 species and is divided into a number of sub-genera including *Aedes* and *Stegomyia*. *Ae. aegypti* (also known as the yellow fever mosquito) is a medium-sized (3-4 mm) blackish mosquito, with a distinctive pattern of silvery scales on its back with white-banded legs.

It has four distinct life stages, from egg, through larvae to pupae and adulthood. The first three life-stages are aquatic. The female adults require a blood meal to lay eggs, which they take almost exclusively from humans. Adult males do not bite humans as they survive on nectar and similar sugar sources.

Eggs

The eggs are laid individually by females in the damp walls of both natural and artificial containers that can hold clean, still water. The female adults lay eggs in a multitude of places, using a strategy of laying a few eggs in a variety of containers (skip oviposition) rather than laying all eggs in one container. Eggs are the long- term survival structures of these mosquitoes, surviving up to 6 months in appropriate conditions, although they are generally non-diapausing (over-wintering). Survival is affected by larval nutrition, temperature and humidity.

Larvae and pupae

The larvae and pupae prefer relatively clean water typically found in types of container: water storage containers, flowerpots and waste materials such as tires, cans, bottles etc. The waste material containers are usually only sources of mosquitoes during the rainy season in other countries but in tropical climates this tends to be year round. The duration of the larval stages is approx 7-9 days and pupae 2-3 days but this is temperature-dependent. Larvae also need to reach a certain mass if they are to complete pupation.

Adults

The preferred sites for adults are domestic urban environments in sheltered dark spaces within houses/ apartments. *Ae. aegypti* is a day-biting mosquito with two peaks, one mid-morning and one mid-afternoon. The average adult lifespan is 8-15 days for female mosquitoes and 3-6 days for male mosquitoes. Spontaneous flight of adults is limited to around 200 m depending on availability of breeding sites, and hosts from which to take a blood meal. However the species is dispersed by passive transport on boats, trains etc. and International Sanitary Regulations require ports and airports to be clear of *Ae. aegypti* for 400 m. Climate and the availability of breeding sites are the two main factors that regulate the populations of *Ae. aegypti* in urban environments.

Reproduction

Ae. aegypti reproduces sexually with internal gametes. Key mating behaviors are essential to successful coupling between males and females (Cator et al., 2009), which means mating is extremely species-specific.

Male mosquitoes are not sexually mature until up to 24 hours post emergence. The reproductive potential of females is determined by both larval and adult nutrition, as they require a bloodmeal to provide sufficient nutrients for egg laying. Females each produce 18-116 eggs (Breigel, 1990), with the average around 70 eggs.

E. Regulatory Authority

APHIS has the regulatory authority for regulation of genetically engineered mosquitoes under the Animal Health Protection Act (7 U.S.C. 8301–8317) section 8308: Detection, control, and eradication of diseases and pests, (a) In general The Secretary may carry out operations and measures to detect, control, or eradicate any pest or disease of livestock (including the drawing of blood and diagnostic testing of animals), including animals at a slaughterhouse, stockyard, or other point of concentration.

This EA was conducted under the authority of the National Environmental Policy Act (NEPA) 42 U.S.C 4321 and 7 CFR §372 NEPA implementing procedures. Except for actions that are categorically excluded, approvals and

issuance of permits for proposals involving genetically engineered or non-indigenous species normally require environmental assessments, but not necessarily environmental impact statements.

II. Need for the Proposed Action

Proposed Action

The proposed action is for APHIS Veterinary Services to issue a permit for movement and field testing of *Ae. aegypti* mosquitoes modified to contain a conditional lethality and a fluorescent marker gene. The study will use genetically engineered *Ae. aegypti* to determine if their deployment can suppress a target population of local *Ae. aegypti*.

Ae. aegypti is the primary vector of dengue fever, a viral disease of humans. Dengue is increasing worldwide, is endemic in Puerto Rico and local transmission has recently been reported in Florida. There is no vaccine or therapeutic treatment for dengue disease, and control is by population reduction of *Ae. aegypti*. Current control methods for the vector are increasingly ineffective and new solutions are required (Guzman et al., 2010; Ooi et al., 2006; WHO-TDR, 2006).

The engineered *Ae. aegypti* for the proposed field release in Key West have previously been released in the Cayman Islands (**Appendix II**) and Malaysia (**Appendix III**) and has been granted an approval for field release in Brazil (**Appendix IV**).

Need for an Environmental Assessment

The need for this Environmental Assessment (EA) is to assess potential adverse environmental and human health impacts of a field research study to be held in Key West, Monroe County, Florida. The application for a permit was filed with APHIS Veterinary Services on 15th March 2010. It was submitted to APHIS Veterinary Services by Oxitec Ltd (a UK-based Company) acting through a US regulatory agent. The application number is 09211044, and the application is appended (**Appendix V**). It should be noted that the scope of the application has been reduced to a trial of the one species - *Ae. aegypti* - in one location, compared to the broad application submitted.

A.   Similar EA

Environmental Assessments (EAs) have been published in 2001 and 2006 for the confined studies and open release of genetically engineered pink bollworm (*Pectinophora gossypiella*) expressing fluorescent marker genes similar to those in this application. In both cases a Finding of No Significant Impact (FONSI) was issued and published in the Federal Register (66 FR 33226 and 71 FR 35408, respectively).

Furthermore, in compliance with the National Environmental Policy Act of 1969 (NEPA) as amended, an Environmental Impact Statement (EIS) on the Use of Genetically Engineered Fruit Fly and Pink Bollworm in APHIS Plant Pest Control Programs. A Record of Decision was subsequently published was prepared and published in the Federal Register (73 FR 31115), followed by a public comment period that closed in August 2008. The final EIS (FEIS) was published in the Federal Register in Oct 2008, (73 FR 67511), again followed by a waiting period, in accordance with NEPA implementing regulations (40 CFR 1506). A Record of Decision was subsequently published in the Federal Register (74 FR 21314) in May 2009, recommending use of genetically engineered fruit flies and pink bollworm in APHIS pest control programs, as the *"environmentally preferred alternative"* as it *"minimized potential impacts to human health, nontarget species and environmental quality"*.

This draft EA addresses the use of *Ae. aegypti* mosquitoes expressing the same genetic traits of conditional lethality and fluorescent marker genes that were examined in the FEIS for fruit flies and pink bollworm in plant protection programs. Technical aspects of this modification are described in detail in **Appendix VI**.

B. Purpose and Need for this EA

Under APHIS regulations, the receipt of a permit application to introduce a genetically engineered organism requires a response from the Administrator: (CHECK)

III. Alternatives

A. No Action

Under APHIS Veterinary Services regulations, the Administrator must either grant or deny permits properly submitted. For the purposes of this EA, the No Action alternative would be to deny the permit 09211044.

B. Issue a Permit

Issuance of the permit would allow the following research to proceed in Key West, Monroe County, Florida. **Appendix VII** contains details of the research protocol, including procedures on handling, transport and waste disposal. Experience gained in the Cayman Islands with field release of genetically engineered *Ae. aegypti* of the same strain indicates that protocol flexibility is required due to the changing nature of *Ae. aegypti* populations and, therefore, some minor protocol changes are envisaged. These will be reported at the end of the trial. The results of the trials conducted in Grand Cayman in 2009 and 2010 are in **Appendix II.**

Purpose of the Research

The purpose of the research is to determine the effectiveness of genetically engineered male *Ae. aegypti* (also known as RIDL® *Ae. aegypti*) to reduce the local *Ae. aegypti* population when released into test sites in Key West, Florida and ultimately to provide new mosquito control tools for *Ae. aegypti*, which vectors transmission of dengue fever, chikungunya and yellow fever viruses.

As this mosquito species is peri-domestic and intrinsically co-located with humam habitations, a public engagement program is planned for Key West prior to and during the trial. This will include various levels of engagement including broadcast and print media, community meetings and interactions with key stakeholders at State and community level.

Description of the Research

There are three phases proposed for the evaluation:
1. Range finding evaluation of RIDL *Ae. aegypti* mating with local female *Ae. aegypti* population
2. Suppression of a field population of *Ae. aegypti* mosquitoes by means of RIDL technology
3. Pilot implementation trial to evaluate operational parameters for the use of RIDL technology.

The range finding study is used to determine the size of the local *Aedes* population in Key West using a limited release over a short period (approximately 6-8 weeks). The data from this study informs the scale of release required for an evaluation of suppression potential using RIDL *Ae. aegypti* mosquitoes in Key West.

The suppression trial evaluates the potential of RIDL *Ae. aegypti* mosquitoes to suppress wild *Ae. aegypti* populations in parts of Key West

The pilot implementation study also evaluates the potential of RIDL *Ae. aegypti* mosquitoes to suppress wild *Ae. aegypti* populations in Key West, but on a larger scale and with a greater focus on operational issues and integrated vector management.

These three phases are described below.

Phase 1: Range finding study evaluation of RIDL *Aedes aegypti* mating with local female population

The range finding study will determine the feasibility and resource requirements for suppressing a target local *Ae. aegypti* population using RIDL technology in Key West, Monroe County, Florida. The study will involve a limited release of genetically sterile[3] RIDL *Ae. aegypti* in two sites in Key West with the explicit aim of demonstrating mating with local females in the field. The specific objectives of this stage of the trial are:

1. Determine the relationship between putative RIDL male release numbers and RIDL (versus local type) paternity of eggs in two locations in Key West.
2. Determine the relationship between RIDL male release numbers and observed adult male sex ratio.
3. Determine lifespan (daily survivorship) of RIDL males in the field.
4. Estimate dispersal of RIDL males in the field.
5. Establish a laboratory scale production and dispersal system for limited sustained release of sterile males RIDL mosquitoes in field trial sites.

Sufficient RIDL *Ae. aegypti* eggs will be shipped by Oxitec Ltd UK laboratories to FKMCD facilities, where they will be hatched and reared to pupae, sorted and then released either as pupae or adults. At the pupal stage the females will be removed by mechanical separation – females are larger than males, allowing efficient sex-separation by this method (Ansari et al., 1977; Focks, 1980). Sterile males will be dispersed into the environment either as pupae or adults in specially designed pupal or adult release devices. Sterile males will be released 2-3 times weekly for a period of approximately 4-8 weeks in an area of up to 50 acres. Ovitraps will be used to collect eggs laid by local females. Field-collected eggs will be hatched and parentage assessed to establish proportion of eggs with RIDL construct. The RIDL construct includes a fluorescent genetic marker that is visible in the larval stage *via* fluorescent microscopy.

