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| **From:** | Eric B |
| **To:** | DLNR.BLNR.Testimony |
| **Subject:** | [EXTERNAL] Oppose C6 |
| **Date:** | Thursday, May 9, 2024 8:54:15 AM |

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I oppose increase in funding for the release of mosquitoes in Hawaii. It is a trespass on the population and a reckless experiment.

Regards,
Eric Bjerke
Honokaa, HI

blnr.testimony@hawaii.gov

Sent May 8, 2024 via email

**Hearing BLNR May 10th at 9:00am testimony Comments on Agenda Item C Division of Forestry and Wildlife #6**

1151 Punchbowl St. Room 132 (Kalanimoku Building), Honolulu.

From Cynthia Groves Current resident on Oahu, Former resident on Maui and Kauai

Honorable Board of Natural Resources Chair and members.

Thank you for the opportunity to testify. My comments relate to the following reasons for opposition to the BLNR 5/10/24 re: agenda item C6 I propose it is far more reasonable to:: **Make the Environmental Impact Study (EIS) conditional on receiving funding and have whoever does the EIS report put in benchmarks that have to be reached for additional funding, rather than automatically giving out money**. **Make coordination with Emergency Management and HI Department of Health part of those benchmarks, including a EM Plan and Health Department Coordination Plan, A Bird Monitoring Plan and documentation, Plan for further research, Plan for unintended consequences, and a Financial accountability plan. The word "safe" and "effective" need to be clearly defined and documented with any proposal for funding. Proof that the IIT actually works and comparing it to other approaches or in concert with other approaches ie mosquito predators. (Are dragonflies even being partially considered as a modality?) Needs a justification of impacts on other sectors of the economy prior to funding. What are the protections for biodiversity? IIT doesn't just happen in a vaccum! It's not a license for industry to get free money from DLNR for their bigger agenda. Hawaii is not a petri dish.**

A lawsuit on the need for an Environmental Impact Study (EIS) brought to light important considerations which are definitely needed prior to huge mosquito releases!. These are my concerns coming from a health and environmental perspective.

All mosquitoes are invasive species and non-native for that reason alone, we need to make sure due diligence occurs **BEFORE** release into the environment so an Environmental Impact Statement is essential, **not just an Environmental Assessment in approving any funding**.

1) There are **Containment and unresearched issues** given introducing billions of the Wolbachia per week which impacts not just the particular bird population, but the human population and numerous cascading effects on the environment.

2) **Too many unanswered questions prior to mosquito release** into the wild that impacts the public and Hawaii long term, let alone Kauai.

I found the below concerns on line:

- There are no published safety studies showing that the lab mosquitoes won't be better at transmitting West Nile Virus (WNV) to humans and birds, should WNV become established in Hawaii. One study has already shown that *Wolbachia* enhances West Nile Virus infection in one species of Culex mosquito.
- Some *Wolbachia* infections can increase the probability of pathogen infection or transmission by mosquitoes (Hughes et al., 2014, Dodson et al., 2014), and there is a risk that the release of *Wolbachia*-infected mosquitoes could increase, rather than prevent, disease.
- Horizontal transmission of the introduced bacteria (non-hereditary spread of an infectious agent from one group or individual to another, directly or indirectly) has been documented in peer-reviewed studies
- Horizontal transmission may cause the creation of introduced-strain-infected females in the wild
- Unexpected, dangerous evolutionary events may occur
- The capacity for evolutionary offspring to spread disease is unknown
- Horizontal transmission and evolutionary events are documented in a 2020 study out of Singapore, "*Wolbachia* infection in wild mosquitoes (Diptera: Culicidae): implications for transmission modes and host-endosymbiont associations in Singapore" – Huicong Ding, Huiqing Yeo, Nalini Puniamoorthy (BMC,

12/09/2020) https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-020-04466-8

- Through evolution, the existing wild mosquitoes could be replaced by the lab-bred mosquitoes, thereby establishing the lab-bred invasive population in the wild (this is called "population replacement")
- Horizontal gene transfer of *Wolbachia* DNA to other invertebrates may occur (the movement of genetic information between organisms – a process that includes the spread of antibiotic resistance genes among bacteria, fueling pathogen evolution)

**3) There should be reasonable caution regarding mutations, outbreaks, and another pandemic and a clearly defined plan** due to an uncontrolled experimental release with a dramatic increases of billions more mosquitoes weekly that could backfire. Mosquitoes are an invasive species and bite people with serious health consequences. The Mosquito-borne diseases that may cause serious illness in humans, include dengue, chikungunya, and Zika virus diseases transmitted by the day-biting *Aedes (Stegomyia)* mosquitoes, which are found here in Hawaii. Specifically, *Aedes albopictus* may be found on all islands. While we want to save our endemic species, I am concerned about minimizing a variety of impacts of this widespread release of billions of mosquitoes in the process with untoward consequences **without an EIS**. Mosquitoes have caused billions of deaths worldwide. While *Aedes aegypti* has only been found in some areas on the Big Island. Not only can mosquitoes carry diseases that afflict humans, but they also can transmit several diseases and parasites that dogs and horses are very susceptible to. These harms include dog heart worms, eastern equine encephalitis and West Nile virus.

