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June 9, 2022

To: Advisory Committee on Plants and Animals

From: Suzanne Case, Chairperson
Hawaii Department of Land and Natural Resources
1151 Punchbowl Street, Honolulu, HI 96813

David G. Smith, Administrator
Hawaii Department of Land and Natural Resources
Division of Forestry and Wildlife
1151 Punchbowl Street, Room 325, Honolulu, HI 96813

Cynthia King, Entomologist
Hawaii Department of Land and Natural Resources
Division of Forestry and Wildlife
1151 Punchbowl Street, Room 325, Honolulu, HI 96813

Through: Christopher Kishimoto
Entomologist
Plant Quarantine Branch
Department of Agriculture

Subject: Request to: (1) Preliminarily Review the Currently Unlisted Southern House Mosquito, *Culex quinquefasciatus* (Diptera: Culicidae), for Future Placement on the List of Restricted Animals (Part A) by Board Order, For Immediate Field Release to Suppress Wild Populations of *Culex quiquefasciatus*, by the State of Hawaii Department of Land and Natural Resources (DLNR);

(2) Provided the Southern House Mosquito, *Culex quinquefasciatus,* is Placed on the List of Restricted Animals (Part A), Allow the Importation of Lab-Reared Strains of the Mosquito, *Culex quinquefasciatus* (Diptera: Culicidae), inoculated with Strains of *Wolbachia* Bacteria, by Permit, For Immediate Field Release to Suppress Wild Populations of *Culex quiquefasciatus*, by the DLNR; and

(3) Provided the Southern House Mosquito, *Culex quinquefasciatus,* is Placed on the List of Restricted Animals (Part A), Establish Permit Conditions for the

Importation and Immediate Field Release of Lab-Reared Strains of the Mosquito, *Culex quinquefasciatus* (Diptera: Culicidae), inoculated with Strains of *Wolbachia* Bacteria to Suppress Wild Populations of *Culex quinquefasciatus* by the DLNR.

I. Summary Description of the Request

PQB NOTES: *The Plant Quarantine Branch (PQB) submittal for requests for import or possession permits, as revised, distinguishes information provided by the applicant, Suzanne Case, from procedural information and advisory comment and evaluation presented by PQB. With the exception of PQB notes, hereafter "PQB NOTES," the text shown below in section III from page 4 through page 18 of the submittal was taken directly from the applicant's application and subsequent written communications provided by the applicant. For instance, the statements on pages 13 through 15 regarding effects on the environment are the applicant's statements in response to standard PQB questions and are not PQB's statements. This approach for PQB submittals aims for greater applicant participation in presenting import requests in order to move these requests to the Board of Agriculture (Board) more quickly, while distinguishing applicant provided information from PQB information. The portion of the submittal prepared by PQB, including the procedural background, summary of proposed list additions, environmental assessment, proposed permit conditions and advisory review, are identified as sections II, IV, V, VI, and VII of the submittal, which start at pages 3, 18, middle of page 19, ending of page 19, and 25 respectively.*

COMMODITY: Various Shipments of the Southern House Mosquito, *Culex quinquefasciatus* (Diptera: Culicidae), inoculated with Strains of *Wolbachia* Bacteria.

SHIPPERS: Stephen Dobson
MosquitoMate, Inc.
2520 Regency Road,
Lexington, Kentucky, 40503

Verily Life Sciences
269 E Grand Avenue,
South San Francisco, California 94080

IMPORTER: Suzanne Case, Chairperson
Hawaii Department of Land and Natural Resources
1151 Punchbowl Street, Honolulu, HI 96813

CATEGORY: *Culex quinquefasciatus* is currently an unlisted animal. Animals not found on any list are considered prohibited until placed on a list. Additionally, Chapter 4-71, Hawaii Administrative Rules (HAR), allows importation of unlisted animals into Hawaii under special permit for the purpose of remediating medical emergencies or ecological disasters, or conducting scientific research that is not detrimental to agriculture, the environment, or humans by special permit, on a case-by-case basis, as approved by the Board.

II. Procedural Background

DLNR has requested that one of the lists in Chapter 4-71, Hawaii Administrative Rules (HAR), be amended by Board Order to include the Southern House Mosquito, *Culex quinquefasciatus.* The species may be placed on the List of Conditionally Approved Animals, List of Restricted Animals (Part A or B), or the Prohibited List. Species on the Restricted and Conditionally Approved Lists may enter the State of Hawaii under permits with conditions approved by the Board. Until placement on a list, species are considered prohibited except as provided by Section 150A-6.2(c), Hawaii Revised Statutes (HRS).

Species on the List of Restricted Animals (Part A) are available for research by universities and government agencies, exhibition in municipal zoos and government-affiliated aquariums, and for other institutions for medical and scientific purposes as determined by the Board. All species listed for import require a permit for entry into the State.

Pursuant to HRS §150A-6.6, the Board has the authority to adopt administrative rules to make additions to or deletions from the lists required to be maintained under HRS §150A-6.1 through §150A-6.3, which include the List of Restricted Animals, Part A. Changes to the lists can be made without regard to the notice and public hearing requirements of HRS Chapter 91 provided that there is notice and opportunity for public input regarding additions or deletions to the lists.

HAR §4-71-4.2, “Public Input and Notification for Listing,” details the specific process that the Board must follow to make a change to the lists maintained by PQB. It requires that, thirty days or more prior to the effective date of the Board order, the Hawaii Department of Agriculture (Department) issue a press release and mail a notice to the Environmental Review Program (formerly the Office of Environmental Quality Control) for publication and to all persons who have made a timely written request of the department for advance notice of the order or the Department's rulemaking proceedings.

Provided the Board acts favorably on this request for list placement by Board Order, the species will have been placed on a respective list and be eligible for import and/or possession. PQB can then process a permit application by having the Board approve the future importation and establishment of appropriate permit conditions for the organism and proposed purpose.

III. Information Provided by the Applicant in Support of the Application

Summary Description of the Requests
In accordance with the provisions of Chapter 150A, Hawaii Revised Statutes, we are requesting to import the following animal commodities:

Commodity	Scientific Name	Quantity
Southern House Mosquitoes (Male Adults)	*Culex quinquefasciatus*	Continued shipments for immediate release.

Additionally, we are requesting the listing of *Culex quinquefasciatus* mosquito species on the Hawaii Department of Agriculture's (HDOA) List of Restricted Animals Part A given that specific conditions, as outlined and enforced by HDOA, are met at the time of importation. Suggested conditions for importation are included within this application.

Reason for importation:
For immediate field release applications to suppress mosquito populations in areas where Hawaii fauna are at risk of disease transmission due to the presence of these mosquitoes.

Shippers:
1) Stephen Dobson, MosquitoMate, Inc.
   2520 Regency Rd., Lexington, KY, 40503

2) Verily Life Sciences
   269 E Grand Ave, South San Francisco, CA 94080

Importers:
1) DLNR Waimano Baseyard – Hawaii Invertebrate Program - Oahu
   2680 Waimano Home Road, Pearl City, HI 96782, (808) 266-7989
2) Kaua'i Branch, Division of Forestry & Wildlife, 3060 Eiwa Street Rm. 306, Lihue, HI 96766. (808) 274-3433
3) O'ahu Branch, Division of Forestry & Wildlife, 2135 Makiki Heights Drive, Honolulu, HI 96822. (808) 973-9778
4) Maui (& Moloka'i) Branch, Division of Forestry & Wildlife, 1955 Main Street, Room 301, Wailuku, HI 96793. (808) 984-8100

5) Hawai'i Branch, Division of Forestry & Wildlife, 19 E. Kawili Street, Hilo, HI 96720. (808) 974-4221

Project:
This is an application for:

- A permit to import male *Culex quinquefasciatus* mosquito species.
- The listing of *Culex quinquefasciatus* mosquito species on the Hawaii Department of Agriculture's (HDOA) List of Restricted Animals Part A given that specific conditions, as outlined and enforced by HDOA, are met at the time of importation. Suggested conditions for importation are included within this application.

As outlined in the suggested conditions for importation, these mosquitoes will either contain the same wild type bacterium (*Wolbachia* spp.) which is already endemic in *Culex quinquefasciatus* mosquitoes in Hawaii, or will be inoculated with an incompatible bacterium (*Wolbachia* spp.) that is not native to the wild mosquito's current internal fauna. The presence of this different strain of bacteria within the male mosquito's reproductive system will render the imported male mosquitoes unable to successfully mate with wild females found within Hawaii, a process called cytoplasmic incompatibility. Cytoplasmic incompatibility has been used with much success in other parts of the world to reduce mosquito populations and thus reduce the potential of transmission of mosquito vectored diseases. We intend to import male, sexually incompatible mosquitoes for direct release onto the environment. This process uses cytoplasmic incompatibility to reduce current populations of this pest mosquito species, which are vectors for pathogens to Hawaii's fauna, including pathogens such as avian malaria, and which can vector West Nile virus, and lymphatic filariasis to humans. Importing Hawaii lineage mosquitoes which contain the wild type bacterium, will ensure that we can conduct genetic analysis to confirm that the wild *Culex quinquefasciatus* is the wild type originally provided to the collaborators, and that the inoculated mosquitoes are indeed incompatible.

Culex quinquefasciatus is an invasive, disease-spreading mosquito that has dispersed across the Hawaiian islands since its accidental introduction in the 1800s. The species is present on Hawaii, Maui, Molokai, Lanai, Kahoolawe, Oahu, Kauai, and the northwest Hawaiian islands. *Culex quinquefasciatus* can thrive at sea-level to 4800ft in elevation. In Hawaii, the mosquito is able to transmit pathogens to native forest birds. The spread of avian malaria, in particular, has contributed to the extinction of more than half of Hawaii's endemic honeycreepers and continues to pose a risk to the remaining species. *Culex quinquefasciatus* is also known to transmit dog heartworm within pets found throughout Hawaii, and is a concern to human health given its ability to vector West Nile virus on the US mainland and lymphatic filariasis in other Pacific nations.

Efforts to suppress *Culex quinquefasciatus* through utilization of traditional vector

control methods (*e.g.,* pesticides) are inadequate at a landscape scale, and may be problematic for other non-target state and federally protected invertebrate species including Hawaiian picture-wing flies (*Drosophila* spp.), damselflies (*Megalagrion* spp.), yellow-faced bees (*Hylaeus* spp.) and anchialine pond shrimps (*Vetericaris chaceorum* and *Procaris hawaiana*). Current efforts to control mosquito-vectored disease outbreaks are limited to reducing mosquito breeding site locations and localized applications of various larvicides and adulticides.

On September 6-7, 2016, local, national, and international experts gathered in Hawaii to discuss how to mitigate mosquito-borne diseases. The strategy deemed most favorable in terms of its effectiveness, technical readiness, and safety was *Wolbachia*-based cytoplasmic incompatibility. Cytoplasmic incompatibility results from the presence of a bacterium, *Wolbachia*, in the cells of the mosquito. Many arthropod species, including several native species here in Hawaii, naturally contain strains of *Wolbachia.* Bacteria in the genus *Wolbachia* are a type of arthropod endosymbiont that do not occur in humans orother vertebrates. Approximately 50% of insect species naturally have the bacteria, although many of these insects can survive without *Wolbachia*. Conversely, *Wolbachia* cannot persist outside of insect cells, as it is an obligate endosymbiont. The largest effect of *Wolbachia* is on mating compatibility between individual insects that carry the bacteria. However, there are secondary effects that are being studied by many labs. These include altered host insect lifespan and reduced vector competence.

In nature, *Wolbachia* are passed from females to their offspring. Different strains of *Wolbachia* have also been introduced into insects in laboratories. If a male mosquito with one type of *Wolbachia* mates with a female mosquito that has a different strain of *Wolbachia* the resulting offspring can be inviable and not develop into mosquito larvae because of a mismatch of cellular signals (loss of the male parental chromosomes) originating from *Wolbachia*. If sufficient numbers, on the order to 10 times the wild population size, of male mosquitoes of a different *Wolbachia* type are released, wild females are more likely to mate with males of a different *Wolbachia* type and are predicted to have far fewer viable offspring. With subsequent releases, this process can significantly suppress the wild population numbers of mosquitoes over the following generations over a geographic area. *Wolbachia* male-based insect control programs have beenhighly successful for reducing local mosquito populations around the world. Results of initial trials in Fresno, California showed decrease of biting *Ae. aegypti* females by 68%, 95%, and 84% during the peak mosquito seasons in 2017, 2018, and 2019 respectively. *Wolbachia* cannot be spread by the released males, because *Wolbachia* are only passed from mother to offspring. It is also worth noting that male mosquitoes do not bite or vector disease.