Each site will be monitored in three phases:
- Pre-trial baseline monitoring
- Monitoring throughout the trial duration
- Post-trial monitoring.

Monitoring will employ a variety of methods explained below.

Ovitraps

Ovitrapping is a well-established mosquito monitoring method (Silver, 2008). Ovitraps will be used to monitor changes in relative abundance of the field population, and collect eggs for evaluation of matings between released RIDL males and local females. Ovitraps provide an indirect measure of female abundance allowing assessment of field population, without interference from released RIDL males. The protocol for ovitrap monitoring is as follows:

Glass jars (or similar container) painted black are part-filled with clean water and include an oviposition substrate (such as a fiberboard paddle). Other similar ovitraps may be used. Ovitraps are typically deployed for one week, and returned to laboratory for assessment of number of eggs laid on the substrate. Field-collected

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[3] Use of the term "sterile" or "genetically sterile" is explained in more detail in the following excerpt from Alphey, Benedict et al (2010) Vec. Zoo. Dis. 10:295-311:

"Despite the name, the insects used in SIT are not strictly sterile, in the sense of agametic sterility. Rather, they are capable of mating, but some or all of the progeny of mating between the sterile insects and wild insects are nonviable. Several sterilizing methods are available... The terms "sterile," "sterility," and the like [encompass] all of these methods...

These include:
- Radiation, which is used in all current agricultural programs. Radiation generates random dominant lethal mutations in the affected gametes.
- *Wolbachia*-induced cytoplasmic incompatibility, in which sperm from Wolbachia-infected males fail to function correctly after fertilizing eggs from uninfected females.
- Recombinant DNA methods, for example, the use of engineered repressible dominant lethal mutations (RIDL) that lead to the progeny of any cross involving an RIDL parent being nonviable unless provided with a suitable antidote (repressor) from the lethal genetic system. In one embodiment of this system, the lethal effect is female specific, so that only female progeny die.

Other methods have been used historically, including chemosterilants, or incompatible matings, through the use of either sibling species or else the use of artificially induced chromosome rearrangements."

eggs from the ovitraps will be hatched and parentage assessed to establish proportion of eggs with RIDL construct (see larval screening below).

Ovitrapping will be initiated at least four weeks before the proposed release and continue for at least 2 weeks after releases have ceased.

Larval screening

Larval screening will take place for the duration of the trial and for the ovitrapping period post trial. Eggs collected from ovipots will be matured, by allowing them to dry at room temperature for 2-3 days before hatching and larvae will be identified with appropriate taxonomic keys (Bangs and Focks, 2006). *Ae. aegypti* larvae will be screened for the presence of genetic material from RIDL. The RIDL strain has a fluorescent marker gene (Phuc et al., 2007), which is visible at wavelength excitation of 520-550 nm, emission 580+ nm under a fluorescence microscope (e.g. Leica MZ10F) in a darkened room. All larvae from the ovitraps will be screened for fluorescence and PCR performed on subsets to check the fluorescence results and to confirm any uncertain results.

Adult Sampling

Adult sampling will begin at least two weeks before the release and continue for the trial duration, providing a direct measure of changing adult populations. Captured adults will include local females and males in addition to sterile released males. Comparison in the change in sex ratio, from catches before and during the release phase, can be used to estimate the ratio of sterile released to local males.

The BG-Sentinel trap is a trap specifically targeting adult *Aedes* mosquitoes. BG-traps are specially designed suction traps that use a combination of visual and host mimic olfactory cues to attract adult *Aedes* mosquitoes. These traps have been field tested in direct comparison with other live adult sampling methods including the CDC backpack aspirator (Dame et al., 1981; Krockel et al., 2006; Maciel-De-Freitas et al., 2007; Maciel-De-Freitas et al., 2006). They collect both males and females in comparable numbers and are also effective at sampling *Ae. albopictus* (Ritchie et al., 2006).

BG traps and ovipots will be located predominately by domestic dwellings, although alternative sites such as schools, shops may also be included. All trap sites will be geo-referenced using GPS and data managed with a GIS system. Appropriate consent for the placing and servicing of the traps will be sought.

Phase 2: Suppression phase

The purpose of the suppression phase is to determine the biological parameters of mosquito population suppression using RIDL technology in parts of Key West. In essence, this will test and refine predictions based on simulation models (Atkinson et al., 2007; Phuc et al., 2007; White et al., 2010; Yakob et al., 2008), previous experience elsewhere (e.g. Cayman Islands, see Appendix VI) and on the range-finding study (Phase 1 above). This phase will test whether RIDL technology in its present form is a biologically and technically feasible approach to *Aedes aegypti* suppression in Key West. If it is, Phase 3 (below) will go on to investigate operational feasibility in terms of integration with existing methods, resource requirements and scale-up to an area large enough to be of potential epidemiological significance.

The size and scale of a suppression trial will mainly be determined by the rangefinder study. However other factors such as production capacity with current facilities and manpower availability may change these parameters. A suppression trial will be conducted over a longer period than the range-finder trial, with an anticipated release period of 3-9 months. Release areas will be one or more blocks with a combined area of no more than 200 acres (c.f. total area of Key West approx 2000 acres). Control areas, without release of RIDL mosquitoes, will also be monitored to provide comparative data against which to assess the effect of RIDL releases on *Ae. aegypti* population dynamics. The key endpoint is statistically significant suppression of a target population relative to controls (control sites and/or historical data). It is anticipated that RIDL will be used in the context of ongoing conventional control initiatives.

Experimental Goal

- Attempt to suppress, in Key West, a target wild *Ae. aegypti* population using RIDL° technology

Specific objectives

1. Determine the change in time of mosquito population density in an area into which RIDL males are periodically released, relative to untreated areas.
   Therefore suppression endpoint is 'statistically significant suppression' relative to control areas, not suppression to zero, which is unachievable in a small, poorly isolated site.
2. Determine the relationship between OX513A male release numbers and OX513A (vs wild type) paternity of eggs
3. Determine the relationship between OX513A male release numbers and observed adult male sex ratio
4. Determine lifespan (daily survivorship) of OX513A males in the field
5. Estimate dispersal of OX513A males in the field
6. Establish a laboratory scale production and dispersal system for limited sustained release of sterile males OX513 mosquitoes in a field trial site.

Monitoring

The monitoring for this trial (ovitraps and BG sentinels) will be similar to the range finding trial. Eggs from Ovitraps will hatched and the percentage of fluorescent larvae will be determined. This percentage will be used as a guide for mating success in the field and to adjust the levels of release required. A GIS monitoring system will be used to track data and analyse population control.

BG traps will be monitored periodically for adult mosquitoes. Post-trial monitoring will continue until two consecutive trap periods have recovered no GE mosquitoes, unless Phase 3 of the research protocol continues immediately after phase 2. The over-flooding ratio from these will be used in conjunction with the ovitrap data to adjust the levels of release required to maintain the required release ratio.

Rearing and sex-separation

The scale of rearing required will be determined by estimates of the size of the population from the range finder trial and the size of the area to be treated. All eggs needed for the trial will be sent from Oxitec in UK and rearing and sex-separation will be performed essentially as described for the range finder study but on a scale relative to the size of the wild *Ae. aegypti* population and the size of the trial site.

Size of trial

Key West is an area of approximately 2000 acres and has a population of approximately 28,000 people. Historical surveys of Key West show that Old Town has a larger population of *Ae. aegypti* than elsewhere. Release areas will be one or more blocks with a combined area of no more than 200 acres. Control areas, without release of RIDL mosquitoes, will also be monitored to provide comparative data against which to assess the effect of RIDL releases on *Ae. aegypti* population dynamics.

Release

Release numbers will be proportional to the size of the field population as determined by the range finder study. Therefore absolute numbers of the released mosquitoes cannot be determined in advance of the estimation of the size of the field population. Releases will be conducted in a similar manner to the range finder trial, either as pupae or adults.

Other variables to be recorded

Meteorological data will be gathered from a weather station located in the field site. This will include temperature, rainfall, wind speed and direction. Communication will be maintained with local health workers to address any concerns that might arise during the period of up to 1 year post-release.

Retention of data

Raw data will be retained by the investigator for a period of 5 years.

Data analysis

Release points and trap positions will be geographically referenced using global positioning systems (GPS). Data collected will be analysed using appropriate statistical methodology with the aid of professional statisticians.

Due to the fluctuating nature of *Ae. aegypti* populations, changes to release numbers and the protocol may be required during the trial. All protocol changes will be recorded and reported in the trial report.

Phase 3: Pilot implementation trial

The purpose of the Pilot Implementation Trial is to evaluate operational use of RIDL technology, integrated with other methods, for the control of *Ae. aegypti* in Key West. In terms of releases, this will involve a geographic expansion of the suppression trial to cover a larger proportion of Key West (up to 100%). While the methods remain essentially as described for the suppression trial, the emphasis is more on operational integration, systematisation and efficiency, and an assessment of the potential for integrated vector management with a RIDL technology component for sustainable *Ae. aegypti* control, especially in Key West. In contrast the primary emphasis of the suppression trial is on the biological side of the question, i.e. to analyze the interaction between release of RIDL male mosquitoes and perturbation of the wild mosquito population.

The size and scale of the pilot implementation trial will mainly be determined by the suppression trial. However other factors such as production capacity with current facilities and manpower availability may affect these parameters. A pilot implementation trial will be conducted over a larger area than the suppression trial, up to the whole of Key West. Logistical and other considerations may lead to a smaller initial release area within Key West, with gradual or stepwise roll-out to a larger area. The pilot implementation trial may follow on directly from the suppression trial, e.g. represent expansion or roll-out from the suppression trial release areas. The minimum anticipated release duration is 6 months. This may be extended, either to achieve greater suppression or to maintain an adequate level of suppression once achieved. It is anticipated that RIDL will be used in the context of ongoing conventional control initiatives as part of an integrated vector control program.