4) **Preparation to prevent mosquito impacts in Hawaii in coordination with the HI Dept of Health**. Two species of mosquito, *Aedes aegypti* and *Aedes albopictus*, are the chief vectors for diseases that affect humans, including malaria, which raised alarms around the world in 2016. Hawai'i's Big Island suffered an outbreak of dengue fever in 2016 that sickened more than 260 people. Not only can mosquitoes carry diseases that afflict humans, but they also can transmit several diseases and parasites that dogs and horses are very susceptible to. These include dog heart worms, eastern equine encephalitis and West Nile virus. **Worst case scenario of course would be like swarms like locusts taking over and causing harm to other vegetation, humans, dogs, horses**. What is the plan in these instances to prevent this? An EIS would take this into consideration.

On the Dept of Hawaii website, there are several cases between Dengue and Malaria reported in Hawaii in 2024. On releases, we must also learn our lesson from the pandemic lab release that began with a few releases in China that spread throughout the world.

**4) Our Future**. With a dramatic increase in mosquito populations from the experimental mosquito release of billions of mosquitoes, that can likely dramatically change the risk factor and complications to health and other economic sectors in Hawaii.

How would **mosquito release perception direction**, **and activity** affect Hawaii's tourism industry, the health of her diverse population of people, local businesses, natural farming industry and outdoor ventures, residence desirability should outbreaks occur or another pandemic happen from experimental mosquito releases? And particularly if Hawaii becomes a major hub for the biotech insect agenda in their approach by the introduction of billions of mosquitoes?

5) **Concern for balance of interests and unintended consequences.**. This project is not limited to the attempt to cure our population of birds endemic to Hawaii, its forestry, or limited to the ag islands. Do we really want Hawaii to be discovered to be the biotech insect center for the world? How will that affect other industries on the islands and their populations? This project could easily appears to be a dangling carrot to the idea of building out the biotech insect agenda that sounds economically enticing to one industry at the expense of other industries on the island—tourism industry the local businesses, home buying, natural farming industry,  let alone overwhelm to hospitals as did the pandemic should the lab mosquito experiment go awry.

6. **Bioprospecting and bioprosperity enticements and consequences**. I pray our legislators are not enticed by nebulous claims of safety to further the agenda of biotech industry.  In theory, expansion plans economically and biologically can look viable on paper and to the "horsetrading" mentality. Environmental consequences have real world effects. Experimental proliferation of mosquitos may render a benefit to some, yet be destabilizing to others depending on the degree and overall priorities. Look at the results in China, Asian and African countries where insect carriers of disease have survived. The consequences to human health and economy from building out the biotech insect agenda have the larger harms of unintended releases just like with Wuhan Labs experiments. Lab altered mosquitos and mutations can breed bacterial infections, Dengue fever, Zika virus in humans, and other anomalies risk harm to those who live here. This can't just be glossed over and can ensue when experiments go awry..

**Balancing impacts on biodiversity.**..Modifying biotechnology experiments already have in numerous places across the planet overtaken natures balance and wiped out biodiversity in the name of bioprosperity and bioprospecting. Locust-like spreads as mentioned would be a worst case scenario. Further, I am a perfect example of someone with known genetic DNA predisposed to being bitten by mosquitos which when infected become serious risks such as in my case. Many may not have been DNA tested to even know how widespread this risk is or could become. Our human population on Hawaii is already compromised, particularly since the pandemic.

I am further noting research here that Hawaii Unites discovered and has I understand put forth in a lawsuit: “These bacteria-infected mosquito releases are a dangerous experiment on our islands. Serious concerns about this plan have not been adequately addressed. The agencies involved in this project have lied about the introduction of foreign organisms into the islands and about the release of female mosquitoes that bite, breed, and spread disease. On Maui, these agencies are also flagrantly deviating from the approved plan, increasing the risks of helicopter fire and accident incidents.

Southern house mosquitoes transmit diseases to people and animals, and pathogen screenings are not being disclosed. *Wolbachia* bacteria can cause mosquitoes to become more capable of spreading diseases. The agencies releasing these lab-altered mosquitoes have admitted that the **plan does not include monitoring the effects of the experimental mosquitoes on forest birds.** This project has the potential to cause the extinction of the native birds it is meant to protect, and it could impact the health of the people. This organization is demanding an Environmental Impact Study (EIS).”