One way to generate mosquitoes with a different *Wolbachia* type, is by clearing the naturally-occurring *Wolbachia* strain from the mosquitoes using the antibiotic tetracycline. Then *Wolbachia* can be harvested from cells of another insect species (this can be another mosquito or a non-mosquito species) and introduced into the cleared

mosquitoes via microinjection. Another method to establish new *Wolbachia* strains is to mate a *Wolbachia*-carrying female insect to males that have been cleared of their naturally-occurring *Wolbachia* via antibiotic treatment. Because *Wolbachia* are maternally inherited (described above), this cross results in all of the offspring inheriting whichever *Wolbachia* strain is contained in the female parent. Incompatible Wolbachia strains can also be naturally present in populations of mosquitoes.

The first shipper listed within this import application, MosquitoMate Inc., holds the US patent, Patent No.: US 7,868,222 B1, for the method of producing an artificial infection in Culicidae species.
(https://patentimages.storage.googleapis.com/55/da/ae/d7cb8b9cb44599/US7868222.pdf)
Additionally, MosquitoMate Inc. offers a commercially available, *Wolbachia* infected male mosquito product for purchase to suppress *Aedes albopictus* mosquito populations via cytoplasmic incompatibility. This product, ZAP Males®, has been reviewed and registered under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). ZAP Males® are a labeled pesticide product with the EPA registration number 89668-4. This product currently has a restriction that only allows for its application in specific states, which does not currently include Hawaii. We reference this, as it is the only registrant in the US currently with a *Wolbachia* mosquito product currently in process of registration with the EPA.
(https://www3.epa.gov/pesticides/chem_search/ppls/089668-00004-20171103.pdf)

The second shipper listed is Verily Life Sciences, a CA based company which is in the process of working with a different incompatible *Culex quinquefasciatus.* This company is initiating consultations with the EPA relating to this different *Wolbachia* mosquito and will provide additional information directly to HDOA as needed.

Culex quinquefasciatus mosquito eggs originating from Hawaii stock (aka collected from field sites in Hawaii) have been provided to MosquitoMate and Verily for development and testing of cytoplasmic incompatibility. These mosquitoes have been crossed with female mosquitoes carrying a different *Wolbachia* species as outlined above. These mosquitoes have then been backcrossed with a separate population of mosquitoes originating from Hawaii stock over at least seven generations to ensure Hawaii's wild mosquito genetics are >99% contained within a commercially available product to be applied within Hawaii.

Generations	HI Mosquito Genetics	Crossed MosquitoMate Genetics
0	100.00%	100.00%
1	50.00%	50.00%
2	75.00%	25.00%
3	87.50%	12.50%
4	93.75%	6.25%
5	96.88%	3.13%
6	98.44%	1.56%
7	99.22%	0.78%
8	99.61%	0.39%
9	99.80%	0.20%
10	99.90%	0.10%

On January 17, 2017, the Hawaii Invasive Species Council, an inter-departmental collaboration of the Departments of Land and Natural Resources (DLNR), Agriculture (HDOA), Health (HDOH), Transportation (DOT), Business, Economic Development & Tourism (DBEDT), and the University of Hawaii (UH) passed resolution17-2, specifically pertaining to mosquitoes. Resolution 17-2, entitled, "Supporting Evaluation and Implementation of Technologies For Landscape-Scale Control of Mosquitoes, With a Focus On Mitigating Both Human and Wildlife Health Risks," recognizes that mosquitoes in the State of Hawaii are non-native and an important pest species to control. The resolution supports the implementation of evaluated technologies that are scientifically demonstrated as safe, effective control measures for mosquitoes. (https://dlnr.hawaii.gov/hisc/files/2013/02/HISC-Reso-17-2-signed.pdf).

House Resolution (HR) 297 passed the Hawaii State House in 2019 and further directed "DOA to review the *Aedes aegypti* mosquito with *Wolbachia* bacteria, including *Aedes aegypti* mosquitoes originating from Hawaii stock that could be imported for landscape scale mosquito control, and render a determination to place it on the appropriate animal import list. Requires DOA, DOH, and DLNR to collaborate on a report to the Legislature with recommendations for appropriate vector control programs." (https://www.capitol.hawaii.gov/session2019/bills/HB297_SD1_.htm)

Additionally, House Resolution (HR) 95 passed the Hawaii State House in 2021 urging DLNR, DOA, DOH and UH to implement a mosquito control program using *Wolbachia* to reduce mosquito population levels throughout the state. (https://www.capitol.hawaii.gov/session2021/bills/HR95_HD1_.htm)

It should be noted that this project has been developed with the full support of, and will be implemented in close coordination with, the Hawaii Department of Health Vector Control Branch. Per Hawaii Revised Statutes §26-13, the Department of Health "shall administer programs designed to protect, preserve, care for, and improve the physical and mental health of the people of the State." DOH has the authority in Hawaii relating

to mosquitoes and public health, and their staff have decades of expertise to implement mosquito surveillance, control and abatement programs.

Proposed Required Conditions for Importation via HDOA List of Restricted Animals Part A

Included are proposed conditions, suggested in collaboration with the HDOH Vector Control Branch, that could be required for importation if *Culex quinquefasciatus* mosquitoes are added to the HDOA List of Restricted Animals Part A to ensure any future imports meet safeguards to preserve public health, the environment, and the long-term efficiency of the IIT tool. All of the following suggested requirements would need to be met to obtain importation permitting.

Culex quinquefasciatus

1. Only mosquitoes originating from a Hawaii stock are allowed for importation.
2. Only mosquitoes containing the same wild-type bacteria as is already present in Hawaii, or a sexually incompatible *Wolbachia* bacteria compared against Hawaii's wild mosquito populations are allowed for importation.
3. Only adult male mosquitoes are allowed for importation.
4. Only individuals or organizations who have conducted work for EPA registration trials for mosquito biopesticide products and who can provide data on rearing and sorting methodologies are allowed to ship these mosquitoes to Hawaii.
5. Only individuals or organizations listed on the import application are allowed to import/receive these mosquitoes.
6. Only islands with established or incipient wild mosquito populations, as determined by the Hawaii Department of Health's Vector Control Branch, are allowed to import these mosquitoes.
7. All environmental review processes, including potential Environmental Impact Statements, Environmental Assessments, or other environmental compliance requirements as outlined by State Law and OEQC, must be completed or cited prior to importation.

PQB NOTES: *PQB has taken DLNR's proposed permit conditions and incorporated them into the conditions in section VI.*

Specific details for importation
This is an application for:

- A permit to import male, mosquito species: *Culex quinquefasciatus*.
- The listing of these mosquito species on the Hawaii Department of Agriculture's (HDOA) List of Restricted Animals Part A given that specific conditions, as outlined and enforced by HDOA, are met at the time of importation. Suggested conditions for importation are included within this application.

Within *Culex quinquefasciatus*, the strain of incompatible bacterium will be *Wolbachia wAlbA, Wolbachia wAlbB,* or *Wolbachia wPip4.* These *Wolbachia* bacterium are not present within the corresponding species of Hawaii's established mosquito population. The presence of this bacterium will make these males sexually incompatible with the wild, established female mosquitoes. Once imported, the male, sexually incompatible males will be released according to EPA and HDOA label directions to suppress the population of the established mosquito populations. Based on the prior use of this technology in California, Florida, and Kentucky, there are no data to suggest releases of these male mosquitoes to have a negative impact on agriculture, the environment, or public health and safety. Existing wild-type bacteria strain that may be imported is wPipV, which is already found on all of the main Hawaiian islands.

DISCUSSION:

1. <u>Persons Responsible:</u>

DLNR Chairperson, Suzanne Case
DOFAW Administrator, David Smith
DOFAW Entomologist, Cynthia King
Department of Land and Natural Resources – Oahu
1151 Punchbowl Street, Honolulu, HI 96813

DLNR-DOFAW, Hawaii Invertebrate Program Captive Propagation Facility - Oahu
779 Ulukahiki Street, Kailua, Honolulu, HI 96813, (808) 266-7989

DLNR Waimano Baseyard – Oahu
2680 Waimano Home Road, Pearl City, HI 96782, (808) 266-7989

Kaua'i Branch Manager, Sheri Mann, Division of Forestry & Wildlife, 3060 Eiwa Street Rm. 306, Lihue, HI 96766. (808) 274-3433

O'ahu Branch, Division of Forestry & Wildlife, 2135 Makiki Heights Drive, Honolulu, HI 96822. (808) 973-9778

Maui (& Moloka'i) Branch, Division of Forestry & Wildlife, 1955 Main Street, Room 301, Wailuku, HI 96793. (808) 984-8100

Hawai'i Branch, Division of Forestry & Wildlife, 19 E. Kawili Street, Hilo, HI 96720. (808) 974-4221

2. <u>Locations and Safeguards:</u>

All mosquitoes for import will originate from Hawaii biotypes collected from

Hawaii. All mosquitoes will be backcrossed for at least 7 generations to ensure >99% Hawaii genetics are contained within the commercially available products to be applied within Hawaii. This backcrossing will also mitigate the risks of infections microorganisms and parasites to the mosquitoes via vertical transmission – thus lowering the risk of the mosquitoes accidentally introducing a new parasite or pathogen. In order for these mosquitoes to acquire and vector a disease, an adult female must blood feed from a disease infected vertebrate, and the pathogen must survive in the mosquito and be injected into another vertebrate during a subsequent blood feeding. As the intended importation of these mosquitoes only includes the importation of male mosquitoes that do not bite or feed on blood, the unintended importation of an acquired pathogen is eliminated. Verification of Hawaii biotypes and *Wolbachia* strains will be conducted on initial shipments of male mosquitoes to verify requirements have been met, in collaboration with University of Hawaii and Department of Health.

These mosquitoes will be imported into Hawaii through the use of commercial cargo flights. Upon reception to Hawaii, the male mosquitoes will be directly released into the laboratory for quality control testing, and into the environment for the purpose of suppressing the wild mosquito populations. These releases will be performed by individuals or organizations certified to apply these mosquito pesticide products to ensures that the product will be applied properly according to the recommended guidelines.

MosquitoMate and Verily will regularly sample release containers by releasing the contents into lab cages and then examining mosquito sex and number. There is an EPA reviewed value of 1 female release per 250,000 males with the MosquitoMate product. A similar value is likely to be estimated for *Culex quinquefasciatus* given that similar automation, engineering and machine learning technology is being applied to sex sorting. MosquitoMate and Verily have not previously identified a female in a single release container during the course of the Puerto Rico or Fresno projects. In another example, a published study estimates the probability at less than 1 female per 200 million males (Crawford JE, Clarke DW, Criswell V, Desnoyer M, Cornel D, Deegan B, et al. Efficient production of male Wolbachia-infected *Aedes aegypti* mosquitoes enables large-scale suppression of wild populations. Nat Biotechnol. 2020;38(4):482-92.) To date, PCR monitoring of mosquitoes collected from release field sites have not identified any ZAP infected females.

At least once per year, MosquitoMate and Verily will also conduct longevity and competitiveness studies, comparing the mosquitoes proposed for releases and wild type males. Data from previous trials demonstrate ZAP mosquito longevity and competitiveness to be at least equal to Wild Type males. In addition to Hawaii's import requirements, the shipper and/or receiver will obtain additional permits as required by federal or state agencies.

Wolbachia is an obligate endosymbiont and cannot survive outside of the host invertebrate. *Wolbachia* strains already exist in Hawaii in a range of invertebrates in the wild, including mosquitoes. The presence of *Wolbachia* endosymbionts is the normal state for 40% to 60% of Arthropods and does not represent an unusual or pathogenic bacterial infection. *Wolbachia* are not capable of infecting human cells. MosquitoMate and Verily will perform PCR testing on the mosquitoes to confirm the presence of the correct *Wolbachia* bacterium within the shipment lineage to ensure cytoplasmic incompatibility.