Experimental Goals

- Attempt to suppress, in a significant fraction of Key West, a target wild *Ae. aegypti* population using RIDL° technology in combination with other approaches
- Develop an integrated vector management system, including RIDL° technology in combination with other approaches, for sustainable control of *Ae. aegypti* in Key West.

Specific objectives

1. Demonstrate a reduction of *Ae. aegypti* in Key West
2. Establish a production and dispersal system for sustained release of RIDL mosquitoes in Key West
3. Refine estimates of costs and requirements of an integrated vector management (IVM) program against *Ae. aegypti* incorporating RIDL technology

Monitoring

The monitoring for this trial (ovitraps and BG sentinels) will be similar to the range finding and suppression trials. Eggs from Ovitraps will hatched and the percentage of fluorescent larvae will be determined. This percentage will be used as a guide for mating success in the field and to adjust the levels of release required. A GIS monitoring system will be used to track data and analyse population control.

BG traps will be monitored periodically for adult mosquitoes. Post-trial monitoring will continue until two consecutive trap periods have recovered no GE mosquitoes. The over-flooding ratio from these will be used in conjunction with the ovitrap data to adjust the levels of release required to maintain the required release ratio.

Rearing and sex-separation

The scale of rearing required will be determined by estimates of the size of the population from the range finder trial and suppression trials, data from the suppression trial on the relationship between release rates and population dynamics (i.e. suppression) and the size of the area to be treated. All eggs needed for the trial will be provided by Oxitec. Rearing and sex-separation will be performed essentially as described for the range finder and suppression trials but on a scale appropriate to the size of the wild *Ae. aegypti* population and the size of the trial site.

Size of trial

Key West is an area of approximately 2000 acres and has a population of approximately 28,000 people. It is likely that a program will be initiated where a proportion of Key West is treated and then a roll-out of control will occur from this area.

Release

Release numbers will be determined by estimates of the size of the population from the range finder trial and suppression trials, data from the suppression trial on the releastionship between release rates and population dynamics (i.e. suppression) and the size of the area to be treated. Releases will be conducted in a similar manner to the range finder and suppression trials, either as pupae or adults.

Other variables to be recorded

Meteorological data will be gathered from a weather station located in the field site. This will include temperature, rainfall, wind speed and direction. Communication will be maintained with local health workers to address any concerns that might arise during the period of up to 1 year post-release.

Retention of data

Raw data will be retained by the investigator for a period of 5 years.

Data analysis

Release points and trap positions will be geographically referenced using global positioning systems (GPS). Data collected will be analysed using appropriate statistical methodology with the aid of professional statisticians.

Due to the fluctuating nature of *Ae. aegypti* populations, changes to release numbers and the protocol may be required during the trial. All protocol changes will be recorded and reported in the trial report.

IV. Consequences of the Proposed Action and Alternative

The test sites proposed in Key West, Monroe County, Florida is an area that has local populations of *Ae. aegypti* mosquitoes. The local populations are increasingly difficult to control as there is widespread insecticide resistance and removal of all the breeding sites is difficult. Recently the area has also been subject to locally acquired transmission of the dengue virus. Consequently new tools are required to control the *Ae. aegypti* population in the area. The proposed field trial will test the utility of the use of sterile male *Ae. aegypti* to reduce the numbers of the local population of the mosquito. The proposed research will have no impact on the numbers or biting potential of other species of mosquito that might be in the area (such as *Ae. albopictus* or *Culex* mosquitoes).

Areas of concern identified include potential for risks to the environment, vector control workers, public and animal health and they are summarized in A below:

A. Summary of the Consequences

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Concern	Potential issues	No action alternative	Issue permit
Potential risks to the environment	Change in host range	No effect	No effect
	Change in environmental tolerances	No effect	No effect
	Changes in mosquito feeding behavior	No effect	Males don't bite
	Change in development time	No effect	No effect
	Change in habitat range	No effect	No effect
	Changes in survival or fitness of the organism	No effect	Mosquitoes will die
	Mating with other organisms	No effect	No effect
	Stability of the introduced genetic components (genotype and phenotype)	No effect	No effect
	Gene transfer to other organisms	No effect	No effect
	Potential for adverse effects on non-target organisms including threatened and endangered species	No effect	No effect
	Persistence in the environment	No effect	No effect
	Change in susceptibility to insecticides	No effect	Potential benefit
	Effects on chemical load on the environment	No effect	Potential benefit
	Possibility of unanticipated changes, resulting in risk to the environment	Negligible	Negligible
	Population crash of *Aedes aegypti*	No effect	Potential for other vector species to occupy niche
Potential risks to public health	Potential impacts on humans, including minorities, low income populations and children	Potential for increases in dengue fever cases	Potential benefit to reduce vector that transmits dengue.
	Physical and /or biological confinement of the mosquito	No effect	Genetic sterility
	Changes in the potential to control local mosquito populations	No effect	Potential benefit
	Potential for the increase in female mosquitoes	Not applicable	No effect
	Potential for lethality trait to exhibit incomplete penetrance	Not applicable	Negligible effects.
Potential risk to animal health	Ability to transmit zoonotic diseases	No effect	No effect/potential benefit
Potential risk to vector control workers	Occupational exposure to mosquito bites by workers	No effect	No effect/potential benefit

B. Analysis of Issues, Consequences, and Theoretical Risks of Field Research with RIDL *Aedes aegypti*

i. Environmental safety

Summary

Overall risk is negligible in comparison to the no action alternative of continuing current control methods.

Change in host range

A potential hazard is that the mosquitoes could feed on other hosts. A larger or modified host range could include other mammals, including livestock, causing the potential zoonotic transmission of animal viral diseases. In the proposed experiment male mosquitoes will be released; male mosquitoes do not bite or transfer diseases as their mouthparts are adapted to nectar feeding. Further information is given in the section on the potential for transmission of zoonotic diseases.

Changes in environmental tolerances (temperature/humidity)

The potential hazard is the genetically engineered mosquitoes could change their environmental tolerances to temperature and/or humidity. This could lead to an increase in mosquito geographic range and/or survivability. The scenarios that could lead to potential for a change in environmental tolerances would be if the introduced genetic traits changed the diapausing ability of the eggs, or the introgression of the traits into different genetic backgrounds triggered a pleiotropic effect. *Ae. aegypti* already have a cosmopolitan geographic range, and are distributed globally around the world, particularly where the temperature is above 10°C and below 44°C and there is sufficient humidity. *Ae. aegypti* does not diapause (Chang et al., 2007; Urbanski et al., 2010). The genetically engineered mosquitoes have been introduced to a variety of *Aedes* genetic backgrounds (Asian, Latin American – Oxitec unpublished information) with no genotypic or phenotypic instability noted. Consequently, the probability of the genetically engineered *Ae. aegypti* strains changing their environmental tolerances is negligible.

Changes in mosquito feeding behavior

This has been addressed in the potential to expand the host range above. Male mosquitoes will be released which do not bite or transfer diseases as their mouthparts are adapted for nectar feeding. *Ae. aegypti* does not pollinate crop plants and the only recorded plant dependent on *Aedes* species, although not *Ae. aegypti,* for pollination is one species of bog orchid (Thien, 1969), present in north-western USA.

Changes in development time

No changes in development time have been observed in the mosquitoes in laboratory colonies in the UK or Malaysia. In the UK, the *Ae. aegypti* strain has been reared constantly for over 60 generations and in quantities of over 2 million/week with no change in development times observed (Oxitec, unpublished data). The Malaysia laboratory examined comparative life history parameters of a wild-type laboratory strain of *Ae. aegypti* and the genetically modified strain OX513A (referred to as LA513 in some published information). They found that the following parameters were indistinguishable in both strains: the number of eggs laid, the number of unhatched eggs, the egg-hatching rate, the duration of the larval period in all four instars, larval survivorship, pupation, adult eclosion rate, gonotrophic cycle, adult fecundity, adult lifespan and offspring sex-ratio. This study has been published in the 2009 Dengue Bulletin (Lee et al., 2009).

Changes in habitat range

Ae. aegypti mosquitoes are an invasive mosquito species introduced into the United States with human migrations and international trade. It is regarded as uniquely domestic species of mosquito, tied closely to human habitations and urban areas and has limited interactions with ecological systems outside the domestic settings. The presence of suitable breeding sites, along with the availability of a blood meal, strongly influences the range of the mosquito. As the proposed release site has plenty of breeding sites and a human population there would be no advantage in the adaptation of the mosquitoes to a different habitat range. Additionally the introduced genetic traits, of conditional lethality and fluorescent marker genes are unlikely to exert any physiological influence on the mosquitoes that would lead to a habitat range change.

Changes in survivability or fitness of the organism

The introduced traits confer a strong selective disadvantage to the mosquito – the mosquitoes are engineered to express a dominant lethal gene, which at a sufficiently high level of expression adversely affects the cell cycle and leads to cell death. The origin and characteristics of the expressed protein have been described in Appendix C of the Final Environmental Impact Statement on the Use of Genetically Engineered Fruit Fly and Pink Bollworm in APHIS Plant Pest Control Programs (73 FR 67511). Consequently, the released RIDL male *Ae. aegypti* mate with the local female *Ae. aegypti* and the progeny do not survive to adulthood. If the dominant lethal gene is not 100 percent effective then a small number of transgenic insects could occur in the field. The survival of a small number of progeny is unlikely to pose additional risk, as the large majority of the insects in the next and subsequent generations would inherit the construct and will not survive or reproduce.

In the case of the strain OX513A, proposed for release in this trial the penetrance of the lethality trait has been described by Phuc et al. (2007) and it exhibits approximately 97% penetrance. This figure has been obtained under laboratory conditions designed to be optimal for survival, where even under such conditions the rare survivors are weak and short-lived. Evidence from other experiments with different insect species carrying the similar constructs indicates that penetrance in the field is likely to be significantly higher in the field due to the harsher conditions and predation.

Fitness factors that relate to persistence and establishment of populations in the environment do not apply to conditional lethal traits, such as that expressed in *Ae. aegypti* as the released insects will die and their progeny will not survive to adulthood.