**Current direction**. “ These mosquito releases on Maui and Kaua‘i are being presented as conservation actions, but the potential significant impacts to our environment have not even been studied**.** Not only are these projects experimental, but these releases are just the beginning of a much bigger biotech insect agenda planned for all islands. The Department of Land and Natural Resources (DLNR) – proposing agency for the projects – has a lab in Hawai‘i, and they’ve been funded to build out their insectary where they intend to mass produce lab-altered mosquitoes for release on the islands into perpetuity (forever)**.**

Millions of dollars in federal funding continue to pour in, and these agencies are already making future plans to expand into dangerous mosquito gene drives, synthetic biology control tools, and CRISPR gene-edited mosquitoes (pgSIT)**.** The Hawai'i Department of Health is also running a parallel program where they intend to release lab-altered mosquitoes throughout the islands to "control mosquitoes of public health concern."

**These experimental approaches should be run in "contained settings" prior to release into the wild. Replacing nature with synthetic varieties may not be acceptable in the mating dance with mosquitoes, nor in repair to health once bitten. This should be discovered prior to putting anything into the wild.**

**Sincerely,**

**Cynthia Groves**

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**Health and environmental consultant for 20 years, retired**

**Kailua, HI**

Parasites & Vectors

RESEARCH Open Access

# *Wolbachia* infection in wild mosquitoes (Diptera: Culicidae): implications for transmission modes and host-endosymbiont associations in Singapore

Huicong Ding†, Huiqing Yeo† and Nalini Puniamoorthy*

## Abstract

**Background:** *Wolbachia* are intracellular bacterial endosymbionts found in most insect lineages. In mosquitoes, the influence of these endosymbionts on host reproduction and arboviral transmission has spurred numerous studies aimed at using *Wolbachia* infection as a vector control technique. However, there are several knowledge gaps in the literature and little is known about natural *Wolbachia* infection across species, their transmission modes, or associations between various *Wolbachia* lineages and their hosts. This study aims to address these gaps by exploring mosquito-*Wolbachia* associations and their evolutionary implications.

**Methods:** We conducted tissue-specific polymerase chain reaction screening for *Wolbachia* infection in the leg, gut and reproductive tissues of wild mosquitoes from Singapore using the *Wolbachia* surface protein gene (*wsp)* molecular marker. Mosquito-*Wolbachia* associations were explored using three methods—tanglegram, distance-based, and event-based methods—and by inferred instances of vertical transmission and host shifts.

**Results:** Adult mosquitoes (271 specimens) representing 14 genera and 40 species were screened for *Wolbachia*. Overall, 21 species (51.2%) were found positive for *Wolbachia*, including five in the genus *Aedes* and five in the genus *Culex*. To our knowledge, *Wolbachia* infections have not been previously reported in seven of these 21 species: *Aedes* nr. *fumidus*, *Aedes annandalei*, *Uranotaenia obscura*, *Uranotaenia trilineata*, *Verrallina butleri*, *Verrallina* sp. and *Zeugnomyia gracilis*. *Wolbachia* were predominantly detected in the reproductive tissues, which is an indication of vertical transmission. However, *Wolbachia* infection rates varied widely within a mosquito host species. There was no clear signal of cophylogeny between the mosquito hosts and the 12 putative *Wolbachia* strains observed in this study. Host shift events were also observed.

**Conclusions:** Our results suggest that the mosquito-*Wolbachia* relationship is complex and that combinations of transmission modes and multiple evolutionary events likely explain the observed distribution of *Wolbachia* diversity across mosquito hosts. These findings have implications for a better understanding of the diversity and ecology of *Wolbachia* and for their utility as biocontrol agents.

**Keywords:** *Wolbachia*, *Wolbachia* surface protein gene, Reproductive endosymbiont, Tissue-specific polymerase chain reaction, Transmission modes, Host-endosymbiont association

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## Background

*Wolbachia* are intracellular endosymbiotic bacteria that alter host reproduction [1]. They are widespread in arthropods, infecting a wide range of insect, crustacean, and nematode species [2, 3]. In some cases, *Wolbachia* exist in a mutualistic relationship with their hosts [4–6]. However, *Wolbachia* are most often recognised as reproductive manipulators that bias the sex ratio of the host offspring towards the production of more infected females [7, 8]. This reproductive manipulation is commonly achieved through four phenotypes—male killing [9], feminisation [10, 11], parthenogenesis [12, 13], and cytoplasmic incompatibility [14, 15]—which increase the endosymbiont's reproductive success [16]. Owing to their strong influence on host reproduction, an increasing amount of research is being dedicated to exploring the impacts of reproductive endosymbionts on host population dynamics and evolution [17, 18], especially in medically important insects such as mosquitoes. The promising use of *Wolbachia* to alter both mosquito reproduction [19] and arboviral transmission [20] has prompted the deployment of novel *Wolbachia*-infected mosquitoes for population replacement and suppression [21].