The likelihood that introduced strains of *Wolbachia* would become the dominant strains in the environment is highly unlikely. Replacing the dominant *Wolbachia* strain has been done purposefully in the environment for projects that are separate from the approach we are proposing (such as by the World Mosquito Program in Australia and other nations). To clarify, DLNR is NOT proposing a World Mosquito Program type project where the goal is to intentionally force a different dominant *Wolbachia* strain into the wild mosquitoes in the environment and change vector competence of the wild population. However, in these types of programs, they have to release 4 million mixed male AND female mosquitoes in a given location to force a new *Wolbachia* strain to become the dominate strain over an area of 66 km^2. Given the aforementioned EPA reviewed value of 1 female release per 250,000 males with the MosquitoMate product, such an outcome is not expected to occur.

If, somehow population replacement were to occur (despite the estimated 1 female release per 250,000 males) DLNR would cease releases as the released males would then be able to mate with the wild females with the established *Wolbachia* species. The outcome of this would be that the mosquito species that already exists in Hawaii would continue to exist in the wild, just with a different *Wolbachia* bacteria. We do not anticipate a different *Wolbachia* bacteria having any new or negative effects on the environment.

DLNR and DOH feel comfortable utilizing these mosquitoes at a very small scale (in remote forest habitat) or at a very large scale (across urban areas and island wide) so long as recommended application guidelines are followed. The scale and scope of the project will likely vary across time based on the funding available and mosquito prevalence. As with any pesticide product, if you do not eradicate the species of concern, they will rebound if you stop using the pesticide product. However, we view this as a beneficial aspect of the project as we also know we can stop the process at any time. Unfortunately, due to the critical nature of the declines of Hawaiian forest birds, we anticipate mosquito control becoming a long-term management action to be performed (similar to rat control and invasive weed control) annually.

Data collection will occur during releases using the State general funds as well as federal funds from partner agencies (USFWS, USGS, NPS), depending on who is performing the releases. As the application of the pesticide product is intended for the reduction of *Culex quinquefasciatus* mosquito populations, this monitoring will include extensive mosquito population surveillance following releases to ensure that populations are reduced. DLNR is already conducting this type of monitoring in preparation for incompatible mosquito releases. *Wolbachia* genetic monitoring will also occur, likely in partnership with USGS, throughout the release program.

In addition to Hawaii's import requirements, the shipper and/or receiver will obtain additional permits as required by federal or state agencies.

3. Method of Disposition

Any dead imported mosquitoes will be disposed of as municipal waste.

4. Abstract of Organism

Culicidae species are sexually reproducing species. Minimum generation times vary but are approximately three weeks. Mature adults are up to approximately a centimeter in length and can live for a month to a few months. Adult mosquitoes range from 2.0 to 10.0 mm in size with males being smaller than females on average. Mosquito life cycles are well understood for most species, including all those established in Hawaii.

Larvae feed on organic material found in pools of water. Both adult males and females feed on water that contains carbohydrates (water with sap or nectar). Only mature females of certain species seek out and feed on vertebrate blood prior to egg laying. This blood feeding process allows for the transmission of pathogens and parasites.

Culex quinquefasciatus rely on pools of water with organic material for the growth of larvae. Only adult females bite, as they require blood meals from vertebrate hosts to develop their eggs.

5. Potential Impact to the Environment

Culex quinquefasciatus are already well established in the wild on all of the main islands in Hawaii from sea-level to ~6,000 feet in elevation. and *Culex quinquefasciatus* are established statewide and is well establish on Hawaii's Big Island. An additional five other "biting" non-native mosquito species have also become established: *Ae. albopictus, Ae. aegypti Ae. japonicus*, *Ae. vexans*, and *Wyeomyia mitchelli*.

Wolbachia are not infectious to humans and are vertically transmitted through the eggs from one generation to another. The *Wolbachia* bacteria are obligate endosymbionts and can only survive inside the insect host's cytoplasm. A mosquito transinfected with a different strain of *Wolbachia* that results in cytoplasmic incompatibility would not be able to successfully reproduce with a wild mosquito due to cytoplasmic incompatibility. Therefore, if individual mosquitoes did become temporarily established, then they will quickly die off over the following generations because of cytoplasmic incompatibility with wild mosquitoes of the same species, with which they would be expected to encounter and mate.

Through the importation we intend to only import male mosquitoes. The sex separation can be performed in a variety of manners including through computer recognition and separation of males and females or through pupal sorting of males and females. However, if both sexes of transinfected mosquito were to be accidently released, they are unlikely to maintain a breeding population of a transinfected mosquito. *Wolbachia* invasions into populations require a critical threshold frequency of infection that needs to be overcome before a novel *Wolbachia* infection can spread into a population. The *Wolbachia* infection rate must exceed 20-45% before it can spread and become established. This is evident in large scale releases such as in Cairns, Australia, where millions of transinfected mosquitoes (both sexes) with *Wolbachia* are released into the environment to control disease transmission, yet they do not easily reach fixation in the wild. If transinfected mosquitoes were to become established, the establishment is likely to be spatially localized due to incompatibility with neighboring mosquito populations.

6. Potential Impacts of Importation

pro: Importation of male mosquitoes will allow the implementation of an evaluated technology that has been scientifically demonstrated as a safe and effective control method for mosquitoes on a landscape-scale. These are mosquitoes that are widespread in Hawaii and which have negative impacts to humans, wildlife, and pets, and are causing the extinction of native forest birds. Thirty species of main Hawaiian forest birds have become extinct since European contact, and another 11 of the 21 remaining species are federally listed as threatened or endangered. The remaining 21 forest bird species remain at great risk as a result of avian pox and avian malaria. Four honeycreeper species (Akikiki, *Oreomystis bairdi*; Akekee, *Loxops caeruleirostris*; Kiwikiu, *Pseudonestor xanthophrys* and Akohekohe, *Palmeria dolei*) are of particular concern – each are federally endangered, single-island endemics with highly restricted ranges, number fewer than 1,800 individuals, and display recent alarming population declines. DLNR and USFWS have previously attempted to address these declines through bold conservation actions, such as translocations

and establishment of captive populations; however agencies have met with only limited success due to rapidly changing disease-transmission conditions on the landscape. There is an urgent need to develop new conservation tools, including landscape-level mosquito control in order to prevent further extinctions.

The application of traditional chemical controls for mosquitoes in both natural areas is impractical and causes unacceptable non-target impacts, whereas IIT carries no non-target risks to native species, humans or the environment. Furthermore, mosquitoes were first introduced to the Hawaiian Islands in the 1800s, and while they are used opportunistically as prey items, no species native to Hawai‘i are dependent on their presence for survival. The control of mosquito populations in Hawaiian forests would thus cause no negative impacts on Hawaiian species.

Demonstrated application of this approach in Hawaii would have also have a wide range of potential positive effects in that it may facilitate the incompatible insect technique approach being used for human health.

con: It is hard to imagine any negative effects since the species is already established in Hawaii. Importing these organisms will not have any foreseeable beneficial effect to this mosquito species already found in Hawaii. The introduction of, for example, increased genetic variation within the mosquito species will be minimized by crossing the lines to mosquitoes originating from Hawaii.

The presence of unintended accompanying microbiota is minimized by the sterile laboratory rearing conditions used. These mosquitoes have been maintained for many generations in the lab environment and have not had the opportunity to obtain pathogens from the wild from blood feeding. The presence of intended microbiota, the *Wolbachia,* potentially has very positive effects on the societal health, the suppression of human disease vectored by mosquitoes, the environment, via population suppression of mosquitoes that vector avian pathogens, and the economy, through the potential increased tourism and lessened disease burden.

This mosquito species is already well established in Hawaii, as are many different strains of *Wolbachia*. MosquitoMate and Verily have a demonstrated track record of success utilizing sex-sorting methods which are highly effective. In the event that technical difficulties did occur during sex-sorting methods, because of cytoplasmic incompatibility, the escape of female mosquitoes carrying a new *Wolbachia* strain is not expected to be stable over the following generations. Laboratory reared females outcrossing to locally established wild male mosquitoes will result in cytoplasmic incompatibility and the failure of offspring to develop.

There is an extensive body of literature surrounding this mosquito species, its impact upon Hawaii, and Wolbachia-mediated cytoplasmic incompatibility.

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IV. Summary of Proposed Additions to the List of Restricted Animals, Part A

The DLNR permit application is requesting the following addition to the List of Restricted Animals (Part A) in Chapter 4-71, HAR:

§4-71-6.5, HAR, List of Restricted Animals (Part A)

Adds "Scientific Name: "Culex quinquefasciatus" and Common Name "mosquito, Southern house".

See Attachment 2 for proposed changes in Ramseyer Format. All other sections in Chapter 4-71, HAR will remain unchanged.

V. Environmental Assessment (EA):

Pursuant to a May 2008 Hawai'i Intermediate Court of Appeals decision ('Ohana Pale Ke Ao v. Board of Agriculture, 118 Haw. 247 (Haw. App. 2008), the Department of Agriculture's (Department's) import permit process is subject to the requirements of the Hawai'i Environmental Protection Act, chapter 343, Hawai'i Revised Statutes (HRS). Under this decision, the requirement for an EA as a condition of the import permit or related authorization applies in those circumstances where the underlying permit activity for the importation initiates a "program or project" and where the use of state or county funds or state or county lands is involved. When those circumstances are present, as they appear to be when a new organism is used in a new program or project located at a facility located at UHM or UHH (state lands), an EA is required to determine whether the proposed project or program is likely to have a significant impact on the environment. However, certain activities may be eligible for "exemption" under provisions established through the Environmental Advisory Council, provided that the project or program is determined to have little or no impact on the environment.

Analysis of Application re EA: Under the above-cited court decision, the EA requirement is triggered under certain circumstances, including when an applicant proposes an action on state lands that requires agency approval and is not specifically exempted under Chapter 343, HRS. That is the case here. The applicant's request in this instance involves the field-release of *Culex quinquefasciatus* for field release to suppress wild populations of *Culex quiquefasciatus* in the environment. So, agency approval is required for the applicant's proposed action/activity on state lands or sensitive habitats. As PQB understands the court's analysis in the 'Ohana Pale decision, the activity proposed under this permit application would initiate a project that may use state lands and/or sensitive habitats, initially triggering the EA requirement.

DLNR has indicated that they will be issuing their own exemption, with an EA to be completed at a future date.

VI. Proposed Permit Conditions:

1. The restricted article(s), Hawaiian biotype Southern House Mosquito, *Culex quinquefasciatus* (Say, 1823), inoculated with a foreign *Wolbachia* bacteria species, shall be used for field-release for area-wide mosquito suppression, a purpose approved by the Board of Agriculture (Board). Live sale or transfer of the restricted article(s), including progeny, is prohibited, except as approved by

the Board. Transport to or release on any island that does not have a population of *Cu. quinquefasciatus* is prohibited.

2. Only male restricted article(s) shall be imported and released.

3. Only Hawaiian biotype *Cu. quinquefasciatus* that have been backcrossed with mosquitos collected in Hawaii for at least 7 generations or 100% Hawaii-collected *Cu. quinquefasciatus* and their progeny, shall be imported.

4. Only restricted article(s) inoculated with *Wolbachia pipientis* bacteria strains already occurring in Hawaii mosquitos or strains wAlbA, wAlbB, wPip4, and wPip5 shall be imported.

5. The permittee, Suzanne Case, Chairperson, State of Hawaii Department of Land and Natural Resources (DLNR), 1151 Punchbowl Street, Honolulu, Hawaii, 96813 shall be responsible and accountable for all restricted article(s) imported, including progeny, from the time of receipt until their final disposition.

6. The restricted article(s) shall be maintained by the responsible DLNR personnel, Suzanne Case, David Smith, or Cynthia King or by trained or certified personnel designated by the permittee.

7. The restricted article(s), including progeny, shall be safeguarded at the following sites listed below, inspected and approved by the Plant Quarantine Branch (PQB) prior to importation. Movement of the restricted article(s), including progeny, to another site shall require a site inspection and approval by the PQB Chief prior to movement.

    a. DLNR Waimano Baseyard – Hawaii Invertebrate Program - Oahu 2680 Waimano Home Road, Pearl City, HI 96782, (808) 266-7989

    b. Kaua'i Branch, Division of Forestry & Wildlife, 3060 Eiwa Street Rm. 306, Lihue, HI 96766. (808) 274-3433

    c. O'ahu Branch, Division of Forestry & Wildlife, 2135 Makiki Heights Drive, Honolulu, HI 96822. (808) 973-9778

    d. Maui (& Moloka'i) Branch, Division of Forestry & Wildlife, 1955 Main Street, Room 301, Wailuku, HI 96793. (808) 984-8100

    e. Hawai'i Branch, Division of Forestry & Wildlife, 19 E. Kawili Street, Hilo, HI 96720. (808) 974-4221

8. The restricted article(s), including progeny, shall be maintained by Suzanne Case, State of Hawaii Department of Land and Natural Resources (DLNR), 1151 Punchbowl Street, Honolulu, Hawaii, 96813, or by trained or certified personnel designated by the permittee(s).