RIDL systems are repressible in the presence of the antidote tetracycline and its analogues, allowing the switching of the conditional lethality trait in the laboratory to allow rearing in the laboratory or the rearing facilities. If RIDL mosquitoes are exposed to tetracycline at a sufficient concentration in the environment there is a small, but remote risk that the conditional lethality could be switched off, making the released mosquitoes fertile. *Ae. aegypti* breeds in containers of clear, stagnant water, either rainwater-filled or human-filled water storage containers. Such containers do not contain exogenous tetracycline. Even if tetracycline was introduced into these breeding sites, it is sensitive to heat and light and has a short half-life in the environment. In the extremely unlikely event that tetracycline of sufficient concentration could be accessed by mosquito larvae then only those larvae that survived (not predated, etc) could become adults, but as they carry the transgene for lethality their progeny would not survive, unless they had further access to tetracycline and its analogues in sufficient concentration and at the right developmental stage.

Routes of exposure to tetracycline are likely to be from intensive animal rearing operations, prophylactic use by humans or for companion animals. *Ae. aegypti* does not occupy containers that could be contaminated by tetracycline from these routes of exposure, as it prefers clear stagnant waters.

Mating with other organisms

Ae. aegypti has complex mating behaviors and mating is extremely species-specific. There are reports of potential cross-mating in the laboratory with the related *Ae. albopictus*, but even with forced matings in the laboratory the eggs fail to develop (Nasci et al., 1989; Nazni et al., 2009).

ii.   Stability of the introduced genetic components (genotype and phenotype)

Genotype stability

The genetic modification of the strain is described (Phuc et al., 2007). Transformation was effected with a non-autonomous *piggyBac* transposable element, or transposon, co-injected with a non-integrating source of *piggyBac* transposase. A non-autonomous transposon is prevented from moving within or outside the genome of its host because it does not encode or produce the transposase enzyme that is necessary for such movement. Previously it has been shown that mobilization of donor *piggyBac* transposon could be induced in *Drosophila melanogaster* in the presence of exogenous transposase (Thibault et al., 2004). However, recently it has been shown that the non-autonomous *piggyBac* vector is very stable in the *Aedes* genome even when

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exposed to exogenous transposase under a wide variety of conditions (O'Brochta et al., 2003; Sethuraman et al., 2007).

One exceptional insertion event in *Ae. aegypti* was documented by Adelman et al. (2004). This non-canonical insertion event led to the integration of a multicopy array of linearized plasmids, including the helper plasmid (which does not contain the *cis*-acting elements required for *piggyBac*-mediated insertion) as well as the *piggyBac* plasmid. Consequently the inserted DNA contained multiple tandem repeats and its instability may be attributable to instability *via* recombination between these repeats. Such rare insertion events are readily identified as they contain plasmid backbone sequence that does not integrate in a canonical event, furthermore the DNA adjacent to the *piggyBac* ends ('flanking sequence') is derived from the injected plasmid, rather than from *Ae. aegypti* genomic sequence, at least one end. Such insertions may be of some scientific interest but will be eliminated from the product development pipeline at a very early stage.

Published data (O'Brochta et al., 2003; Sethuraman et al., 2007) and additional unpublished work at Oxitec indicate that inserted *piggyBac* elements are completely refractory to germline remobilization, even when deliberately re-exposed to *piggyBac* transposase under a wide variety of conditions. The consequences of such remobilization were considered in detail in the USDA's Final Environmental Impact Statement (FEIS[4]) on the use of autocidal methods (RIDL) in fruit flies and pink bollworm, as *piggyBac* is known to remobilizable in these species. The issues discussed there are essentially the same as for any hypothetical remobilization event in *Ae. aegypti*.

The homozygous OX513A strain has been reared at Oxitec UK since 2005 and at the Institute for Medical Research Malaysia since 2006 (about 60 and 40 generations, respectively). Molecular analysis has found no evidence for loss or movement of the transgene from its original insertion site within the genome.

Phenotype stability
The two key phenotypic traits introduced into the mosquitoes are the expression of a conditional lethal sterile trait and a fluorescent marker gene. The conditional lethal sterile trait prevents the progeny of a mating with the wild type surviving to adulthood. This is the same concept as making insects sterile with irradiation (Sterile Insect Technique) but avoids radiation damage to insects, costs of the process and the need for a radioactive source. SIT has been widely used as a successful control tool in plant pest species for over 50 years, but has been largely unsuitable for mosquitoes as the dose required to achieve sterility was too damaging to the fitness of the mosquito. However, radiation-based SIT in mosquitoes is now being used in the Sudan for *Anopheles arabiensis* (Helinski et al., 2008).

Monitoring of the two key phenotypes or novel traits expressed by the OX513A insertion, (i) fluorescent marker and (ii) lethality when reared without tetracycline, has been conducted on a regular basis in both UK and Malaysian laboratories. None of these tests has produced evidence of breakdown, suppression or separation of these phenotypes of the RIDL system. In addition to Malaysia and the UK, this strain is also being reared in independent laboratories in other parts of the world (e.g. Thailand, India, Brazil, Vietnam, Grand Cayman and Singapore) and there have been no reports from these laboratories of any changes or instability in the phenotype.

Phenotypic characteristics that might be considered to be hazard-related are also contingent on the biological fitness characteristics and/or the potential transfer of introduced genes into other organisms of the genetically engineered mosquitoes.

Biological fitness characteristics
The released mosquitoes are sterile and progeny from matings with populations of *Ae. aegypti* mosquitoes in the environment are unable to survive to adulthood. Consequently the biological fitness of both male and

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[4] Available at http://www.aphis.usda.gov/plant_health/ea/geneng.shtml

female sterile genetically engineered mosquitoes could be considered as approximately 0% as neither gender can reproduce successfully. In reality, male mosquitoes will be released to mate with local populations of *Ae. aegypti* mosquitoes. Fitness factors that relate to persistence and establishment of populations in the environment do not apply to conditional lethal traits, such as that expressed in *Ae. aegypti* as the released insects will die and their progeny will not survive to adulthood. Lee et al, (2009) found that the following parameters were indistinguishable between wild-type *Ae. aegypti* and the genetically engineered OX513A strain: the number of eggs laid, the number of unhatched eggs, the egg-hatching rate, the duration of the larval period in all four instars, larval survivorship, pupation, adult eclosion rate, gonotrophic cycle, adult fecundity, adult lifespan and offspring sex ratio. The mating competitiveness of the strain has also been examined in independent laboratories in USA and Thailand (Clark et al., 2010; Khongtak et al., 2009) and the strain was found to be equally competitive in mating ability compared to wild-type. Any fitness advantage that did conceivably exist would have to compete for survival against overwhelming reproductive sterility, i.e. a selective disadvantage.

iii. Gene transfer to other organisms

Vertical gene transfer

Vertical gene transfer refers to the ability of genes to move via sexual reproduction. *Ae. aegypti* reproduces sexually, usually in flight via internal gametes and a variety of mating cues. Mating cues include such a wing beat "song", the potential of a pheromone or kairomone attractant, and the size of the mosquito (Alongkot and Harrington, 2009), however these are not all well understood. Because of these highly developed mating behaviors mating with other insect species does not take place and *Aedes* mating is extremely species-specific. As discussed in mating with other organisms section, there have been reports of mating with the related species *Ae. albopictus* in both the laboratory and the field (Nasci et al., 1989), but even these matings, if successful, fail to produce embryos capable of developing to adulthood.

Horizontal gene transfer

Horizontal gene transfer describes the movement of genes between independent co-existing organisms from different species. It does not include the transfer of genes through sexual reproduction mechanisms i.e. breeding.

Horizontal gene transfer (HGT) between certain bacteria and other single-celled (prokaryotic) organisms can occur at a detectable frequency. HGT from multicellular (eukaryotic) organisms, such as insects, to bacteria is remarkably rare, occasionally being detected under optimized laboratory conditions, but never in natural or field conditions. HGT between eukaryotes has never been observed.

Specifically with regard to recombinant insects, different insect species rarely interbreed with each other in the wild, and even in laboratory conditions cannot form fertile hybrids. These mating barriers restrict any introduced genes to species that contains them. This is in contrast to many other higher organisms that release genetic material into the surrounding environment, such as pollen or spores, or sperm in fish. Insects do not freely release their gametes.

The potential for horizontal gene transfer from transgenic insects has been extensively reviewed in the Final EIS on the use of genetically engineered fruit flies and pink bollworm (Appendix C and D of that document (USDA, 2009). In summary, there are two hypothetical scenarios that can be envisaged:

a) *The transposable elements that are used during the transformation of the mosquitoes, which are capable of transferring segments of DNA from one site to another, could potentially transfer genetic materials to other organisms.*

Transformation was effected with a non-autonomous *piggyBac* transposable element, or transposon, co-injected with a non-integrating source of *piggyBac* transposase. A non-autonomous transposon is prevented from moving within or outside the genome of its host because it does not encode or produce

the transposase enzyme that is necessary for such movement. Previously it has been shown that mobilization of donor *piggyBac* transposon could be induced in *Drosophila* in the presence of exogenous transposase (Thibault et al., 1999). However, recently it has been shown that the non-autonomous *piggyBac* vector is very stable in the *Aedes* genome even when exposed to exogenous transposase under a wide variety of conditions (O'Brochta et al., 2003; Sethuraman et al., 2007). As far as we can tell from published data (O'Brochta et al., 2003; Sethuraman et al., 2007) and additional unpublished work at Oxitec, inserted *piggyBac* elements are completely refractory to germline remobilization, even when deliberately re-exposed to *piggyBac* transposase. The consequences of such remobilization were considered in detail in the USDA's Final Environmental Impact Statement (FEIS[5]) on the use of autocidal methods (RIDL) in fruit flies and pink bollworm, as *piggyBac* is known to remobilizable in these species. The issues discussed there are essentially the same as for any hypothetical remobilization event in *Ae. aegypti*. Most of the relevant information is in Appendices C and D of the FEIS, especially pages C-2 to C-9. Note that the post-integration stabilization method developed by Oxitec [(Dafa'alla et al., 2006) and FEIS Appendix C Figure 1] is not applicable to *Ae. aegypti* as it depends on remobilization (excision) of integrated non-autonomous *piggyBac* elements at a reasonably frequency when re-exposed to *piggyBac* transposase, which does not occur in *Ae. aegypti* (O'Brochta et al., 2003; Sethuraman et al., 2007).

b) *Transfer of the introduced genetic elements could be transferred to other organisms by ingestion of the mosquitoes by predators and prey.*

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

- Millwood et al. (2010) Fluorescent Proteins in Transgenic Plants. Reviews in Fluorescence 2008:387-403.
- Stewart (2006) Go with the Glow: Fluorescent Proteins to light transgenic organisms. Trends in Biotechnology 24(4):155-162

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

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

Similarly the conditional lethal element, known as the tTA system developed by Gossen and Bujard (1992) has been widely used both *in vitro* and *in vivo* for over a decade. Low-level expression of tTA has been widely used and thought to be innocuous; whereas a high level expression is thought to be deleterious to cells, likely due to transcriptional "squelching" (Gill and Ptashe, 1988) and /or interference with ubiquitin-dependent proteolysis.