Several countries, including Singapore, have started to employ *Wolbachia* as biocontrol agents of mosquitoes by releasing infected mosquitoes [22–24]. However, the presence of naturally occurring endosymbionts in wild mosquito populations has not been adequately assessed. The release of mosquitoes artificially infected with *Wolbachia* might have a profound impact on closely interacting wild mosquito populations through various transmission modes. For instance, horizontal transmission of an introduced *Wolbachia* strain may result in manipulation of the reproductive biology of non-target species, which could potentially result in an unintentional population crash, opening up niches for other vector species [25]. Another possible effect of this type of biocontrol method is the increased likelihood of co-infections with other naturally occurring *Wolbachia* strains or other endosymbionts, such as *Cardinium*, *Rickettsia*, and *Spiroplasma*. These co-infections may result in a synergistic effect on mosquito host fitness and future transmission of endosymbionts [26–29]. Without a detailed characterisation of *Wolbachia* prevalence and diversity among wild mosquitoes, the ecological risk of releasing artificially infected mosquitoes might be overlooked. Therefore, bearing the precautionary principle in mind, it is important to investigate the natural occurrences of *Wolbachia*.

There is also a need to discern the main mode of infection transmission among mosquitoes. Although *Wolbachia* are mainly thought to be vertically transmitted [15, 30], there have been accounts of horizontal transmissions into wild populations through parasitism [31, 32], or through proximity to infected individuals [33]. *Wolbachia* may not be strictly localised in germline tissues, as they have also been detected in somatic tissues such as the gastrointestinal tract and haemolymph [34–36]. The detection of *Wolbachia* in the gastrointestinal tract suggests that they could be horizontally transmitted through uptake from the environment or host sharing [34, 37, 38], whereas their detection in non-gastrointestinal somatic tissues, such as those of jointed appendages, could indicate horizontal bacterial genome integration into the host genome [36]. Currently, detection of *Wolbachia* in mosquitoes is mostly achieved through conventional polymerase chain reaction (PCR) methods using DNA extracted from an entire individual or its abdomen [39–47]. This limits our ability to identify the site of endosymbiont infection within an individual (tissue tropism). Tissue-specific screening of *Wolbachia* is necessary to provide insights and infer the extent of vertical and horizontal transmission.

It has been proposed that host mitochondrial DNA (mtDNA) and *Wolbachia* are maternally co-transmitted within the cytoplasm [17, 48], which suggests a congruency between host mtDNA and *Wolbachia* phylogenies—a consequence of cytoplasmic hitchhiking driven by endosymbiont transmission [17]. In insect systems such as bedbugs where vertical transmission has been established to be the main mode of transmission, *Wolbachia* exhibit clear patterns of cophylogeny with their hosts, with few instances of host shifting or multiple infections within a single host species [49, 50]. In contrast, cophylogeny is not apparent among nematodes and bees, and numerous acquisitions of *Wolbachia* infections through horizontal transmission as well as losses have been shown in these diversified host lineages [51, 52]. The modes of *Wolbachia* transmission among mosquitoes have not been well established, nor has the extent of multiple infections within mosquito hosts or host shifting of these bacteria.

There is presently no comprehensive analysis of the evolutionary associations between *Wolbachia* and their mosquito host species. An understanding of host-endosymbiont associations will not only further our ability to discern the mode of transmission which influences *Wolbachia* diversity, but will also allow for an evaluation of *Wolbachia* host specificity, speciation, and their ability to establish in new hosts. All of this is key to understanding the diversity and ecology of *Wolbachia*, and their utility in biocontrol methods.

This study has three major research objectives. First, to examine the prevalence and diversity of *Wolbachia* among wild mosquitoes from Singapore. Second, to determine the tissue tropism of *Wolbachia* infection

in mosquitoes using a tissue-specific PCR screening method. Finally, to reconstruct the evolutionary associations between *Wolbachia* and their mosquito hosts to provide a basis for an understanding of host-endosymbiont evolution.

## Methods

### Adult mosquito collection and identification

Mosquito samples were collected from 12 localities across Singapore between March 2018 and November 2019 (Fig. 1a). Three methods were employed to collect the samples: $CO_2$-baited Centers for Disease Control and Prevention traps, sweep-netting using hand-held fan traps, and larval sampling [53]. For the latter, dipping was carried out at streams and ponds and pipettes were used to collect larvae from various microhabitats, including tree holes, plant axils, and artificial containers. Thereafter, the field-collected larvae were reared to adults in an incubator maintained at 26 °C and 70% relative humidity, under a 12:12-h (day:night) photoperiod. Larvae were fed with pulverised fish food (TetraMin Granules) daily. Mosquitoes were identified using relevant taxonomic keys and descriptions [54–59]. A subset of individuals from commonly sampled species was selected and preserved in phosphate-buffered saline solution at – 80 °C for subsequent dissection step.

### Tissue-specific dissection

Tissue-specific dissection was carried out on each adult mosquito sample to isolate the leg, gut, and reproductive tissues (Fig. 1b–d). To prevent the contamination of tissues with bacteria on the external surface of the mosquito, the leg was removed first before isolating the gut and reproductive tissues. All dissection equipment and microscope slides were thoroughly wiped with 70% ethanol before commencing dissection of the next sample. Dissected tissues were individually placed into a 96-well plate on ice to prevent DNA degradation.