9. The permittee shall submit samples of the restricted article(s) prior to importation to the PQB upon request.

10. Prior to the arrival of each shipment containing the restricted article(s), the permittee shall provide to the PQB Chief the following information in writing:

    a. Expected arrival date;

    b. A copy of the shipping waybill or tracking numbers for each parcel;

    c. A copy of the invoice, packing list or other similar PQB approved document that states the quantity of the restricted article(s), the scientific and common name(s) of the restricted article(s), the shipper, and the consignee for the restricted article(s);

    d. The names and addresses of the shipper and permittee; and

    e. The total number of parcels.

11. The restricted article(s) shall be imported only through the port of Honolulu, except as designated by the Board. Entry into Hawaii through another port is prohibited unless designated by the Board.

12. At least four sides of each parcel containing the restricted article(s) shall be clearly labeled in plain view with “Live Animals” and “This Parcel May be Opened and Delayed for Agriculture Inspection”, in 1/2” minimum-sized font.

13. The restricted article(s) shall be shipped in sturdy PQB-approved containers designed to be escape-proof and leak-proof.

14. Each shipment of the restricted article(s) shall be accompanied by a complete copy of the PQB permit with permit conditions for the restricted article(s), and an invoice, packing list or other similar PQB approved document listing the scientific and common names of the restricted article(s), the quantity of the restricted article(s), the shipper, and the permittee(s) for the restricted article(s).

15. The permittee(s) shall immediately notify the PQB Chief in writing under the following circumstances:

    a. If any escape, theft, accidental release, disease outbreaks, pest emergence and/or mass mortalities involving the restricted article(s), including progeny, under this permit occurs. The department may confiscate or capture the restricted article(s) and any progeny that escapes or is found to be free from confinement at the expense of the owner, pursuant to the Hawaii Revised Statutes (HRS), §150A-7(c).

    b. If any changes are made to the approved sites, facilities or containers used to hold the restricted article(s), including progeny.

    c. If a shipment of the restricted article(s) is delivered to the permittee without a PQB "Passed" stamp, tag or label affixed to the article, container or delivery order that indicates that the shipment has passed inspection and is allowed entry into the State. Under this circumstance, the permittee shall not open or tamper with the shipment. Additionally, the permittee(s) shall secure all restricted article(s), shipping containers, shipping documents and packing materials for the PQB.

    d. If the permittee(s) are found in violation of any municipal, state or federal policies, rules and/or laws, pertaining to the restricted article(s).

    e. If the permittee(s) will no longer import and/or possess the restricted article(s) authorized under this permit. Under this circumstance, the permittee shall inform the PQB Chief of the final disposition for the restricted article(s), including progeny, and the permit will be canceled.

16. In the event that the restricted article(s) become parasitized or infected by disease, all restricted article(s), including progeny, from which the parasitized or infected restricted article(s) originated shall be considered compromised and immediately subjected to a treatment(s) approved by the PQB Chief. All shipping containers, packing materials, equipment, and any other items used in conjunction with the compromised restricted article(s), shall also be subjected to a treatment(s) approved by the PQB Chief.

17. Prior to interisland transport, all restricted article(s) shall be presented to the PQB for inspection. The permittee shall also follow Permit Conditions Nos. 12, 13, and 14 for each interisland shipment. The PQB inspector shall affix an interisland certificate of inspection to the shipment as verification of a completed inspection.

18. The permittee(s) shall submit an annual report to the PQB on the results of all research including post-release monitoring programs. The report shall be submitted by the 31st of January of each year and shall cover the prior 12-month period. Information reported shall include:

    a. Number of mosquito releases per site.

    b. Number of mosquitoes released per site.

    c. Impact on wild mosquito populations.

    d. Detections of introduced Wolbachia strains in wild mosquito populations.

    e. Impact of mosquito releases on native bird populations.

19. The permittee(s) shall adhere to the use, facility, equipment, procedures, and safeguards described in the permit application, and as approved by the Board and the PQB Chief.

20. Any approved site, restricted article(s), progeny, and records pertaining to the restricted article(s) or progeny under permit may be subject to post-entry inspections by the PQB, upon arrival at the permittees facility. The permittee shall make the approved site, restricted article(s), progeny, and records pertaining to the restricted article(s) or progeny available for inspection upon request by a PQB Inspector.

21. The permittee shall have a biosecurity manual available for review and approval by the PQB, at the time of the initial site inspection and any subsequent post-entry inspections, which identifies the practices and procedures to be adhered to by the permittee, to minimize the risk of theft, escape, or accidental release of the restricted article(s), including progeny, including minimizing the risk of introduction and spread of diseases and pests associated with the restricted article(s) to the environment. The permittee shall adhere to all practices and procedures as stated in this biosecurity manual.

22. The permittee shall submit to the PQB Chief a copy of all valid licenses, permits, certificates, or other similar documents required by other agencies for the restricted article(s). The permittee shall immediately notify the PQB Chief in writing when any of the required documents are suspended, revoked, or terminated. This permit may be amended, suspended, or canceled by the PQB Chief in writing, upon suspension, revocation, or termination of any required license, permit, certificate or similar document for the restricted article(s).

23. It is the responsibility of the permittee to comply with any and all applicable requirements of municipal, state, or federal law pertaining to the restricted article(s).

24. The permittee shall be responsible for all costs, charges, or expenses incident to the inspection, treatment, or destruction of the restricted article(s) or progeny under this permit, as provided in Act 173, Session Laws of Hawaii 2010, Section 13, including, if applicable, charges for overtime wages, fixed charges for personnel services, and meals.

25. Any violation of the permit conditions may result in citation, permit cancelation, and enforcement of any or all of the penalties set forth in HRS §150A-14.

26. A canceled permit is invalid and upon written notification from the PQB Chief, all restricted article(s) listed on the permit shall not be imported. In the event of permit cancelation, any restricted article(s) imported, including progeny, may be moved, seized, treated, quarantined, destroyed, or sent out of State at the discretion of the PQB Chief. Any expense or loss in connection therewith shall be borne by the permittee.

27. The permit conditions are subject to cancelation or amendment at any time due to changes in statute or administrative rules restricting or disallowing import of the restricted article(s) or due to Board action disallowing a previously permitted use of the restricted article(s).

28. These permit conditions are subject to amendment by the PQB Chief in the following circumstances:

    a. To require disease screening, quarantine measures, and/or to place restrictions on the intrastate movement of the restricted article(s), as appropriate, based on scientifically validated risks associated with the restricted article(s), as determined by the PQB Chief, to prevent the introduction or spread of diseases and/or pests associated with the restricted article(s).

    b. To conform to more recent Board approved permit conditions for the restricted article(s), as necessary to address scientifically validated risks associated with the restricted article(s).

29. The permittee shall agree in advance to defend and indemnify the State of Hawaii, its officers, agents, and employees for any and all claims against the State of Hawaii, its officers, agents, employees, or Board of Agriculture members that may arise from or be attributable to any of the restricted article(s) that are introduced under this permit. This permit condition shall not apply to a permittee

that is a federal or State of Hawaii entity or employee, provided that the State or federal employee is a permittee in the employee's official capacity.

VII. **ADVISORY SUBCOMMITTEE REVIEW:** This request was submitted to the Advisory Subcommittee on Entomology for its review and recommendation. Advisory Subcommittee recommendations and comments are as follows:

1. I recommend approval____/____disapproval of future placement of the currently unlisted Southern House Mosquito, *Culex quinquefasciatus* (Diptera: Culicidae) on the List of Restricted Animals (Part A) by Board Order, for immediate field release to suppress wild populations of *Culex quiquefasciatus*, by the State of Hawaii Department of Land and Natural Resources (DLNR);

Dr. Daniel Rubinoff: Recommends Approval.

Ms. Janis Matsunaga: Recommends Approval.

Comments: "I recommend and support future placement of the currently unlisted southern house mosquito, Culex quinquefasciatus (Diptera: Culicidae) on the List of Restricted Animals (Part A) by Board Order, for immediate field release to suppress wild populations of C. quiquefasciatus, by DLNR. This is a necessary step to move this project forward."

Dr. Mark Wright: Recommends Approval

Comments: "The potential benefits for taking this action are extensive."

2. Provided *Culex quinquefasciatus* is placed on the list of Restricted Animals (Part A), I recommend approval____/____disapproval to allow the importation of lab-reared strains of the mosquito, *Culex quinquefasciatus* (Diptera: Culicidae), inoculated with strains of *Wolbachia* bacteria for immediate field release to suppress wild populations of *Culex quinquefasciatus* by the DLNR.

Dr. Daniel Rubinoff: Recommends Approval.

Ms. Janis Matsunaga: Recommends Approval.

Comments: "Provided *Culex quinquefasciatus* is placed on the list of Restricted Animals (Part A), I recommend approval to allow the importation of lab-reared strains of the mosquito, *C. quinquefasciatus* (Diptera: Culicidae), inoculated with strains of *Wolbachia* bacteria for immediate field release to suppress wild

populations of *C. quinquefasciatus* by the DLNR given questions I provide in the word document are addressed."

Dr. Mark Wright: Recommends Approval.

Comments: "The applicants have provided documentation showing the stringent quality control systems they have in place to ensure that only male mosquitoes are released. There are data showing that this mosquito suppression method can be highly effective. There is enormous potential benefit of implementing this technology in Hawaii."

3. Provided *Culex quinquefasciatus* is placed on the list of Restricted Animals (Part A), I recommend approval____/____disapproval to establish permit conditions for the importation and immediate field release of lab-reared strains of the mosquito, *Culex quinquefasciatus* (Diptera: Culicidae) inoculated with strains of Wolbachia bacteria for immediate field release to suppress wild populations of *Culex quinquefasciatus* by the DLNR.

Dr. Daniel Rubinoff: Recommends Approval.

Comments: "This technology is low risk and should be fast tracked ASAP."

Ms. Janis Matsunaga: Recommends Approval.

Comments: "Provided *Culex quinquefasciatus* is placed on the list of Restricted Animals (Part A), I recommend approval to establish permit conditions for the importation and immediate release of lab-reared strains of the mosquito, *C. quinquefasciatus* (Diptera: Culicidae) inoculated with strains of *Wolbachia* bacteria for immediate field release to suppress wild populations of *C. quinquefasciatus* by the DLNR given the following questions are addressed:

What and where are these EPA and HDOA label directions?

What and where are the recommended guidelines for releasing these mosquitoes?

Will these Wolbachia inoculated mosquitoes be classified as a bio-pesticide product in Hawaii?

What quality control testing will be done in the laboratory in Hawaii just prior to release of the imported mosquitoes into the environment?

-How will the results be reported?
-How will the results affect releases into the environment?

Will there be routine monitoring of traps for incompatible adult females or larvae as proposed by Verily Life Sciences?

The applicant is applying for 1 million mosquitoes. How many mosquitoes will be in each shipment? What is the duration of time between shipments?

What is the plan and procedure for releases into the environment?
-How will it be decided how many mosquitoes are leased at each time at each site?
-How will each site of release be determined?
-Is there a potential release site list available?"

Dr. Mark Wright: Recommends Approval.

Comments: "This application makes a strong case for these releases to be made. Environmental risks of taking this action are minimal."

ADVISORY COMMITTEE REVIEW: May we request your recommendation and comments at the next meeting of the Advisory Committee on Plants and Animals.

PQ-7 (01/04)

State of Hawaii
Department of Agriculture
PLANT QUARANTINE BRANCH
1849 Auiki Street, Honolulu, HI 96819-3100
Phone: (808) 832-0566, FAX: (808) 832-0584

For Office Use Only

Fee: $_____ Receipt No._____

☐ Approve Permit No._____ Date:_____

☐ Disapprove ☐ Other _____

Processed by:_____ Date:_____

PERMIT APPLICATION FOR RESTRICTED COMMODITIES INTO HAWAII

Date: *4/1/22*

In accordance with the provision of Chapter 4-71 , Hawaii Administrative Rules of the Division of Plant Industry, Department of Agriculture, a permit is requested for the following commodities:

Please type or print clearly.