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

- Zhou et al. (2009) Developing tTA transgenic rats for inducible and reversible gene expression. Int J Biol Sci; 5(2):171-81.
- Barton et al. (2002) Modified GFAP promoter auto-regulates tet-activator expression for increased transactivation and reduced tTA-associated toxicity. Brain Res Mol Brain Res 101(1-2):71-81.
- Chen et al. (1998) Transgenic animals with inducible targeted gene expression, in brain. Mol Pharmacology 54(3) 495-503.

Acquisition of genes through oral ingestion or blood feeding

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[5] Available at http://www.aphis.usda.gov/plant_health/ea/geneng.shtml

One potential hazard could be that the blood meal taken by the female mosquito in the laboratory might provide an opportunity for horizontal gene transfer. However, mosquitoes have been feeding on humans and other mammals for millennia, probably more than 100 million years. Complete genome sequences are now available for several mammalian species, including humans, and several mosquito species, including *Ae. aegypti*; there is no evidence of horizontal gene transfer via blood feeding. If this occurred, even at extremely low frequency, one would see DNA sequences from humans in human-feeding mosquitoes, from birds in bird-feeding mosquitoes and so forth and *vice versa* under the even more implausible hypothesis of DNA transfer from mosquito to host. More generally, animals do not incorporate DNA from their food into their genomes.

If the organism does acquire a gene through horizontal gene transfer then it must maintain it to have an impact or consequence in the environment. To have an impact, a significant number of organisms must acquire the new gene. This depends on the rate of HGT, the nature of the gene, the incorporation of the gene into heritable cells and environmental influences. If maintaining the new gene has no advantage for an organism, the gene will not persist. The strain of *Ae. aegypti* to be released contains no DNA that would confer a selective advantage to an organism; in fact the contrary is true as they confer a strong selective disadvantage. Consequently any hypothetically transferred genetic material would be rapidly lost from the recipient population.

Potential for adverse effects on non-target organisms including threatened and endangered species
Ae. aegypti is a peri-domestic species closely associated with human habitation and urban habitats, with limited interactions with organisms outside of the domestic habitat. It is an invasive species in the USA, introduced with the movement of humans and trade routes. It is present in the largest densities in the Gulf Coast States. It is continually suppressed by control methods such as the use of insecticides and breeding site source reduction, these methods already reduce the *Ae. aegypti* population to low levels and also the use of chemical control methods may be considered to have a greater environmental impact than the result of the suppression of *Ae. aegypti*. For example, pyrethroid-based sprays are considered an extreme toxicity hazard to aquatic organisms (Wheelock et al., 2008).

A threatened and endangered species habitat analysis has been carried out for Monroe County (attached – **Appendix VIII**) and none of the habitats overlap with the peri-domestic/domestic environment of *Ae. aegypti*. There are no Indian Tribal Lands in the vicinity of Key West or the trial site. The nearest Indian tribe (Miccosukee) is located in Broward County 40 miles west of Miami, bordering on Monroe County, at the Tamiami Trail Reservation[6].

In summary, because *Ae. aegypti* is an invasive, peri-domestic/domestic species, is currently controlled by chemical methods, and has limited interactions with natural ecosystems it is considered highly unlikely that non-target organisms would rely on it as either as a food source or as a provider of ecosystem services (such as pollination, decomposition, etc).

Persistence in the environment
Factors that relate to persistence and establishment of populations in the environment do not apply to conditional lethal traits, such as that expressed in *Ae. aegypti* as the released insects will die and their progeny will not survive to adulthood. However the introduced lethal trait, is conditional on the absence of a dietary antidote, tetracycline. A potential hazard could be that the environmental concentration of tetracycline may be high enough to "switch-off "the conditional lethality and allow the mosquitoes to survive in the environment. The proposed potential release sites represent peri-domestic habitats, the preferred habitat of *Ae. aegypti* mosquitoes. Uses of tetracycline in such habitats are likely to be at very small scale and intermittent (human therapeutic use, or local veterinary treatments of companion animals). From our survey, the areas around the proposed sites include managed vegetation, such as gardens and cemeteries, and

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[6] http://www.miccosukeetribe.com/ Accessed 10 Feb 2011

houses. We did not find any large scale animal or fish farming site that could use larger quantities of tetracycline within 500 m of the proposed locations. Additionally there are no industries using tetracyclines within the vicinity (>500 m) of the proposed locations. The *Ae. aegypti* mosquito does not fly very far providing there are human hosts available (Maciel-de-Freitas et al., 2010; Suwonkerd et al., 2006). Besides, roads, water courses and vegetation represent significant barriers to the movement of *Ae. aegypti* (Hemme et al., 2010). Tetracycline is known to be rapidly degraded by ultra-violet radiation (Bautiz and Nogueira, 2006) in the presence of iron (Fe) or other metal catalysts (Reyes et al., 2006), with total deactivation occurring in 70 minutes. The use of tetracycline in the environment was reviewed by Sarmah et al. (2006) and again tetracycline was found to have rapid degradation (with the bulk of degradation taking place on day 1) and a short half-life in the environment (15-30 days in water and up to 9 days in animal manure). A recent study (Kim et al., 2010) simulating the effect of rainfall on transport of veterinary antibiotics (VAs) showed that tetracycline and chlortetracycline were present in aqueous run-off up to 45 minutes and sediment up to 10 minutes and then was below the level of quantification (LOQ) for the rest of the study. It is likely that the complex nature of the environmental conditions, daily rain intensity, temperature, solar radiation, soil type and size and type of soil microflora will have an impact on the degradation times, most likely decreasing the degradation time compared to those in controlled laboratory conditions.

Consequently tetracycline concentrations in the environment are unlikely to reach a suitable concentration to allow the survival of OX513A mosquitoes in the field. It should also be borne in mind that the *Ae. aegypti* mosquito is a container breeder which prefers to breed in clear, stagnant water, and not in sewers or drains. Rain and stored water (from tap or well) usually constitutes the containers in which *Aedes* breeds, and these sources are unlikely to contain tetracycline.

Potential for lethality trait to exhibit incomplete penetrance
In the OX513A mosquito strain, penetrance of the lethal phenotype is about 97-98% in both male and female heterozygotes (Phuc et al., 2007). This means that, in the laboratory, 2-3% of the progeny of RIDL males and wild type females will survive, if reared without the antidote.

What is the potential impact of such reduced penetrance?

- This does not represent resistance, as the survivors show the same 2-3% survival in their progeny; it is genuine incomplete penetrance.

- These tests were performed in the laboratory, under conditions ideal for survival. For a similar strain of pink bollworm (an agricultural pest), survival was much lower for RIDL heterozygotes reared in the field than in the lab, so this figure of 2-3% is probably a considerable overestimate of the value likely to be observed in the field.

- Modeling shows that above a threshold level of about 90%, incomplete penetrance has little effect on program effectiveness; OX513A is well above this level, even in the laboratory.

- If a few heterozygotes can survive in the field, the RIDL construct will not disappear from the wild population within one generation after cessation of releases, as would be the case if 100% penetrant. However, even 2-3% survival will still lead to rapid elimination of the RIDL construct, reducing in frequency by more than 30-fold each generation (one generation for this mosquito is a few weeks, depending on temperature).

- Some of these rare survivors will be females, and therefore potentially capable of biting. However, a RIDL control program would only be conducted in an area that had a substantial (and problematic) mosquito population already. The small number of female RIDL heterozygotes generated by this method would have negligible consequences. Mass-reared insects in conventional SIT programmes are known to be shorter lived than equivalent wild insects. The reasons for this universal trait are not entirely clear, but are likely to involve a combination of genetic and environmental effects of artificial rearing and handling.

This is significant as shorter-lived females are much less effective as disease vectors, i.e. in their ability to transmit disease. The reason is due to the biology of the disease. Female mosquitoes emerge as adults without the pathogen (e.g. dengue virus). If they bite an infected human, they may themselves become infected. The pathogen invades the mosquito and replicates inside it, but the mosquito only becomes infectious after 7-12 days (Watts et al., 1987). The mosquito then remains infectious (to humans) for the rest of her life. Average female lifespan is only around 7-10 days, depending on location (Focks et al., 1993). Therefore, most disease transmission is due to a relatively small number of older females; even a modest reduction in average life expectancy results in quite a considerable reduction in the number of such females. RIDL females (or any mass-reared females) are therefore likely to be significantly less effective disease vectors, i.e. less dangerous, than wild females.

Overall, we conclude that incomplete penetrance at the level observed for OX513A poses no threat to human health or the environment, nor would it have a significant negative impact on a control program based on the use of this strain.

Change in susceptibility to insecticides

Comprehensive data on insecticide resistance status of mosquitoes in the proposed release area do not appear to be available at time of writing. *Aedes* mosquitoes in the proposed release area, and elsewhere in Florida, have been subject to chemical control for many generations, so a degree of insecticide resistance is to be expected. The OX513A *Ae. aegypti* mosquitoes to be released are sensitive to the insecticides used in the current control programs (Cayman Islands MRCU, unpublished data). The OX513A insertion has also been found not to impair the response to a range of irritants and repellents (Kongmee et al., 2010). This has the potential therefore to introduce susceptibility alleles into the population of the *Ae. aegypti* in the area, with the potential outcome of enhancing existing chemical control methods. However, the lethal effect of the transgene greatly reduces the survival of hybrids between the released transgenic mosquitoes and wild type. Therefore, little or no introgression of insecticide susceptibility alleles into the wild population is anticipated. To the extent that this occurred, it would be a desirable outcome (Alphey et al., 2009; Alphey et al., 2007). Even without consideration of introgression of susceptibility alleles, longer-term use of RIDL would be expected to slow the rate of appearance of heritable resistance to other interventions (e.g. insecticides) used in parallel, as has been reported for the use of irradiated sterile pink bollworm (*P. gossypiella*) in conjunction with *Bt* cotton in Arizona (Tabashnik et al., 2010).