### DNA extraction, PCR amplification, and sequencing

DNA extraction of each dissected tissue was performed using 7 µl of QuickExtract DNA Extraction Solution (Lucigen, Madison, USA) in a thermocycler (Eppendorf, Hamburg, Germany) with the following protocol: 65 °C for 18 min, followed by 98 °C for 2 min, ending with cooling on ice for at least 10 min. All dissected tissues were screened for *Wolbachia* infections following single-primer PCR protocols described by Martin et al. [26] with slight modifications to the cycle conditions. The *Wolbachia* surface protein gene (*wsp*) general primers, wsp81F (5′-TGGTCCAATAAGTGATGAAGAAAC TAGCT-3′) and wsp691R (5′-AAAAATTAAACGCTA CTCCAGCTTCTGCAC-3′), were used in this study [60]. In addition, a fragment of the cytochrome c oxidase subunit I (*cox*1) gene of the mosquito hosts was also amplified using primers LCO1498 (5′-GGTCAACAA ATCATAAAGATATTGG-3′) and HCO2198 (5′-TAA ACTTCAGGGTGACCAAAAAATCA-3′) [61]. This served to confirm host identity and acted as an internal control. We used DNA from known *Wolbachia*-infected *Nasonia* specimens as positive controls for this study.

All PCR procedures were performed in reaction mixtures consisting of 12.5 µl of GoTaq G2 Green Mastermix (Promega, Madison, USA), 1 µl of 1 mg $ml^{-1}$ bovine

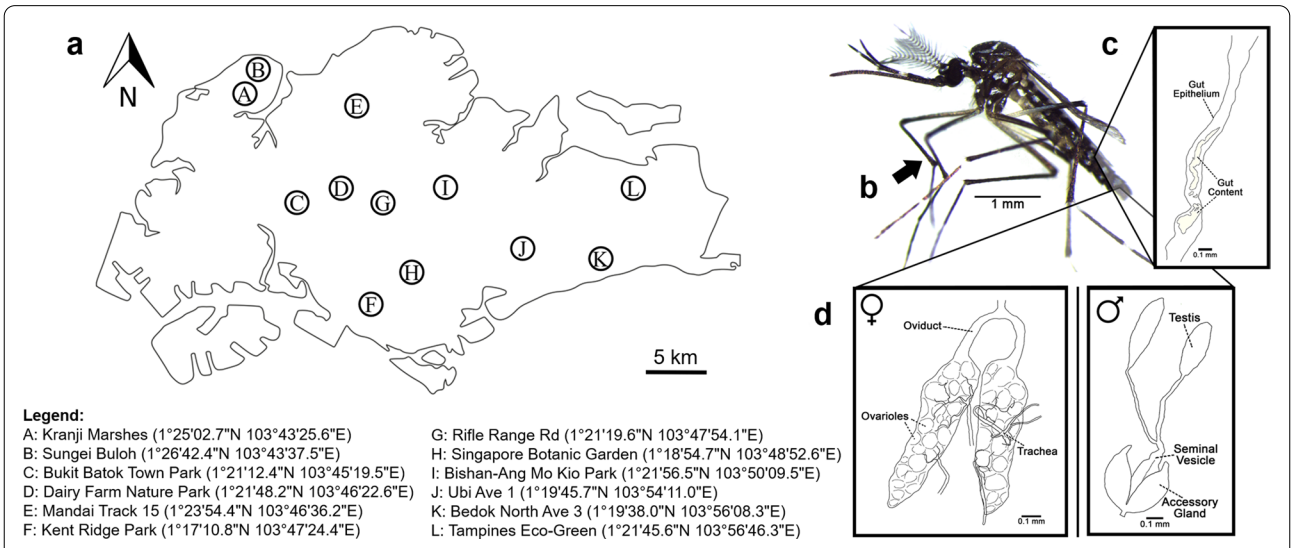


**Fig. 1a–d** Map of sampling sites and diagrammatic images of *Aedes aegypti* and its dissected tissues. **a** Various mosquito collection localities across Singapore and their respective coordinates, **b** mosquito leg, **c** gut, **d** female reproductive tissue (*left*) and male reproductive tissue (*right*)

serum albumin, 0.184 µl of 25 mM magnesium chloride, 1.5 µl of extracted DNA, and 1.5 µl each of 5 µM *wsp* forward and reverse primers for *Wolbachia* PCR screens or 1.0 µl each of 5 µM LCO1498 and HCO2198 primers for *cox*1 PCRs. Double-distilled water was used to top up the reaction mixture to a final volume of 25 µl. PCR amplification of positive and negative controls was also conducted simultaneously.