Quantity	Commodity	Scientific Name
1,000,000	Southern House Mosquito	Culex quinquefascatius

Name and address of shipper: Department of Land and Natural Resources, Division of Forestry and Wildlife
1151 Punchbowl Street, Honolulu, HI 96813

(Mainland or Foreign address)

Approximate date of arrival: TBD

Mode of Shipment: ☑ Mail ☐ Air Freight ☐ Boat

Type of Permit:
- --- Import
  - ☐ one time only ☑ multi-shipments
- --- Intrastate shipment
  - ☐ one time only ☑ multi-shipments
- ☑ Possession

Object of importation:
- ☐ Kept caged at all time
- ☐ Used for propagation
- ☐ Imported for exhibition
- ☑ Imported for liberation
- ☐ Other purposes - specify _____

Please type or print clearly.

Applicant's Name Suzanne Case

Company Name Department of Land and Natural Resources
(if applicable)

Hawaii Mailing Address 1151 Punchbowl Street, Honolulu, HI 96813

Telephone number 808-587-0400

Facsimile number _____

Fee Amount Enclosed (cash, check or mail order) $ Journal Voucher

(complete reverse side)

PLEASE COMPLETE THE FOLLOWING INFORMATION (attach extra sheet if necessary)

1. State in detail the reasons for introduction (include use or purpose).

   See attached application for 1-5.

2. Person responsible for the organism (include name, address and phone number).

3. Location(s) where the organism will be kept and used (include address, contact and phone number).

4. Method of disposition.

5. Give an abstract of the organism with particular reference to potential impact on the environment of Hawaii (include impact to plants, animals and humans).

****

I request permission to import the articles as listed on the permit application and further, request that the articles be examined by an authorized agent of the Department of Agriculture upon arrival in Hawaii.

I agree that I, as the importer, will be responsible for all costs, charges or expenses incident to the inspection or treatment of the imported articles.

I further agree that damages or losses incident to the inspection or the fumigation, disinfection, quarantine, or destruction of the articles, by an authorized agent of the Department of Agriculture, shall not be the basis of a claim against the department or the inspectors for the damage or loss incurred.

Signature Sgame Q. Case _____ **Date** Apr 1, 2022

(Applicant)

Subcommittee on Entomology
G. Simmons & L. Wells – Hawaii Department of Health
D. Smith & C. King – Hawaii Department of Land and Natural Resources

https://hdoa.hawaii.gov/wp-content/uploads/2019/08/Plant-and-Non-Domestic-Animal-Quarantine-Non-Domestic-Animal-Import-Rules.pdf

Date:4/1/2022

To:
Advisory Subcommittee on Entomology

From:
Suzanne Case
Department of Land and Natural Resources
1151 Punchbowl Street, Honolulu, HI 96813

David G. Smith
Hawaii Department of Land and Natural Resources, Division of Forestry and Wildlife
1151 Punchbowl Street, Room 325, Honolulu, HI 96813

Cynthia King
Hawaii Department of Land and Natural Resources, Division of Forestry and Wildlife
1151 Punchbowl Street, Room 325, Honolulu, HI 96813

Summary Description of the Requests
In accordance with the provisions of Chapter 150A, Hawaii Revised Statutes, we are requesting to import the following animal commodities:

Commodity	**Scientific Name**	**Quantity**
Southern House Mosquitoes (Male Adults)	*Culex quinquefasciatus*	Continued shipments for immediate release.

Additionally, we are requesting the listing of *Culex quinquefasciatus* mosquito species on the Hawaii Department of Agriculture's (HDOA) List of Restricted Animals Part A given that specific conditions, as outlined and enforced by HDOA, are met at the time of importation. Suggested conditions for importation are included within this application.

Reason for importation:
For immediate field release applications to suppress mosquito populations in areas where Hawaii fauna are at risk of disease transmission due to the presence of these mosquitoes.

Shippers:
1) Stephen Dobson, MosquitoMate, Inc.
   2520 Regency Rd., Lexington, KY, 40503

2) Verily Life Sciences
   269 E Grand Ave, South San Francisco, CA 94080

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D. Smith & C. King – Hawaii Department of Land and Natural Resources

Importers:

1) DLNR Waimano Baseyard – Hawaii Invertebrate Program - Oahu
   2680 Waimano Home Road, Pearl City, HI 96782, (808) 266-7989
2) Kaua'i Branch, Division of Forestry & Wildlife, 3060 Eiwa Street Rm. 306, Lihue, HI 96766. (808) 274-3433
3) O'ahu Branch, Division of Forestry & Wildlife, 2135 Makiki Heights Drive, Honolulu, HI 96822. (808) 973-9778
4) Maui (& Moloka'i) Branch, Division of Forestry & Wildlife, 1955 Main Street, Room 301, Wailuku, HI 96793. (808) 984-8100
5) Hawai'i Branch, Division of Forestry & Wildlife, 19 E. Kawili Street, Hilo, HI 96720. (808) 974-4221

Project:
This is an application for:

- A permit to import male *Culex quinquefasciatus* mosquito species.
- The listing of *Culex quinquefasciatus* mosquito species on the Hawaii Department of Agriculture's (HDOA) List of Restricted Animals Part A given that specific conditions, as outlined and enforced by HDOA, are met at the time of importation. Suggested conditions for importation are included within this application.

As outlined in the suggested conditions for importation, these mosquitoes will either contain the same wild type bacterium (*Wolbachia* spp.) which is already endemic in *Culex quinquefasciatus* mosquitoes in Hawaii, or will be inoculated with an incompatible bacterium (*Wolbachia* spp.) that is not native to the wild mosquito's current internal fauna. The presence of this different strain of bacteria within the male mosquito's reproductive system will render the imported male mosquitoes unable to successfully mate with wild females found within Hawaii, a process called cytoplasmic incompatibility. Cytoplasmic incompatibility has been used with much success in other parts of the world to reduce mosquito populations and thus reduce the potential of transmission of mosquito vectored diseases. We intend to import male, sexually incompatible mosquitoes for direct release onto the environment. This process uses cytoplasmic incompatibility to reduce current populations of this pest mosquito species, which are vectors for pathogens to Hawaii's fauna, including pathogens such as avian malaria, and which can vector West Nile virus, and lymphatic filariasis to humans. Importing Hawaii lineage mosquitoes which contain the wild type bacterium, will ensure that we can conduct genetic analysis to confirm that the wild *Culex quinquefasciatus* is the wild type originally provided to the collaborators, and that the inoculated mosquitoes are indeed incompatible.

Culex quinquefasciatus is an invasive, disease-spreading mosquito that has dispersed across the Hawaiian islands since its accidental introduction in the 1800s. The species is present on Hawaii, Maui, Molokai, Lanai, Kahoolawe, Oahu, Kauai, and the northwest

Hawaiian islands. *Culex quinquefasciatus* can thrive at sea-level to 4800ft in elevation. In Hawaii, the mosquito is able to transmit pathogens to native forest birds. The spread of avian malaria, in particular, has contributed to the extinction of more than half of Hawaii's endemic honeycreepers and continues to pose a risk to the remaining species. *Culex quinquefasciatus* is also known to transmit dog heartworm within pets found throughout Hawaii, and is a concern to human health given its ability to vector West Nile virus on the US mainland and lymphatic filariasis in other Pacific nations.

Efforts to suppress *Culex quinquefasciatus* through utilization of traditional vector control methods (*e.g.,* pesticides) are inadequate at a landscape scale, and may be problematic for other non-target state and federally protected invertebrate species including Hawaiian picture-wing flies (*Drosophila* spp.), damselflies (*Megalagrion* spp.), yellow-faced bees (*Hylaeus* spp.) and anchialine pond shrimps (*Vetericaris chaceorum* and *Procaris hawaiana*). Current efforts to control mosquito-vectored disease outbreaks are limited to reducing mosquito breeding site locations and localized applications of various larvicides and adulticides.

On September 6-7, 2016, local, national, and international experts gathered in Hawaii to discuss how to mitigate mosquito-borne diseases. The strategy deemed most favorable in terms of its effectiveness, technical readiness, and safety was *Wolbachia*-based cytoplasmic incompatibility. Cytoplasmic incompatibility results from the presence of a bacterium, *Wolbachia*, in the cells of the mosquito. Many arthropod species, including several native species here in Hawaii, naturally contain strains of *Wolbachia.* Bacteria in the genus *Wolbachia* are a type of arthropod endosymbiont that do not occur in humans orother vertebrates. Approximately 50% of insect species naturally have the bacteria, although many of these insects can survive without *Wolbachia*. Conversely, *Wolbachia* cannot persist outside of insect cells, as it is an obligate endosymbiont. The largest effect of *Wolbachia* is on mating compatibility between individual insects that carry the bacteria. However, there are secondary effects that are being studied by many labs. These include altered host insect lifespan and reduced vector competence.

In nature, *Wolbachia* are passed from females to their offspring. Different strains of *Wolbachia* have also been introduced into insects in laboratories. If a male mosquito with one type of *Wolbachia* mates with a female mosquito that has a different strain of *Wolbachia* the resulting offspring can be inviable and not develop into mosquito larvae because of a mismatch of cellular signals (loss of the male parental chromosomes) originating from *Wolbachia*. If sufficient numbers, on the order to 10 times the wild population size, of male mosquitoes of a different *Wolbachia* type are released, wild females are more likely to mate with males of a different *Wolbachia* type and are predicted to have far fewer viable offspring. With subsequent releases, this process can significantly suppress the wild population numbers of mosquitoes over the following generations over a geographic area. *Wolbachia* male-based insect control programs have beenhighly successful for reducing local mosquito populations around the world. Results of initial trials in Fresno, California showed decrease of biting *Ae. aegypti* females by 68%, 95%, and 84% during the peak mosquito seasons in 2017, 2018, and

2019 respectively. *Wolbachia* cannot be spread by the released males, because *Wolbachia* are only passed from mother to offspring. It is also worth noting that male mosquitoes do not bite or vector disease.

One way to generate mosquitoes with a different *Wolbachia* type, is by clearing the naturally-occurring *Wolbachia* strain from the mosquitoes using the antibiotic tetracycline. Then *Wolbachia* can be harvested from cells of another insect species (this can be another mosquito or a non-mosquito species) and introduced into the cleared mosquitoes via microinjection. Another method to establish new *Wolbachia* strains is to mate a *Wolbachia*-carrying female insect to males that have been cleared of their naturally-occurring *Wolbachia* via antibiotic treatment. Because *Wolbachia* are maternally inherited (described above), this cross results in all of the offspring inheriting whichever *Wolbachia* strain is contained in the female parent. Incompatible Wolbachia strains can also be naturally present in populations of mosquitoes.

The first shipper listed within this import application, MosquitoMate Inc., holds the US patent, Patent No.: US 7,868,222 B1, for the method of producing an artificial infection in Culicidae species.
(https://patentimages.storage.googleapis.com/55/da/ae/d7cb8b9cb44599/US7868222.pdf)
Additionally, MosquitoMate Inc. offers a commercially available, *Wolbachia* infected male mosquito product for purchase to suppress *Aedes albopictus* mosquito populations via cytoplasmic incompatibility. This product, ZAP Males®, has been reviewed and registered under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). ZAP Males® are a labeled pesticide product with the EPA registration number 89668-4. This product currently has a restriction that only allows for its application in specific states, which does not currently include Hawaii. We reference this, as it is the only registrant in the US currently with a *Wolbachia* mosquito product currently in process of registration with the EPA.
(https://www3.epa.gov/pesticides/chem_search/ppls/089668-00004-20171103.pdf)

The second shipper listed is Verily Life Sciences, a CA based company which is in the process of working with a different incompatible *Culex quinquefasciatus.* This company is initiating consultations with the EPA relating to this different *Wolbachia* mosquito and will provide additional information directly to HDOA as needed.