Effects on chemical load on the environment

The use of engineered sterile males is a non-chemical approach to the control of *Ae. aegypti* which could be use in addition to existing chemical control or as a complete or partial substitute. If used strictly in addition then the chemical load would be unchanged. In more likely event of partial substitution, i.e. re-orientation of an integrated vector management (IVM) program now heavily reliant on chemicals to one with a substantial biological (RIDL) component, chemical load on the environment would decrease. In the context of trials it is likely that current non-genetic control methods will continue unchanged, i.e. neither increase nor decrease; in the longer term, introduction of a non-chemical method is expected to lead to a degree of substitution of chemical insecticide and consequent reduction in chemical insecticide load on the environment. In addition to chemical insecticides, one biological insecticide is widely used to control *Ae. aegypti* – *B. thuringiensis israelensis*. This microbial larvicide – as larvicides in general – is expected to combine well with RIDL technology, however the introduction of RIDL technology does not have direct implications for the choice of larvicide.

Possibility of unanticipated changes, resulting in risk to the environment

Ae. aegypti is an alien invasive species with respect to the Americas. It is not known to form fertile hybrids with other species present in the US (or elsewhere). The OX513A transgene is highly deleterious, in terms of biological fitness, to mosquitoes which carry it. Were the transgene to become less deleterious, through an unknown mechanism, a potential consequence would be that release of OX513A might be less effective at

suppression wild populations than anticipated. However, apart from a potential and indirect effect of opportunity cost, this class of unanticipated change poses no additional risk to the environment beyond the no action alternative. If, alternatively, the transgene were to become more deleterious, the effect would be similar in that the OX513A mosquitoes would become harder to rear and/or less effective in the field, perhaps reducing the degree of mosquito control achievable for a given resource expenditure, but with no significant additional risk to the environment.

The strain background genetics might also change over (e.g. through genetic drift or selection in rearing). This will typically lead to reduced field performance; performance in rearing may be improved (selection), worsened (inbreeding/drift) or not significantly affected. The effect of reduced field performance is described above.

Population crash of Aedes aegypti

In the context of *Ae. aegypti*, the long-term intended goal of the use of RIDL technology – in combination with other approaches – is to suppress *Ae. aegypti* populations, i.e. to reduce the long-term population density relative to the *status quo ante* or to the no action alternative. This is the declared intention of existing vector control programs, and is widely considered to be an acceptable, indeed desirable and intended outcome. Nonetheless the longer-term reason (beyond trial purposes) for introducing the use of RIDL technology is to facilitate or improve the reduction of *Ae. aegypti* populations and to the extent this ambition is realized then population reduction of *Ae. aegypti* in the treatment area will correspondingly become more likely. This raises the question as to whether such reduction is an unmitigated public good (e.g. in respect of reduced biting rate and reduced risk of transmission of *Ae. aegypti*-borne diseases) or whether it might also have associated risks to the environment or public health.

The effect of removing *Ae. aegypti* is not specific to RIDL technology but would apply to any effective control method and is correspondingly not a new issue. This particular mosquito, *Ae. aegypti*, is native to part of Africa (Mousson et al., 2005), and is an invasive, alien species in most dengue areas - the Americas and Caribbean, India and South-east Asia, for example - where it has been inadvertently introduced by man. It is therefore unlikely that native species would be specialized or dependent on it. Its suppression or local elimination may therefore be considered to be a move towards a pre-introduction state. More generally, mosquitoes do not appear to be keystone species in ecosystems. A study conducted in Germany showed that all mosquito species together comprised only 0.16% of the total diet of an amphibian species in the Rhine Valley (Blum et al., 1997). Campos and Lounibos (2000) found that mosquito larvae made up only between 5 and 6% of the diet of the predatory mosquito, *Toxorhynchites rutilus*, in Southern Florida. Note that *Ae. aegypti* is only one of many mosquito species in Florida; around the world there are 3000 or more described species of mosquito. Fang (2010), writing in *Nature*, concluded that even global elimination of *all* mosquito species would cause minimal ecological harm, with the biggest effect being biomass reduction in arctic regions.

The current distribution of *Ae. aegypti* in the USA covers a narrower geographic range than previously. This is believed to be consequent on the more recent introduction and spread of another mosquito species, *Ae. albopictus* (Hobbs et al., 1991; Moore and Mitchell, 1997; O'Meara et al., 1995). *Ae. albopictus* is also an alien, invasive species in the US, being native to parts of Asia. Where these two species are present, both in Asia and in the Americas, *Ae. albopictus* is typically found in more vegetated areas, e.g. rural or suburban areas, while *Ae. aegypti* is found in more urban areas (Honório et al., 2009; Tsuda et al., 2006). Environmental factors such as temperature and rainfall may also affect the equilibrium distribution, as may competition between these two species for one or more resources such as larval sites. It is also possible that sterile interspecies mating occurs, representing a form of natural sterile insect technique, albeit likely at low frequency (Nasci et al., 1989). This natural reduction in range of *Ae. aegypti* in the US has not led to any observed negative impact on human health or the environment.

Beyond the US, *Ae. aegypti* was eliminated from large parts of South America (Camargo, 1967) as a result of large vector control programs coordinated by the Pan-American Health Organization (PAHO), again with no

observed negative impact on human health or the environment from the absence of the mosquito. Several islands, e.g. in the Caribbean, have had similar experience.

To the extent that a species is excluding others by its presence in a niche ['competitive exclusion' or, in a dynamic context, 'competitive displacement' (Lounibos, 2007)] then suppressing or removing it may allow some expansion in numbers or range of the excluded competitor. In the case of *Ae. aegypti* in the US, the key potential competitor in this context is *Ae. albopictus*. It is not clear to what extent *Ae. albopictus* could or would expand its range into areas currently dominated by *Ae. aegypti* if the latter were removed, but it is reasonable to expect a degree of such expansion if no countervailing activities are undertaken. Two questions arise: (i) what would be the consequences of such expansion and (ii) what could be done to mitigate the expansion and/or negative effects?

Consequences of the spread of Ae. albopictus into areas currently dominated by Ae. aegypti

A recent major review concluded that *Ae. albopictus* is a much less effective vector of dengue than is *Ae. aegypti* (Lambrechts et al., 2010). Though there had been general agreement on this as a qualitative statement, the previous literature had been quite confused as to the magnitude of the effect. Lambrechts et al. (2010) clarify several issues, for example noting that *Ae. albopictus* strains seem to become more susceptible to dengue virus after several generations of laboratory rearing, such that laboratory studies tend to over-estimate the competence of this vector for dengue. Although both *Ae. albopictus* and *Ae. aegypti* can transmit other viruses and pathogens, there seems no reason to think that the replacement of *Ae. aegypti* by *Ae. albopictus*, for example in northern Florida, has had a significant negative effect on human health or the environment (Gratz, 2004; Lambrechts et al., 2010; Moore and Mitchell, 1997). One key aspect of this is that *Ae. aegypti* is much more anthropophilic/anthropophagic than *Ae. albopictus*, which makes it an inherently more effective vector of any human disease. *Ae. aegypti* and *Ae. albopictus* populations have been the subject of long-term monitoring in Florida (Britch et al., 2008) who identified that *Ae. albopictus* might be exploiting unoccupied or more marginal habitat than the habitat containing *Ae. aegypti* and this is particularly correlated with wet and dry periods, with *Ae. aegypti* being favored in dry periods due to its peri-domestic habitat and both species increasing in the wet periods. This conclusion is supported in a further study conducted in urban Florida (Leisnham and Juliano, 2009).

Mitigation

Conventional control methods such a larviciding and source reduction affect both *Aedes* species. Since it is anticipated that RIDL technology would be used with conventional methods, *Ae. albopictus* would be continue to be suppressed by these methods.

RIDL technology could also be applied to *Ae. albopictus* and deployed in conjunction with similar methods against *Ae. aegypti*, if and when this seemed desirable. Although this EA does not specifically address this eventuality, the technology and issues relating to use of the technology against *Ae. albopictus* are likely to be extremely similar to those analyzed here in the context of *Ae. aegypti*. Genetic transformation methods for *Ae. albopictus* have been developed for this purpose (Labbé et al., 2010) and prototype RIDL strains constructed[7].

Cessation of release of RIDL mosquitoes would remove the consequent pressure on the *Ae. aegypti* population, essentially returning to the present situation, or no action alternative. Over time, the two species would re-equilibrate to their present level. The time for this will depend on the extent to which the RIDL-based control has been successful, i.e. the degree of additional suppression of the *Ae. aegypti* population because of the use of RIDL technology. Except in the case of complete elimination of *Ae. aegypti* over a very large area, re-equilibration is likely to take a small number of years and require no special human intervention.

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[7] http://www.oxitec.com/our-products/asian-tiger-mosquito-control/ accessed Jan 21, 2011

iv.   Public health

Potential impacts on humans, including minorities, low income populations and children

Summary
The risk to public health is low, compared to the no action alternative and the increasing burden of locally transmitted dengue in the area. The suppression of the vector of both human and zoonotic diseases has some potential benefits, although in this trial they may not be quantified. There are no indications of special safety measures being required for the general public, to conduct this trial.

Dengue
Dengue is a threat to all inhabitants of affected areas. Some risk factors are higher for low-income populations, e.g. less well screened housing; to the extent that this is the case such groups will benefit more from area-wide dengue control than others, being at higher risk initially. Similarly, dengue is primarily a pediatric disease in many countries though this may relate largely to epidemiological issues such as average time to first infection in high-transmission settings, rather than being inherently a pediatric condition (Guzman et al., 2002; Hammond et al., 2005). To the extent that children are at greater risk from the disease, they will benefit more from area-wide dengue control.