PCR conditions were as follow: 94 °C for 5 min, followed by 35 cycles of 95 °C for 30s, 55 °C for 45s, and 72 °C for 1 min, with a final elongation step of 72 °C for 10 min. Amplicons were separated by gel electrophoresis on 2% agarose gel stained with GelRed (Biotium, Fremont, USA) and visualised under a ultraviolet transilluminator (Syngene, Cambridge, UK). PCR products were purified using SureClean Plus (Bioline, London, UK) following the manufacturer's protocol. Samples were sequenced by First Base Laboratories (Axil Scientific, Singapore), using a 3730XL DNA Analyzer (Applied Biosystems, Waltham, USA). Obtained sequences were edited and aligned using Geneious Prime (version 2019.2.3) (https://geneious.com). Similarities with publicly available sequences were assessed using the Basic Local Alignment Search Tool (BLAST) [62].

#### Statistical analyses

To test if there were significant differences in *Wolbachia* infection across the different mosquito tissues, Cochran's Q-test was carried out. As a follow-up, McNemar's post hoc test was employed to identify the tissue pairs that differed significantly in infection. Individuals for which the internal control (*cox*1 gene) was not amplified successfully for any of the three dissected tissues were excluded from this statistical analysis. The effect of sex on host infection was also tested using binary logistics regression with sex as a categorical dependent variable and infection outcome as a binary independent variable. Logistic regression was conducted on a subset that only included species that had a roughly similar representation of both sexes, i.e. for every species included, the number of individuals of the less common sex was proportionally at least 60% of the number of individuals of the more common sex. This was to prevent a biased analysis due to a dataset with unequal representation of the sexes. Statistical significance was determined as $P < 0.05$. All statistical analyses were performed in R version 3.6.2 [63] with packages nonpar [64], rcompanion [65], and ISLR [66].

#### Sequence analyses

Multiple alignment of consensus sequences was carried out using the ClustalW algorithm with default settings (gap penalty = 15, gap extension penalty = 6.66) [67], in software MEGA X [68]. Mosquito *cox*1 sequences generated in this study were aligned with 61 reference *cox*1 barcodes of identified local mosquitoes from Chan et al. [53]. For *wsp* sequences, the generated sequences were aligned with 54 available *wsp* sequences of known *Wolbachia* strains obtained from GenBank [69]. Short sequence reads (< 500 base pairs) were excluded.

Neighbour-joining (NJ) phylogenetic trees for mosquito hosts and *Wolbachia* were reconstructed using the sequenced *cox*1 gene fragment and the *wsp* gene, respectively. *cox*1 sequences from previous publications were not included because a comparison of the genetic relationships between the hosts was not the aim of this research. Instead, 54 *wsp* sequences from GenBank were included in the construction of the *Wolbachia* NJ tree. The NJ tree reconstruction was performed with the Kimura two-parameter model as the nucleotide substitution model in MEGA X [68]. Internal gaps were treated as indels and terminal gaps as missing for *wsp* sequences. Bootstrap probabilities were estimated by generating 1000 bootstrap replicates. We designated two biting midge species, *Culicoides asiana* (KJ162955.1) and *Culicoides wadai* (KT352425.1), as outgroups for the host NJ tree construction. Due to the lack of an appropriate endosymbiont outgroup [51], the *Wolbachia* NJ tree was midpoint rooted.

When possible, *Wolbachia* strains were classified into supergroups and putative strains using 97% bootstrap probability as a threshold [60]. *Wolbachia* surface protein sequences that did not have 97% bootstrap support were evaluated on a case-by-case basis. For example, sequences which clustered closely together and had a relatively high support value (> 90%) were deemed as originating from the same putative strain.

Putative strains which were infectious to only one host species were categorized as 'specialists' and those which infected two or more hosts as 'generalists'. Then, the standardised phylogenetic host specificity (SPS) score of each generalist strain was calculated by adapting the method outlined by Poulin et al. [70] and Kembel et al. [71]. SPS measures the degree of phylogenetic relatedness among host species infected by the same endosymbiont strain. It also tests for significance by comparing it with null models generated with 999 replicates of random host-endosymbiont associations. A positive SPS value with a high *P*-value ($P > 0.95$) indicates a high degree of host flexibility where *Wolbachia* infect hosts which are phylogenetically even. A negative SPS value with low *P*-value ($P < 0.05$) suggests a low degree of host flexibility where the infected hosts are phylogenetically clustered together. SPS scores were calculated using R package picante [71].

### Evolutionary analyses of the mosquito-*Wolbachia* relationship

Three distinct methods were used to explore the evolutionary associations between mosquito hosts and their *Wolbachia* endosymbionts. The analyses were carried out using pruned phylogenies where each species is represented by a single individual.

First, using the software TreeMap 3.0 [72], a tanglegram was created between host and endosymbiont NJ trees to visualise mosquito-*Wolbachia* associations. A tanglegram is useful as a pictorial representation of the interactions between two phylogenies [73]. TreeMap also seeks to minimise the entanglement between the two trees to provide a clearer visualisation of the phylogenetic relationship between host and endosymbiont [72].