Culex quinquefasciatus mosquito eggs originating from Hawaii stock (aka collected from field sites in Hawaii) have been provided to MosquitoMate and Verily for development and testing of cytoplasmic incompatibility. These mosquitoes have been crossed with female mosquitoes carrying a different *Wolbachia* species as outlined above. These mosquitoes have then been backcrossed with a separate population of mosquitoes originating from Hawaii stock over at least seven generations to ensure Hawaii's wild mosquito genetics are >99% contained within a commercially available product to be applied within Hawaii.

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Generations	HI Mosquito Genetics	Crossed MosquitoMate Genetics
0	100.00%	100.00%
1	50.00%	50.00%
2	75.00%	25.00%
3	87.50%	12.50%
4	93.75%	6.25%
5	96.88%	3.13%
6	98.44%	1.56%
7	99.22%	0.78%
8	99.61%	0.39%
9	99.80%	0.20%
10	99.90%	0.10%

On January 17, 2017, the Hawaii Invasive Species Council, an inter-departmental collaboration of the Departments of Land and Natural Resources (DLNR), Agriculture (HDOA), Health (HDOH), Transportation (DOT), Business, Economic Development & Tourism (DBEDT), and the University of Hawaii (UH) passed resolution17-2, specifically pertaining to mosquitoes. Resolution 17-2, entitled, "Supporting Evaluation and Implementation of Technologies For Landscape-Scale Control of Mosquitoes, With a Focus On Mitigating Both Human and Wildlife Health Risks," recognizes that mosquitoes in the State of Hawaii are non-native and an important pest species to control. The resolution supports the implementation of evaluated technologies that are scientifically demonstrated as safe, effective control measures for mosquitoes. (https://dlnr.hawaii.gov/hisc/files/2013/02/HISC-Reso-17-2-signed.pdf).

House Resolution (HR) 297 passed the Hawaii State House in 2019 and further directed "DOA to review the *Aedes aegypti* mosquito with *Wolbachia* bacteria, including *Aedes aegypti* mosquitoes originating from Hawaii stock that could be imported for landscape scale mosquito control, and render a determination to place it on the appropriate animal import list. Requires DOA, DOH, and DLNR to collaborate on a report to the Legislature with recommendations for appropriate vector control programs." (https://www.capitol.hawaii.gov/session2019/bills/HB297_SD1_.htm)

Additionally, House Resolution (HR) 95 passed the Hawaii State House in 2021 urging DLNR, DOA, DOH and UH to implement a mosquito control program using *Wolbachia* to reduce mosquito population levels throughout the state. (https://www.capitol.hawaii.gov/session2021/bills/HR95_HD1_.htm)

It should be noted that this project has been developed with the full support of, and will be implemented in close coordination with, the Hawaii Department of Health Vector Control Branch. Per Hawaii Revised Statutes §26-13, the Department of Health "shall administer programs designed to protect, preserve, care for, and improve the physical and mental health of the people of the State." DOH has the authority in Hawaii relating to mosquitoes and public health, and their staff have decades of expertise to implement

mosquito surveillance, control and abatement programs.

Proposed Required Conditions for Importation via HDOA List of Restricted Animals Part A

Included are proposed conditions, suggested in collaboration with the HDOH Vector Control Branch, that could be required for importation if *Culex quinquefasciatus* mosquitoes are added to the HDOA List of Restricted Animals Part A to ensure any future imports meet safeguards to preserve public health, the environment, and the long-term efficiency of the IIT tool. All of the following suggested requirements would need to be met to obtain importation permitting.

Culex quinquefasciatus

1. Only mosquitoes originating from a Hawaii stock are allowed for importation.
2. Only mosquitoes containing the same wild-type bacteria as is already present in Hawaii, or a sexually incompatible *Wolbachia* bacteria compared against Hawaii's wild mosquito populations are allowed for importation.
3. Only adult male mosquitoes are allowed for importation.
4. Only individuals or organizations who have conducted work for EPA registration trials for mosquito biopesticide products and who can provide data on rearing and sorting methodologies are allowed to ship these mosquitoes to Hawaii.
5. Only individuals or organizations listed on the import application are allowed to import/receive these mosquitoes.
6. Only islands with established or incipient wild mosquito populations, as determined by the Hawaii Department of Health's Vector Control Branch, are allowed to import these mosquitoes.
7. All environmental review processes, including potential Environmental Impact Statements, Environmental Assessments, or other environmental compliance requirements as outlined by State Law and OEQC, must be completed or cited prior to importation.

Specific details for importation
This is an application for:

- A permit to import male, mosquito species: *Culex quinquefasciatus*.
- The listing of these mosquito species on the Hawaii Department of Agriculture's (HDOA) List of Restricted Animals Part A given that specific conditions, as outlined and enforced by HDOA, are met at the time of importation. Suggested conditions for importation are included within this application.

Within *Culex quinquefasciatus*, the strain of incompatible bacterium will be *Wolbachia wAlbA, Wolbachia wAlbB,* or *Wolbachia wPip4.* These *Wolbachia* bacterium are not present within the corresponding species of Hawaii's established mosquito population. The presence of this bacterium will make these males sexually incompatible with the

wild, established female mosquitoes. Once imported, the male, sexually incompatible males will be released according to EPA and HDOA label directions to suppress the population of the established mosquito populations. Based on the prior use of this technology in California, Florida, and Kentucky, there are no data to suggest releases of these male mosquitoes to have a negative impact on agriculture, the environment, or public health and safety. Existing wild-type bacteria strain that may be imported is wPipV, which is already found on all of the main Hawaiian islands.

Persons Responsible

DLNR Chairperson, Suzanne Case
DOFAW Administrator, David Smith
DOFAW Entomologist, Cynthia King
Department of Land and Natural Resources – Oahu
1151 Punchbowl Street, Honolulu, HI 96813

DLNR-DOFAW, Hawaii Invertebrate Program Captive Propagation Facility - Oahu
779 Ulukahiki Street, Kailua, Honolulu, HI 96813, (808) 266-7989

DLNR Waimano Baseyard – Oahu
2680 Waimano Home Road, Pearl City, HI 96782, (808) 266-7989

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Maui (& Moloka'i) Branch, Division of Forestry & Wildlife, 1955 Main Street, Room 301, Wailuku, HI 96793. (808) 984-8100

Hawai'i Branch, Division of Forestry & Wildlife, 19 E. Kawili Street, Hilo, HI 96720. (808) 974-4221

Locations and Safeguards

All mosquitoes for import will originate from Hawaii biotypes collected from Hawaii. All mosquitoes will be backcrossed for at least 7 generations to ensure >99% Hawaii genetics are contained within the commercially available products to be applied within Hawaii. This backcrossing will also mitigate the risks of infections microorganisms and parasites to the mosquitoes via vertical transmission – thus lowering the risk of the mosquitoes accidentally introducing a new parasite or pathogen. In order for these mosquitoes to acquire and vector a disease, an adult female must blood feed from a disease infected vertebrate, and the pathogen must survive in the mosquito and be

injected into another vertebrate during a subsequent blood feeding. As the intended importation of these mosquitoes only includes the importation of male mosquitoes that do not bite or feed on blood, the unintended importation of an acquired pathogen is eliminated. Verification of Hawaii biotypes and *Wolbachia* strains will be conducted on initial shipments of male mosquitoes to verify requirements have been met, in collaboration with University of Hawaii and Department of Health.

These mosquitoes will be imported into Hawaii through the use of commercial cargo flights. Upon reception to Hawaii, the male mosquitoes will be directly released into the laboratory for quality control testing, and into the environment for the purpose of suppressing the wild mosquito populations. These releases will be performed by individuals or organizations certified to apply these mosquito pesticide products to ensures that the product will be applied properly according to the recommended guidelines.

MosquitoMate and Verily will regularly sample release containers by releasing the contents into lab cages and then examining mosquito sex and number. There is an EPA reviewed value of 1 female release per 250,000 males with the MosquitoMate product. A similar value is likely to be estimated for *Culex quinquefasciatus* given that similar automation, engineering and machine learning technology is being applied to sex sorting. MosquitoMate and Verily have not previously identified a female in a single release container during the course of the Puerto Rico or Fresno projects. In another example, a published study estimates the probability at less than 1 female per 200 million males (Crawford JE, Clarke DW, Criswell V, Desnoyer M, Cornel D, Deegan B, et al. Efficient production of male Wolbachia-infected *Aedes aegypti* mosquitoes enables large-scale suppression of wild populations. Nat Biotechnol. 2020;38(4):482-92.) To date, PCR monitoring of mosquitoes collected from release field sites have not identified any ZAP infected females.

At least once per year, MosquitoMate and Verily will also conduct longevity and competitiveness studies, comparing the mosquitoes proposed for releases and wild type males. Data from previous trials demonstrate ZAP mosquito longevity and competitiveness to be at least equal to Wild Type males. In addition to Hawaii's import requirements, the shipper and/or receiver will obtain additional permits as required by federal or state agencies.

Wolbachia is an obligate endosymbiont and cannot survive outside of the host invertebrate. *Wolbachia* strains already exist in Hawaii in a range of invertebrates in the wild, including mosquitoes. The presence of *Wolbachia* endosymbionts is the normal

state for 40% to 60% of Arthropods and does not represent an unusual or pathogenic bacterial infection. *Wolbachia* are not capable of infecting human cells. MosquitoMate and Verily will perform PCR testing on the mosquitoes to confirm the presence of the correct *Wolbachia* bacterium within the shipment lineage to ensure cytoplasmic incompatibility.

The likelihood that introduced strains of *Wolbachia* would become the dominant strains in the environment is highly unlikely. Replacing the dominant *Wolbachia* strain has been done purposefully in the environment for projects that are separate from the approach we are proposing (such as by the World Mosquito Program in Australia and other nations). To clarify, DLNR is NOT proposing a World Mosquito Program type project where the goal is to intentionally force a different dominant *Wolbachia* strain into the wild mosquitoes in the environment and change vector competence of the wild population. However, in these types of programs, they have to release 4 million mixed male AND female mosquitoes in a given location to force a new *Wolbachia* strain to become the dominate strain over an area of 66 km^2. Given the aforementioned EPA reviewed value of 1 female release per 250,000 males with the MosquitoMate product, such an outcome is not expected to occur.

If, somehow population replacement were to occur (despite the estimated 1 female release per 250,000 males) DLNR would cease releases as the released males would then be able to mate with the wild females with the established *Wolbachia* species. The outcome of this would be that the mosquito species that already exists in Hawaii would continue to exist in the wild, just with a different *Wolbachia* bacteria. We do not anticipate a different *Wolbachia* bacteria having any new or negative effects on the environment.

DLNR and DOH feel comfortable utilizing these mosquitoes at a very small scale (in remote forest habitat) or at a very large scale (across urban areas and island wide) so long as recommended application guidelines are followed. The scale and scope of the project will likely vary across time based on the funding available and mosquito prevalence. As with any pesticide product, if you do not eradicate the species of concern, they will rebound if you stop using the pesticide product. However, we view this as a beneficial aspect of the project as we also know we can stop the process at any time. Unfortunately, due to the critical nature of the declines of Hawaiian forest birds, we anticipate mosquito control becoming a long-term management action to be performed (similar to rat control and invasive weed control) annually.

Data collection will occur during releases using the State general funds as well as federal funds from partner agencies (USFWS, USGS, NPS), depending on who is performing the releases. As the application of the pesticide product is intended for the reduction of *Culex quinquefasciatus* mosquito populations, this monitoring will include extensive mosquito population surveillance following releases to ensure that populations are reduced. DLNR is already conducting this type of monitoring in preparation for incompatible mosquito releases. *Wolbachia* genetic monitoring will also occur, likely in partnership with USGS, throughout the release program.

In addition to Hawaii's import requirements, the shipper and/or receiver will obtain additional permits as required by federal or state agencies.

Method of Disposition
Any dead imported mosquitoes will be disposed of as municipal waste.

Abstraction of Organism
Culicidae species are sexually reproducing species. Minimum generation times vary but are approximately three weeks. Mature adults are up to approximately a centimeter in length and can live for a month to a few months. Adult mosquitoes range from 2.0 to 10.0 mm in size with males being smaller than females on average. Mosquito life cycles are well understood for most species, including all those established in Hawaii.

Larvae feed on organic material found in pools of water. Both adult males and females feed on water that contains carbohydrates (water with sap or nectar). Only mature females of certain species seek out and feed on vertebrate blood prior to egg laying. This blood feeding process allows for the transmission of pathogens and parasites.