Chikungunya
Uninfected genetically modified arthropod vectors fall under Arthropod Containment Level 2 in the USA, provided the modification has no, or only negative effects on viability, survivorship, host range, or vector capacity (ASTMH, 2003). Chikungunya is an arboviral disease spread via mosquitoes of the *Aedes* species, both *Ae. aegypti* and *Ae. albopictus*. It is endemic to Africa and Asia and returning travelers to the USA have brought the disease into the country, although local transmission has yet to be identified, due to a combination of environmental factors, although the vectors are present in south-eastern states (Lanciotti et al., 2007). Chikungunya, like dengue, is a threat to all inhabitants of an area. There is no vaccine and mosquito control remains the best solution to the disease (Pialoux et al., 2007). The proposed trial will target the *Ae. aegypti* mosquitoes that also have the potential to spread this virus in the population.

Potential hazards to human health
Potential hazards to human health that could be envisaged have been described by Beech et al. (2009) and Benedict et al. (2010), and include:
- Released mosquitoes could have changed vector competence for human disease
- Suppression of the target mosquito could lead to increase in other vector species and impact the disease incidence or introduce new diseases
- Increase in the numbers of female mosquitoes in the environment that could bite humans
- An increase in nuisance biting

The consequences and likelihood of each of these potential hazards are addressed:
- Released mosquitoes could have changed vector competence for human disease:
  Male mosquitoes will be released in the trial, which do not bite humans or vector disease. There is the potential for very small numbers of females of the strain to be released (less than 1%) which could bite humans and vector disease. Lee et al. (2009) indicates that the female species of the transgenic strain has the same characteristics as the wild-type when in the laboratory and therefore likely to have the same vectorial capacity. However, females that are laboratory reared are likely to be weaker and more short-lived than their wild type in the environment. Transmission of the dengue virus is related to the age of the female mosquito and the longer-lived the more likely it is to transmit pathogens (Dye, 1992). The potential impact of unintended release of a few (uninfected) females has been analysed and no significant risk identified. There are already far more females in the local

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environment; the engineered *Ae. aegypti* females are shorter-lived than the local ones and any offspring that they produce would die just as the released males. Male mosquitoes cannot bite or spread disease. *Ae. aegypti* is also a vector of chikungunya disease and yellow fever. The control of *Ae. aegypti* through suppression of the population using SIT has the ability to reduce the incidence of these diseases as well to a greater extent than the use of pesticides alone, due to the innate ability of the male mosquito to seek out female mosquitoes for mating.

- Suppression of the target mosquito could potentially lead to increase in other vector species and impact the disease incidence or introduce new diseases:
  Both *Ae. albopictus* and *Ae. aegypti* can transmit other viruses and pathogens, however there seems no reason to think that the replacement of *Ae. aegypti* by *Ae. albopictus*, for example in northern Florida, has had a significant negative effect on human health or the environment (Gratz, 2004; Lambrechts et al., 2010; Moore and Mitchell, 1997). One key aspect of this is that *Ae. aegypti* is much more anthropophilic/anthropophagic than *Ae. albopictus*, which makes it an inherently more effective vector of any human disease.

It has been suggested that, paradoxically, the control of the vector could lead to increased dengue transmission, through the reduced immunity to the infectious disease, as less people are exposed (Goh, 1997; Thammapalo et al., 2008).

The "herd effect" [also known as "herd immunity" (John and Samuel, 2000)] refers to an effect whereby of induced immunity to an infectious disease in a significant proportion of a population provides a degree of protection for individuals who have not developed immunity. This protection is because the chain of infection tends to break if a high proportion of individuals in the population are immune. Immunity can be through vaccination or simply by recovery to an immune state after exposure to the pathogen. Though normally considered for diseases which are transmitted directly from human to human, the same principle applies to mosquito-borne diseases.

It has been suggested in some other contexts that prevention of transmission of a disease for a period of time might reduce the level of herd immunity and therefore the protective herd effect, leaving the population more vulnerable if the pathogen were reintroduced.

As an approximation, in a simple susceptible-infectious-resistant (SIR) model, the effective reproductive rate of a pathogen, R_{eff} is the product of the basic reproductive rate R_0 and S, the fraction of the target population susceptible to disease. Therefore, the herd immunity threshold is $S = 1/R_0$. Estimates (for several reasons these are likely to be underestimates) of the basic reproductive number (R_0) for dengue range from 1.33 to 11.6 (Halstead, 2008). This would imply a herd immunity threshold of 25-91%. Dengue seroprevalence in Key West was estimated to be approximately 5% in 2010 (Hitt, 2010) [c.f. 45% in Singapore, (Wilder-Smith et al., 2004)]. Herd immunity at this level is not expected to be significantly protective. Since the 2009 outbreak in Key West was the first local dengue transmission in Florida for many years, seroprevalence levels elsewhere in Florida, and indeed the continental US, are likely to be generally low. Therefore reduction in herd immunity is not expected to pose a significant hazard. Such reduction would in any case require long-term (multi-year) suppression of transmission, and is therefore not a potential outcome of short-term releases.

It has been suggested that, in countries with very high transmission rates, reduction in transmission could increase the frequency of dengue hemorrhagic fever (DHF) even while decreasing the incidence of dengue fever (DF) (Thammapalo et al., 2008). The proposed mechanism for this is a lower per-infection risk of DHF if a person is infected with a second virus serotype while still cross-immune due to recent infection with a different serotype. This effect requires (a) several virus serotypes to be present and (b) very high transmission rates so that a significant proportion of second infections are within this cross-immune period. The authors assumed a cross-immune period of around 2 years. Effective suppression of *Ae. aegypti* in a high transmission setting should bring transmission rates well below the relevant level; in any case the continental US is very far from this epidemiological situation, with low transmission rates.

Zoonoses

Mosquitoes in general are capable of transmitting several pathogens that can infect both humans and animals, and also several that normally infect only non-human animals leading to animal but not human disease.

- West Nile Virus (WNV) is spread by the bite of an infected mosquito, primarily but not exclusively from the genus *Culex*. Mosquitoes become infected when they feed on infected birds, including crows and magpies. The Centers for Disease Control and Prevention has identified over 250 species of vertebrate animal that can be infected by WNV. Infected mosquitoes can then spread WNV to humans and other animals when they bite. CDC reported that 62 Mosquito species have been found in West Nile-positive mosquito pools in the United States since 1999 (to 2007)[8] including multiple representatives from each of *Culex, Aedes*, and *Anopheles*, and specifically includes *Ae. aegypti* and *Ae. albopictus*.

- Several species of mosquitoes transmit encephalitis viruses to people and animals. In the majority of human cases, these viral infections manifest themselves only as general flu-like symptoms, ending with full recovery. Infection, may, however, lead to encephalitic (inflammation of the brain, which can be fatal or leave permanent neurological damage). *Aedes* spp. mosquitoes transmit, or are thought to be capable of transmitting, several arboviruses, notably those that cause LaCrosse and Eastern equine encephalitis (EEQ).

  o Eastern equine encephalitis virus (EEEV) has reemerged in the northeastern US. This is outside the present range of *Ae. aegypti*. EEEV is perpetuated in an endozootic cycle involving bird-feeding mosquitoes in freshwater swamps, occasionally infecting humans (and horses) via bridge vectors, though to be *Aedes vexans, Coquillettidia perturbans, Ochlerotatus canadensis* and *Oc. sollicitans* (Armstrong and Andreadis, 2010). *Ae. albopictus* was implicated in one isolated incidence of EEQ transmission in Polk County Florida in 1991 (Moore and Mitchell, 1997).

  o LaCrosse (LAC) virus is thought to be transmitted primarily by *Aedes triseriatus*. Several additional species of mosquito, including *Ae. aegypti* and *Ae. albopictus*, are competent vectors in the laboratory but not thought to be epidemiologically significant. Historically, most cases of LAC encephalitis occur in the upper Midwestern states but have been reported through to the east coast (approx 80-100 cases per year in USA reported to CDC[9]). Four cases of California serogroup virus neuroinvasive diseases were reported in Florida between 1964 and 2009[10]. Neuroinvasive disease includes cases reported as encephalitis, meningoencephalitis, or meningitis. Most reported cases of California serogroup virus neuroinvasive disease are due to La Crosse encephalitis virus.

  o St Louis encephalitis (SLE) virus is maintained in an endozootic cycle in birds, vectored by mosquitoes and transmitted from birds to man and other mammals by infected mosquitoes (mainly some Culex species: *Cx. pipiens* and *Cx. quinquefasciatus* in the eastern US, *Cx. nigripalpus* in Florida and *Cx. tarsalis* and *Cx. pipiens* complex in western states[11]). Humans and domestic mammals can acquire SLEV infection but are dead-end hosts. SLE is found throughout the United States, but most often along the Gulf of Mexico, especially Florida. Major SLE epidemics occurred in Florida in 1959, 1961, 1962, 1977, and 1990. The elderly and very young are more susceptible than those between 20 and 50. During the period 1964-1998 (34 years) a total of 4478 confirmed cases of SLE were recorded in the United States[12]. Laboratory transmission by *Aedes* mosquitoes has been reported but is not thought to be epidemiologically significant.

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[8] http://www.cdc.gov/ncidod/dvbid/westnile/mosquitospecies.htm accessed 02 Feb 2011
[9] CDC Fact Sheet http://www.cdc.gov/lac/tech/fact.html accessed 02 Feb 2011
[10] http://www.cdc.gov/lac/tech/epi.html accessed 02 Feb 2011
[11] http://www.cdc.gov/sle/technical/transmission.html accessed 02 Feb 2011
[12] http://www.cdc.gov/sle/technical/epi.html accessed 02 Feb 2011

Limited-scale release of engineered male *Ae. aegypti* would not significantly affect transmission of any of these pathogens relative to the no-action alternative. Larger-scale use, leading to local suppression of the *Ae. aegypti* population, would reduce the ability of such a population to transmit any of these diseases due to reduction to the number of potential vector mosquitoes (adult females). This would be a beneficial effect. However, since each of these pathogens can be transmitted by a number of mosquito species other than *Ae. aegypti*, the overall effect on transmission, though beneficial, is likely to be negligible. In the event of complete or partial niche replacement by *Ae. albopictus* (see above), such change is likely to be further reduced.