Second, ParaFit Global test, a distance-based method, was employed to quantitatively estimate congruence between the host and endosymbiont phylogenetic trees by comparing genetic distances among infected host species and the *Wolbachia* strains [74]. The null hypothesis for this test states that the associations between host and endosymbiont trees are random, whereas the alternative hypothesis suggests that there are strong associations between hosts and parasites, which are indicated by phylogenetic distances. Significance was tested by comparing the observed associations between host and endosymbiont with randomised associations generated with 5000 permutations. The respective host-endosymbiont associations which contributed significantly to the ParaFit Global statistics were also identified by performing a Parafit Link test. ParaFit tests were performed with the Cailliez correction to correct for negative eigenvalues generated [75] using R package ape [76].

Third, an event-based analysis was performed in Jane 4.0 [77] to map out potential evolutionary events of the endosymbiont in relation to the host phylogeny [78]. Five evolutionary events were considered: co-speciation (host and endosymbiont speciate simultaneously), duplication (intra-host speciation), duplication with host shift (endosymbiont host shifts), loss (host speciates but endosymbiont fails to establish in one of the new lineages), failure to diverge (host speciates and endosymbiont remains in both lineages). As each event is expected to have differing likelihoods, default cost values were attached to each of the events. Jane 4.0 determined the best reconstruction of evolutionary events by minimising the overall cost. The following cost-scheme regime was used with 100 generations and a population size of 300: co-speciation = 0, duplication = 1, duplication with host shift = 2, loss = 1, and failure to diverge = 1 [79]. As a follow-up, random tip mapping (randomisation of host-endosymbiont associations) was carried out for 50 iterations, to determine if the overall cost of reconstruction was significantly lower than expected by chance. If 5% or fewer of the random solutions have costs lower than the reconstructed coevolution phylogeny, there is support for the coevolution of the hosts and endosymbionts through co-speciation.

## Results

### Prevalence of *Wolbachia* in wild-caught mosquitoes

A total of 271 adult mosquitoes, representing 40 species and 14 genera, were collected from 12 localities in Singapore (Fig. 1a). Overall, infection prevalence was moderate with 119 out of 271 (43.9%) individuals screening positive for *Wolbachia* (Table 1). In total, 21 (51.2%) species were positive for *Wolbachia*. According to our knowledge, *Wolbachia* infection in seven of these species is reported here for the first time (Table 1). *Wolbachia* were detected in all genera except for *Aedeomyia*, *Anopheles* and *Mimomyia* (i.e. 11 out of 14 genera; 78.6%). Five out of the seven *Aedes* species collected (71.4%) were positive for *Wolbachia*, while in the genus *Culex*, five out of 16 species (31.3%) were positive. Some of the screened species in the genera *Aedes* and *Culex* that were positive for *Wolbachia*, such as *Aedes albopictus* and *Culex quinquefasciatus*, are medically important vector species.

The infection rates varied across the mosquito species. Notably, there was variation in the percentage of infection between species that are epidemiologically related. For instance, *Wolbachia* infection was not detected in *Aedes aegypti*. However, infection was moderately high (56.8%) for *Aedes albopictus*. There was also a difference in the infection rate of two closely related species, *Culex pseudovishnui* (86.4%) and *Culex vishnui* (0%) [53].

Locality did not seem to play a role in the *Wolbachia* infection of mosquito hosts. Among species that have a wide range across Singapore, the percentage of infection was consistent in populations across different habitats. For example, the infection percentage was consistently high for *Cx. pseudovishnui*, while consistently low for *Malaya genurostris*. Based on our results, species identity was a better predictor of infection status than locality.

Based on a data subset containing 153 individuals (45.8% males) representing 12 mosquito species, sex was a significant explanatory variable, and there was a significantly lower infection prevalence in males than females (odds ratio 0.434; binary logistics regression: $Z = -2.48$, $df = 151$, $P = 0.013$).

### Tissue tropism of *Wolbachia* infection in mosquitoes

Among the 159 successfully amplified *cox*1 sequences, *Wolbachia* infection was mainly observed in the reproductive tissues. Among the reproductive tissues of 159 dissected individuals, 42.1% ($n = 67$) were infected. Percentage infection was lower in the gut (5.7%, $n = 9$) and leg (3.1%, $n = 5$) tissues. The difference in