Culex quinquefasciatus rely on pools of water with organic material for the growth of larvae. Only adult females bite, as they require blood meals from vertebrate hosts to develop their eggs.

Potential Impact to the Environment
Culex quinquefasciatus are already well established in the wild on all of the main islands in Hawaii from sea-level to ~6,000 feet in elevation. and *Culex quinquefasciatus* are established statewide and is well establish on Hawaii's Big Island. An additional five other "biting" non-native mosquito species have also become established: *Ae. albopictus, Ae. aegypti Ae. japonicus*, *Ae. vexans*, and *Wyeomyia mitchelli*.

Wolbachia are not infectious to humans and are vertically transmitted through the eggs

from one generation to another. The *Wolbachia* bacteria are obligate endosymbionts and can only survive inside the insect host's cytoplasm. A mosquito transinfected with a different strain of *Wolbachia* that results in cytoplasmic incompatibility would not be able to successfully reproduce with a wild mosquito due to cytoplasmic incompatibility. Therefore, if individual mosquitoes did become temporarily established, then they will quickly die off over the following generations because of cytoplasmic incompatibility with wild mosquitoes of the same species, with which they would be expected to encounter and mate.

Through the importation we intend to only import male mosquitoes. The sex separation can be performed in a variety of manners including through computer recognition and separation of males and females or through pupal sorting of males and females. However, if both sexes of transinfected mosquito were to be accidently released, they are unlikely to maintain a breeding population of a transinfected mosquito. *Wolbachia* invasions into populations require a critical threshold frequency of infection that needs to be overcome before a novel *Wolbachia* infection can spread into a population. The *Wolbachia* infection rate must exceed 20-45% before it can spread and become established. This is evident in large scale releases such as in Cairns, Australia, where millions of transinfected mosquitoes (both sexes) with *Wolbachia* are released into the environment to control disease transmission, yet they do not easily reach fixation in the wild. If transinfected mosquitoes were to become established, the establishment is likely to be spatially localized due to incompatibility with neighboring mosquito populations.

Potential Impacts of Importation

pro: Importation of male mosquitoes will allow the implementation of an evaluated technology that has been scientifically demonstrated as a safe and effective control method for mosquitoes on a landscape-scale. These are mosquitoes that are widespread in Hawaii and which have negative impacts to humans, wildlife, and pets, and are causing the extinction of native forest birds. Thirty species of main Hawaiian forest birds have become extinct since European contact, and another 11 of the 21 remaining species are federally listed as threatened or endangered. The remaining 21 forest bird species remain at great risk as a result of avian pox and avian malaria. Four honeycreeper species (Akikiki, *Oreomystis bairdi*; Akekee, *Loxops caeruleirostris*; Kiwikiu, *Pseudonestor xanthophrys* and Akohekohe, *Palmeria dolei*) are of particular concern – each are federally endangered, single-island endemics with highly restricted ranges, number fewer than 1,800 individuals, and display recent alarming population declines. DLNR and USFWS have previously attempted to address these declines through bold conservation actions, such as translocations and establishment of captive

populations; however agencies have met with only limited success due to rapidly changing disease-transmission conditions on the landscape. There is an urgent need to develop new conservation tools, including landscape-level mosquito control in order to prevent further extinctions.

The application of traditional chemical controls for mosquitoes in both natural areas is impractical and causes unacceptable non-target impacts, whereas IIT carries no non-target risks to native species, humans or the environment. Furthermore, mosquitoes were first introduced to the Hawaiian Islands in the 1800s, and while they are used opportunistically as prey items, no species native to Hawai‘i are dependent on their presence for survival. The control of mosquito populations in Hawaiian forests would thus cause no negative impacts on Hawaiian species.

Demonstrated application of this approach in Hawaii would have also have a wide range of potential positive effects in that it may facilitate the incompatible insect technique approach being used for human health.

con: It is hard to imagine any negative effects since the species is already established in Hawaii. Importing these organisms will not have any foreseeable beneficial effect to this mosquito species already found in Hawaii. The introduction of, for example, increased genetic variation within the mosquito species will be minimized by crossing the lines to mosquitoes originating from Hawaii.

The presence of unintended accompanying microbiota is minimized by the sterile laboratory rearing conditions used. These mosquitoes have been maintained for many generations in the lab environment and have not had the opportunity to obtain pathogens from the wild from blood feeding. The presence of intended microbiota, the *Wolbachia,* potentially has very positive effects on the societal health, the suppression of human disease vectored by mosquitoes, the environment, via population suppression of mosquitoes that vector avian pathogens, and the economy, through the potential increased tourism and lessened disease burden.

This mosquito species is already well established in Hawaii, as are many different strains of *Wolbachia*. MosquitoMate and Verily have a demonstrated track record of success utilizing sex-sorting methods which are highly effective. In the event that technical difficulties did occur during sex-sorting methods, because of cytoplasmic incompatibility, the escape of female mosquitoes carrying a new *Wolbachia* strain is not expected to be stable over the following generations. Laboratory reared females outcrossing to locally established wild male mosquitoes will result in cytoplasmic

incompatibility and the failure of offspring to develop.

There is an extensive body of literature surrounding this mosquito species, its impact upon Hawaii, and Wolbachia-mediated cytoplasmic incompatibility.

Information on *Wolbachia,* with a focus on cytoplasmic incompatibility within mosquitoes:

Atyame, C. M., Cattel, J., Lebon, C., Flores, O., Dehecq, J.-S., Weill, M., Gouagna, L. C. & Tortosa, P. (2015) Wolbachia-based population control strategy targeting*Culex quinquefasciatus* mosquitoes proves efficient under semi-field conditions. *PLoS ONE* 10, e0119288.

Atyame, C. M., Labbé, P., Lebon, C., Weill, M., Moretti, R., Marini, F., Gouagna, L.C., Calvitti, M. & Tortosa, P. (2016) Comparison of irradiation and Wolbachia based approaches for sterile-male strategies targeting *Aedes albopictus*. *PLoS ONE* 11, e0146834.

Barton, N. H., & Turelli, M. (2011). Spatial waves of advance with bistable dynamics:cytoplasmic and genetic analogues of Allee effects. The American Naturalist, 178(3), E48-E75.

Blagrove, M. S., Arias-Goeta, C., Failloux, A. B., & Sinkins, S. P. (2012). Wolbachia strain wMel induces cytoplasmic incompatibility and blocks dengue transmission in*Aedes albopictus*. *Proceedings of the National Academy of Sciences*, *109*(1), 255-260.

Callaini, G., Dallai, R., & Riparbelli, M. G. (1997). Wolbachia-induced delay of paternal chromatin condensation does not prevent maternal chromosomes from entering anaphase in incompatible crosses of *Drosophila simulans*. *Journal of CellScience*, *110*(2), 271-280.

Dobson, S. L., Marsland, E. J., & Rattanadechakul, W. (2001). Wolbachia-induced cytoplasmic incompatibility in single-and superinfected *Aedes albopictus* (Diptera: Culicidae). *Journal of Medical Entomology*, *38*(3), 382-387.

Hamm, C. A., Begun, D. J., Vo, A., Smith, C. C., Saelao, P., Shaver, A. O., ... & Turelli, M. (2014). Wolbachia do not live by reproductive manipulation alone: infection polymorphism in *Drosophila suzukii* and *D. subpulchrella*. Molecular Ecology, 23(19), 4871-4885.

Hoffmann, A. A., Montgomery, B. L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P.H., Muzzi, F., ... & Cook, H. (2011). Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. *Nature*, 476(7361), 454.

Hoffmann, A. A., Ross, P. A., and Rašić, G. (2015) Wolbachia strains for disease control: ecological and evolutionary considerations. *Evolutionary*

Applications, 8(8),751-768.
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RESTRICTED ANIMAL LIST (Part A) §4-71-6.5

SCIENTIFIC NAME	COMMON NAME
FAMILY Culicidae	
Culex quinquefasciatus	mosquito, southern house
FAMILY Drosophilidae	
Drosophila (all species in genus)	flies, pomace Zapriothrica sp.
	biocontrol agent, banana poka
FAMILY Lonchaeidae	
Dasiops curubae	biocontrol agent, banana poka
FAMILY Muscidae	
Musca domestica	house fly
FAMILY Tephritidae	
Ceratitus capitata	fly, Mediterranean fruit
Urophora stylata	biocontrol agent, bull thistle
ORDER Heteroptera	
FAMILY Anthocoridae	
Orius tristicolor	bug, minute pirate
ORDER Homoptera	
FAMILY Eriococcidae	
Tectococcus ovatus	biocontrol agent, strawberry guava
ORDER Hymenoptera	
FAMILY Aphelinidae	
Aphelinus (all species in genus)	parasite, aphid
Cales noacki	parasite, woolly whitefly
Encarsia formosa	parasite, greenhouse whitefly
Encarsia guadeloupae	parasite, spiraling whitefly
Encarsia ?haitiensis	parasite, spiraling whitefly
Encarsia lutea	parasite, sweetpotato whitefly
Encarsia mineoi	parasite, sweetpotato whitefly
Encarsia pergandiella	parasite, greenhouse

Questions from Hawaii Department of Agriculture on Incompatible Culex strain

The following information is being provided by Verily Life Sciences in response to questions from the Hawaiian Department of Agriculture (HI-DoA) about the *w*AlbB-strain *Culex quinquefasciatus* (incompatible i.e. conditionally sterile mosquitoes), which the US Fish and Wildlife Service, the Birds Not Mosquitoes (BNM) coalition and other collaborators wish to import into Hawaii for use in a mosquito control program that uses Sterile Insect Technique (or in this case, an incompatible insect technique). The objective of this program is to protect native Hawaiian birds against avian malaria, which is vectored by invasive *Cx quinquefasciatus* in Hawaiian bird reserves.

Background information:

Debug is a Verily Life Sciences (Verily) project aimed at developing technology to rear and release sterile or incompatible mosquitoes to reduce mosquito populations that transmit disease. Much of the Debug project's work has focused on developing tools for the mass rearing and effective release of male mosquitoes that are conditionally sterile against Wild Type (WT) mosquitoes due to cytoplasmic incompatibility (CI): whereby male insects infected with *Wolbachia* that mate with either females without *Wolbachia* or those infected with different *Wolbachia* strain, produce non-viable progeny, as embryonic development is halted. CI is the basis for the *Wolbachia* Sterile Insect Technique (SIT), also referred to as the Incompatible Insect Technique (IIT). Debug has successfully developed and deployed incompatible male *Aedes aegypti* mosquitoes in several large scale collaborative programs. For reference the following publications provide detail about the efficacy, general approach, rearing, sex sorting, release and field results of some of these projects:

- Crawford et al 2020, Fresno CA, USA (2017-2018)
- Beebe et al 2021, Innisfail QLD, Australia (2018), see also Australian CSIRO website on the Innisfail project.
- Ng et al, 2021, Singapore (2018-current), see also Singapore National Environment Agency website on Project *Wolbachia* Singapore

While there are no recent publications on field programs using incompatible *Cx. quniq*., there is a 1967 publication showing local elimination of *Culex pipiens* using release of incompatible *Wolbachia* males in Myanmar (Laven, H. Eradication of *Culex pipiens fatigans* through Cytoplasmic Incompatibility. Nature 216, 383–384 (1967). https://doi.org/10.1038/216383a0.), and incompatibility in *Cx. pipiens* is widely studied.