None of the effects described in this section are specific to the use of RIDL mosquitoes, rather they would apply to any effective method for reducing dengue transmission by targeting the vector mosquitoes. Vector control is an established paradigm for dengue control, the perceived problem is that it is not adequately effective when using current methods in real-world situations, not improved methods would be hazardous, e.g. for the reasons described in this section.

v.   Animal health

Mosquitoes in general are capable of transmitting several pathogens that can infect both humans and animals, and also several that normally infect only non-human animals leading to animal but not human disease. Pathogens that can cause significant disease in humans are discussed under 'Human health' above. Several mosquito-borne pathogens are known which cause significant animal disease but which do not cause significant human disease, including canine heartworm, Lumpy Skin disease and fowl pox.

- Canine heartworm infects dogs and related canines (foxes, coyotes, and wolves, and cats), but not normally humans, through mosquito-feeding activities. Tiny microfilarial worms, a lifestage of the filarial nematode *Dirofilaria immitis*, enter the animal's blood through the mosquito bite. Once inside the animal, they grow quite large, measuring up to 10 inches in length, and they typically live in the animal's pulmonary artery and "right" heart. The resulting thickening and inflammation of the heart cause symptoms such as difficulty in breathing, chronic cough, and vomiting, and the disease can be fatal. Canine heartworm occurs worldwide and is transmitted by several species of mosquitoes. Sixteen species of mosquitoes (*Aedes albopictus, Ae. canadensis, Ae. cantator, Ae. crucians, Ae. sollicitans, Ae. sticticus, Ae. simulans, Ae. taeniorhynchus, Ae. vexans, Anopheles bradleyi, An. punctipennis, An. quadrimaculatus, Culex nigripalpus, Cx. quinquefasciatus, Cx. salinarius* and *Psorophora ferox*) have been identified as natural hosts of *D. immitis* (dog heartworm) in the United States east of the Mississippi River (Arnott and Edman, 1978; Buxton and Mullen, 1980; Comiskey and Wesson, 1995; Grieve et al., 1983; Johnson and Harrell, 1986; Licitra et al., 2010; Nayar and Knight, 1999; Parker, 1986, 1993; Sauerman and Nayar, 1983; Todaro et al., 1977). Among these, only 11 species are found in any abundance in Florida. Collection of mosquitoes in residential areas in Vero Beach FL, showed that 4 species (*Ae. taeniorhynchus, An. quadrimaculatus, Cx. nigripalpus* and *Cx. quinquefasciatus*) are natural hosts of *D. immitis* (Sauerman and Nayar, 1983). At least seven mosquito species [*An. quadrimaculatus, Ae. taeniorhynchus, Ae. sollicitans, Ae. aegypti, Cx. nigripalpus, Cx. quinquefasciatus* and *Mansonia titillans*] can be infected with *D. immitis* when they are fed on an infected dog (Nayar and Sauerman, 1975).

  o   Humans are exposed to infection by canine heartworm *via* bites from infected mosquitoes. The parasite cannot survive and proliferate in humans but dead parasites are occasionally associated with minor lesions, e.g. detectable by X-ray and potentially confused with other, more severe conditions. Over a 30-year period, approximately 100 cases of human pulmonary dirofilariasis were reported from Florida (Nayar, 1998). No human fatalities are known to have been caused by canine heartworm infection.

- Lumpy Skin disease (LSD) is caused by a capripoxvirus. It is not known to transmit to humans. Epidemiological evidence indicates the involvement of biting insects in the transmission of LSD virus,

including mosquitoes such as *Culex mirificens* and *Aedes natrionus*. *Ae. aegypti* has been shown to be capable as acting as a vector via mechanical transmission (Chinota et al., 2001). Given that this is mechanical transmission, it is likely that a wide range of mosquitoes, and other biting insects, may be able to transmit the virus. The disease affects only cattle. LSD virus does not infect humans[13].

- Fowl pox is a viral disease; infection leads to the formation of wart-like nodules on the non-feathered parts of the head and legs and occasionally to similar lesions or canker in the mouth, nose and throat. The virus can be transmitted directly from infected to susceptible chickens, or *via* a range of biting insects including mosquitoes, e.g. *Culex pipiens* and *Ae. aegypti* (Kligler et al., 1929). A vaccine for fowl pox is available.

Limited-scale release of engineered RIDL male *Ae. aegypti* would not significantly affect transmission of any of these pathogens relative to the no-action alternative. Larger-scale use, leading to local suppression of the *Ae. aegypti* population, would reduce the ability of such a population to transmit any of these diseases due to reduction to the number of potential vector mosquitoes (adult females). This would be a beneficial effect. However, since each of these pathogens can be transmitted by a number of mosquito species other than *Ae. aegypti*, the overall effect on transmission, though beneficial, is likely to be negligible. In the event of complete or partial niche replacement by *Ae. albopictus* (see above), such change is likely to be further reduced. However, in the specific case of canine heartworm, *Aedes albopictus* appears to be a rather poor vector (Nayar and Knight, 1999), so some reduction in transmission might occur, at least where other competent vectors are not present.

Potential risk to vector control workers and staff

Ae. aegypti eggs will be shipped from the UK for the trial and reared to adults at FKMCD facilities. At FKMCD facilities the life stages will include the larval and pupal stages as well as adults. Uninfected genetically modified arthropod vectors fall under Arthropod Containment Level 2 in the USA, provided the modification has no, or only negative effects on viability, survivorship, host range, or vector capacity (ASTMH, 2003). The modification used in this trial has strong negative effects on the survivability and viability of progeny of matings with local mosquitoes and has no effect on the host range or vectorial capacity.

The rearing of *Ae. aegypti* to adults involves the physical separation of male and female pupae, as the female pupae are larger than the male pupae. This is achieved using mechanical sorting, resulting less that 1% of females in the release generation. Male mosquitoes do not bite as they lack the penetrating mouthparts to pierce human or animal skin and consequently cannot transit disease. The staff handling the *Aedes* in the laboratory could be bitten by the few female adults that are present. Oxitec has reared over 2 million *Ae. aegypti* a week in the laboratory in the UK without significant numbers of bites from females recorded (Oxitec, unpublished data). In a 6-month release of the same strain in the Grand Cayman in 2010, involving the release of over 3.3 million adults, with a low percentage of RIDL females (less than 1%), staff conducting the release reported no biting incidence.

Rearing the RIDL eggs to adults will require the use of a dietary supplement, tetracycline in the aquatic life stages, and consequently personal protective equipment will be required and need to be used when weighing powdered antibiotic, for the preparation of stock solutions in the rearing facility.

However, it is recommended that staff and vector control workers are fully trained in handling, rearing, sorting and release activities, and appropriate SOPs are prepared and implemented. Vector control workers/staff will be encouraged to wear long sleeved and legged clothing when releasing the RIDL mosquitoes.

Current mosquito control is conducted as part of an integrated vector control package (see earlier) that uses both cultural and insecticidal methodologies as well as habitat management. The uses of pesticides are subject to strict controls and use in accordance with Federal Insecticide Fungicide and Rodenticide Act (FIFRA) and

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[13] http://www.inspection.gc.ca/english/anima/disemala/lumpderm/lumpdermfse.shtml accessed 03 Feb 2011

various other statutory legislation, including the recent requirements of the Clean Water Act (CWA). However several of the active ingredients, such as organophosphates and pyrethroids have been linked to respiratory sensitization, hypersensitivity and poisoning in humans (Power and Sudakin, 2007; Sudakin and Power, 2007).

Therefore in contrast to the no action alternative the risk to staff and vector control workers from handling and releasing RIDL *Ae. aegypti* is negligible.

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From:	Jiang, Peter
Sent:	Monday, February 14, 2011 4:21 PM
To:	Daiker, Dave; Page, Mike; Dwinell, Steve; Howard, Dennis
Subject:	FW: Draft EA for Aedes aegypti Application # 09211044
Attachments:	Appendix I - Control measures in FKMCD.pdf; Appendix II Cayman results.pdf; Appendix III Malaysia GMACApprovals.pdf; Appendix IV Approval Brazil ParecerLiberacao.pdf; Appendix V Vet Services Application for field release.pdf; Appendix VIII Critical habitat analysis in Monroe Co FL.pdf; Appendix VI Development of GE Aedes aegypti.pdf; Appendix VII Research Protocol.pdf; DRAFT EA Aedes aegytpi V6 14-2-2011.pdf

FYI.

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From: Camilla Beech [mailto:Camilla.Beech@oxitec.com]
Sent: Monday, February 14, 2011 1:35 PM
To: thomas.letonja@aphis.usda.gov
Cc: Gary.Clark@ars.usda.gov; Edsel Fussell; Andrea Leal; Derric Nimmo; Jiang, Peter; Luke Alphey; Neil Morrison
Subject: Draft EA for Aedes aegypti Application # 09211044

Dear Thomas

As we discussed last week on the phone here is a draft of the environmental assessment for the GE *Aedes aegypti* trial in Key West.

There is the main document and 8 separate appendices.
Draft EA for *Aedes aegypti* (as pdf file – please let me know if you need the original word document)
Appendix I – Control methods used in FKMCD
Appendix II – Results of previous releases in the Cayman Islands (Confidential)
Appendix III – Regulatory Approval in Malaysia
Appendix IV – Regulatory Approval in Brazil
Appendix V – development of the GE *Aedes aegypti*
Appendix VI – Research protocol (Confidential)
Appendix VIII – Critical habitat analysis for TES Monroe County.

The trial is envisaged in three distinct phases and hopefully that is clear in the document.   What are the next steps ? Will you send to the other agencies for review ? If so what is an approximate timetable ? The *Aedes* population begins to increase in Key West at the end of April/early May and if possible we would like to get a trial started before the population picks up too much.

I will be away from the office on 15th and 16th Feb this week, so if you need immediate attention from Oxitec, please contact either Luke Alphey or Neil Morrison (email addresses above and office telephone below)
Looking forward to hearing from you soon

1

Camilla

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