In Hawaii, Debug has been requested by Hawaiian conservation groups including the Birds Not Mosquitoes coalition, The Nature Conservancy and the US Fish and Wildlife Service, to participate in a project that attempts to protect native Hawaiian birds from the depredations of Avian Malaria vectored by the invasive *Cx. quniq*. mosquitoes. This project would release

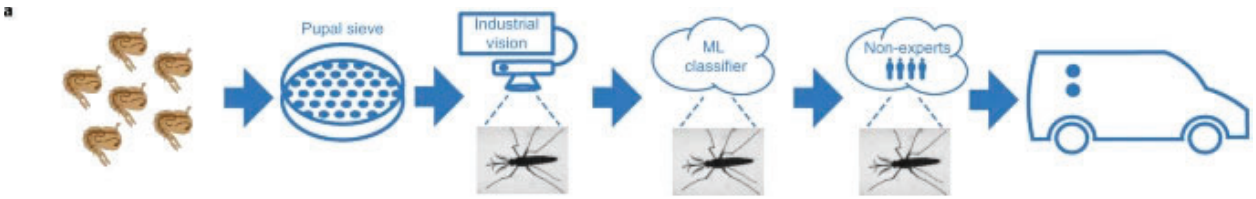
incompatible male *Cx. quinq*. into forest reserves with the goal of reducing wild-type (WT) *Cx. quniq*. mosquitoes and hence reducing malaria infections in birds. These male mosquitoes have a short lifespan (days), can not bite or vector disease, and are conditionally sterile due to the presence of an incompatible strain of the endosymbiont *Wolbachia pipientis* already present in Hawaiian mosquitoes (*w*AlbB sourced from Hawaii.) As noted previously *Cx. quinquefasciatus* are widespread in Hawaii, *Wolbachia pipientis* is present in a majority of insect species, including many endemic to Hawaii, all *Culex* and *Aedes albopictus* mosquitoes in HI have Wolbachia pipientis, and thus there is a long history of human and ecological exposure.

Verily's understanding is that the Hawaiian Department of Agriculture intends to submit an emergency application under FIFRA Section 18 to the EPA for temporary registration of the *w*AlbB in *Cx. quinq*. and that releases will be conducted under appropriate permits.

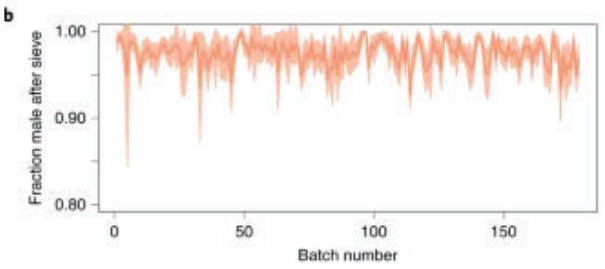
Questions from Christopher Kishimoto (Hawaii Department of Agriculture, Plant Quarantine Branch, Entomologist)

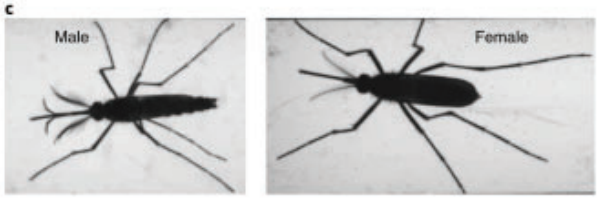
1. Could you please walk us through your procedures on how you eliminate female mosquitos from being released?

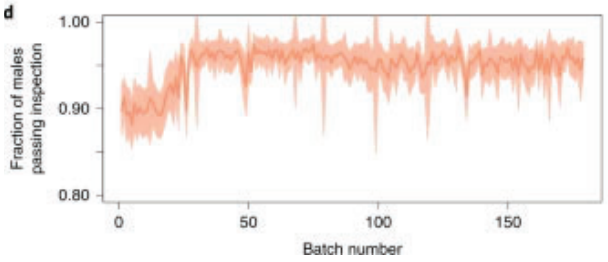
Verily's multi-stage mosquito sex sorting system removes females with extremely high accuracy, while retaining most males for release. As outlined in Crawford et al. 2020 the system comprises 3 stages: 1) sieving of pupae to remove the vast majority of females, as female pupae are larger than males, 2) adult sex sorting using a real-time industrial vision system that uses multiple images of every adult mosquito to identify and remove females, and finally 3) we submit all images of individuals labeled male by the industrial vision system for scoring by a machine learning classifier which acts as a quality control system with further human review to identify and enable removal of any potential females accepted by the adult industrial vision sorter. For *Aedes aegypti* the combined system is expected to release 1 female for every 900 million males with a 95% CI of 1:200 million to 1:26 billion (Crawford et al. 2020). We are currently adapting the Verily sex sorting pipeline to *Culex* mosquitoes but as the basic features used for sex sorting, particularly the adult morphology, are the similar between *Culex* and *Aedes* we hope for extremely high accuracy sorting. As we do for Aedes as a part of EPA permitted manufacturing, we will also perform regular QC assays to confirm absence of females in sample release batches prior to the beginning of shipments and importation into Hawaii. See diagram below from Crawford et al. 2020



Probability of a female contaminant (95% CI)	0.50 (–)	0.03 (0.00 – 0.08)	2.39×10^{-5} $(1.30 \times 10^{-5}) - (3.79 \times 10^{-5})$	1.82×10^{-3} $(3.76 \times 10^{-4}) - (4.38 \times 10^{-3})$	1.13×10^{-4} $(3.08 \times 10^{-9}) - (4.83 \times 10^{-4})$	1.13×10^{-9} $(3.88 \times 10^{-11}) - (4.44 \times 10^{-9})$







a, Illustration of the entire [*Aedes aegypti* mosquito] sex-sorting pipeline, including the mechanical pupal sieve, real-time adult visual inspection, cloud-based machine learning classifier, and [expert human] review. The probability of a female contaminant with 95% CIs for each step is shown along with the estimated overall female contamination rate for the entire pipeline in the final column. [note: the sorting process has been updated since 2018 to include both expert review and other algorithmic improvements] **b**, The fraction of mosquitoes imaged by the sex sorter after the pupal sieve that were male with s.d. intervals shaded for 179 production batches. **c**, Example images from the adult sex sorter (male on the left and female on the right) used by both the industrial vision system and machine learning classifier. **d**, The fraction of true males that were correctly labeled and accepted by the Industrial Vision system with s.d. interval shaded (n = mean of 96, range of 10–140 independent sex-sorter lane measurements per batch). After the industrial vision stage there are further QC inspection steps as noted above.

2. How often are quality control measures implemented?

Every single mosquito Verily provides for release goes through the above sex-sorting pipeline, with multiple stages of independent computer review of each adult mosquito, followed by additional human and computer quality control reviews.

Sieve performance is monitored in every single batch, and in addition as a part of the documented (and EPA reviewed) manufacturing process Verily regularly conducts a "female contamination" assay to ensure that adult sorting runs let through no females, validating that release batches are at <1:250:000 females:males according to Verily's calculations and EPA requirements.

3. How often will quality control checks be implemented in the future?

As noted above, production of *Cx. quinq*. would be under a similar EPA reviewed manufacturing process as used for Verily's *Aedes* manufacturing. Verily is regularly updating sex sorting algorithms and protocols to increase accuracy.

4. Have female mosquitos ever been found in batches of mosquitos destined for field release? If so, how often?
5. Have batches of mosquitos been halted for distribution because of the findings of any female mosquitos or other problems?
6. Have Verily female mosquitos ever been collected from the environment?
7. How often does Verily survey release sites for Verily female mosquitos?

We will provide answers to questions (4-7) together as they are all aimed at identifying the likelihood of *w*AlbB female releases and identification of this in field environments, which could potentially reduce the efficacy of incompatible male releases.

As a part of Verily's *Aedes* manufacturing process as documented in Crawford et al. 2020, a small number of females (~<1:250k) are found in batches *prior* to field release. As noted above a secondary quality control review identifies and removes these resulting in extremely low female contamination rate in released mosquitoes. In our Singapore collaboration we identified a very small number of released females in batches comprising several million males, and have since updated our protocols to reduce the likelihood of this recurring.

Regarding the environmental collection of Verily female mosquitoes: The incompatible *Aedes* programs undertaken by Verily and collaborators have Verily (or our partners) routinely monitor trap collections for incompatible adult females i.e. females positive for the released strain of *Wolbachia* as assessed by molecular assays, and we also test larvae from ovitraps for the presence of this *Wolbachia*. We propose that this be included as a part of any fieldwork and surveillance accompanying a release program of incompatible *Culex quinq*. males in Hawaii. We also note that *Wolbachia pipientis* is present in a majority of insect species, including many endemic to Hawaii and all *Culex* and *Aedes albopictus* mosquitoes in HI have *Wolbachia pipientis,* and thus there is a long history of human and ecological exposure.

ZAP (transfected *Ae. albopictus*) and WB1 are MosquitoMate products, and we are unaware of any Verily manufactured ZAP or WB1 females being discovered in the environment. As noted above (and as reported in Ng et al 2021) Verily discovered the accidental release along with field collection of a very small number of *Ae. aegypti* females in Singapore, which despite ongoing release programs have not spread. As noted in the Singapore paper, in response protocols for sex sorting and QC have been significantly improved.

Exact protocols (e.g. sampling rate, pooling, etc.) for field surveillance to identify incompatible Culex adult females and/or larvae in Hawaii will need to be agreed and finalized.

8. What are the overall results from field releases of Verily mosquitos so far?

As outlined above in the introduction there are several studies showing that incompatible (transfected) male *Ae. aegypti* can cause suppression of wild mosquito populations when operated using Verily's rearing and release systems.

- California (in collaboration with MosquitioMate and others) showed 95% (up to 99%) suppression in treatment areas relative to controls: Crawford, J.E., Clarke, D.W., Criswell, V. et al. Efficient production of male *Wolbachia*-infected *Aedes aegypti* mosquitoes enables large-scale suppression of wild populations. Nat Biotechnol 38, 482–492 (2020). https://doi.org/10.1038/s41587-020-0471-x
- Australia (in collaboration with the Australian CSIRO and others) showed >80% (up to 97%) suppression in treatment areas relative to controls: NW Beebe, D Pagendam, BJ Trewin, A Boomer, M Bradford, A Ford, et al. Releasing incompatible males drives strong suppression across populations of wild and *Wolbachia*-carrying *Aedes aegypti* in Australia. Proceedings of the National Academy of Sciences 118 (41). https://www.pnas.org/doi/full/10.1073/pnas.2106828118
- Singapore Ng. et al 2021 MedRxiv Preprint Paper in collaboration with Singapore National Environment Agency showed 98% suppression in treatment areas relative to controls.

In addition to recent *Aedes* field results there is a 1967 *Culex pipiens* paper showing local elimination (100% suppression) using release of incompatible males in Myanmar: Laven, H. Eradication of *Culex pipiens fatigans* through Cytoplasmic Incompatibility. Nature 216, 383–384 (1967). https://doi.org/10.1038/216383a0.

9. How long does it take for wild mosquito populations to get back to prerelease populations once Verily mosquitos have stopped being released?

It is unknown what would happen with *Cx. quinq*. in the proposed program areas after a successful suppression program, as it will depend on migration rates from outside the treatment area, natural fecundity of wild mosquitoes in the local ecology and a variety of other factors. Any release program would need to maintain an ongoing surveillance program to monitor this.

10. Does Verily have EPA approval to release its mosquitos in Hawaii?
11. Does Verily have Hawaii Department of Agriculture Pesticides Branch approval to release its mosquitos in Hawaii?

In answer to 10-11: As previously discussed with HI-DoA, Verily will support partners and HI-DoA in applying for EPA permits along with any state permits required to undertake this program.

12. How many Verily mosquitos would have to be released to achieve adequate population control in Hawaii's environments?

This will be determined by Verily and local partners based on the results of an initial Mark Release Recapture (MRR) trial, which would give information on wild-type population numbers

and the dispersion and survival of released incompatible males. In general most IIT programs aim to achieve a ratio of 1:10 Wild Type male:sterile male in field traps in the treatment area to ensure strong suppression in each generation.

13. What is the duration of time needed to achieve adequate mosquito population control once releases start?

This will depend on a number of factors including wild type population, release numbers, efficacy of dispersion etc, along with the efficacy of the surveillance program used to measure impact. *Aedes* incompatible male release programs and other SIT projects typically see measurable impact on hatch-rate within several weeks, though it may take months for significant wild-type population reduction. Laven 1967 saw initial reductions after several weeks with incompatible male *Cx. pipiens* releases though this was in a village setting.

14. How will Verily handle a request to specifically manufacture Hawaiian biotype mosquitos, especially if orders for those mosquitos may be inconsistent?

Verily will review with requesting partners as project plans develop.

15. Would Verily be able to show verifiable proof that only Hawaiian biotype mosquitos will be shipped to Hawaii?

Yes, Verily's manufacturing process maintains molecular assays and physical containment to ensure quality controls and biosecurity of shipped mosquitoes.

Sex-sorted male mosquitoes will be shipped to Hawaii from our rearing facility in California and produced using Verily's mosquito manufacturing process which will be reviewed as a part of a HI DoA submitted Section 18 permit application. Males will be transported in line with any issued label and permits.