**Table 1** Percentage infection of *Wolbachia* in 40 mosquito species collected from 12 Singapore localities

| Mosquito species | Localities | | | | | | | | | | | | Total | Infection (%) | Supergroup |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BN | BA | BB | DF | KR | KJ | M | RR | SBG | SBL | T | U | | | |
| *Aedeomyia catastica* | – | 0/1 | – | – | – | – | – | – | – | – | – | – | 0/1 | 0.0 | – |
| *Aedes aegypti* | 0/1 | – | – | – | – | – | – | – | – | – | – | 0/13 | 0/14 | 0.0 | – |
| *Aedes albolineatus* | – | – | – | – | – | – | 0/3 | – | – | – | – | – | 0/3 | 0.0 | – |
| *Aedes albopictus* | – | – | – | 6/10 | 6/10 | 3/6 | 6/11 | – | – | – | – | – | 21/37 | 56.8 | A, B |
| *Aedes annandalei*[a] | – | – | – | – | 3/4 | – | 8/9 | – | – | – | – | – | 11/13 | 84.6 | A |
| *Aedes* nr. *fumidus*[a] | – | – | – | – | – | – | – | – | – | 6/10 | – | – | 6/10 | 60.0 | A |
| *Aedes gardnerii* | – | – | – | – | – | – | 1/1 | – | – | – | – | – | 1/1 | 100.0 | A |
| *Aedes malayensis* | – | – | – | 1/2 | 13/16 | 0/2 | – | – | – | – | – | – | 14/20 | 70.0 | A |
| *Anopheles barbirostris* complex | – | – | – | 0/2 | – | – | 0/2 | – | – | – | – | – | 0/4 | 0.0 | – |
| *Anopheles lesteri* | – | – | – | – | – | 0/2 | – | – | – | – | – | – | 0/2 | 0.0 | – |
| *Anopheles sinensis* | – | 0/12 | – | – | – | – | – | – | – | – | – | – | 0/12 | 0.0 | – |
| *Armigeres kesseli* | – | – | – | – | 3/3 | – | – | – | – | – | – | – | 3/3 | 100.0 | B |
| *Coquillettidia crassipes* | – | – | – | 2/2 | 6/7 | 4/4 | – | – | – | – | – | – | 12/13 | 92.3 | B |
| *Culex* (*Lophoceramyia*) spp.[c] | – | – | – | – | 0/1 | 0/2 | 1/9 | – | – | – | 0/2 | – | 1/14 | 7.1 | B |
| *Culex bitaeniorhynchus* | – | – | – | – | 0/1 | – | – | – | – | – | – | – | 0/1 | 0.0 | – |
| *Culex brevipalpis* | – | – | – | 0/1 | – | – | 0/2 | – | – | – | – | – | 0/3 | 0.0 | – |
| *Culex nigropunctatus* | – | – | – | – | – | 0/1 | 0/2 | – | – | – | – | – | 0/3 | 0.0 | – |
| *Culex pseudovishnui* | – | – | – | – | 11/12 | – | 4/4 | – | 3/5 | 1/1 | – | – | 19/22 | 86.4 | B |
| *Culex quinquefasciatus* | – | 5/8 | – | – | – | – | – | – | – | – | – | – | 5/8 | 62.5 | B |
| *Culex sitiens* | – | – | – | – | – | – | – | – | – | 2/4 | – | – | 2/4 | 50.0 | B |
| *Culex* sp. | – | – | – | – | – | – | 0/2 | – | – | – | – | – | 0/2 | 0.0 | – |
| *Culex tritaeniorhynchus* | – | – | – | – | – | 2/5 | – | – | – | 0/1 | 0/1 | – | 2/7 | 28.6 | UC[b] |
| *Culex vishnui* | – | – | – | – | – | – | 0/2 | – | – | – | 0/3 | – | 0/5 | 0.0 | – |
| *Malaya genurostris* | – | – | 2/4 | – | 0/1 | 4/13 | – | – | 0/1 | – | – | – | 6/19 | 31.6 | B |
| *Mansonia dives* | – | – | – | – | – | – | 0/2 | – | – | – | – | – | 0/2 | 0.0 | – |
| *Mansonia indiana* | – | – | – | – | – | 3/3 | – | – | – | – | – | – | 3/3 | 100.0 | B |
| *Mimomyia luzonensis* | – | – | – | – | – | 0/1 | – | – | – | – | – | – | 0/1 | 0.0 | – |
| *Tripteroides* sp. | – | – | – | – | 0/7 | – | ½ | – | – | – | – | – | 1/9 | 11.1 | UC[b] |
| *Uranotaenia obscura*[a] | – | – | – | 2/4 | – | – | 2/2 | 1/1 | – | – | – | – | 5/7 | 71.4 | A |
| *Uranotaenia* sp. | – | – | – | 1/2 | – | – | – | – | – | – | – | – | 1/2 | 50.0 | A |
| *Uranotaenia trilineata*[a] | – | – | – | – | – | – | 1/1 | – | – | – | – | – | 1/1 | 100.0 | B |
| *Verrallina butleri*[a] | – | – | – | – | – | 1/1 | – | – | – | – | – | – | 1/1 | 100.0 | UC[b] |
| *Verrallina* sp.[a] | – | – | – | – | – | – | – | 1/5 | – | – | – | – | 1/5 | 20.0 | UC[b] |
| *Zeugnomyia gracilis*[a] | – | – | – | 1/2 | – | – | 1/13 | 1/4 | – | – | – | – | 3/19 | 15.8 | B |
| Total | 0/1 | 5/21 | 2/4 | 13/25 | 42/62 | 17/40 | 25/67 | 3/10 | 3/6 | 9/16 | 0/6 | 0/13 | 119/271 | 43.9 | |

*BN* Bedok North Avenue 3, *BA* Bishan-Ang Mo Kio Park, *BB* Bukit Batok Town Park, *DF* Dairy Farm Nature Park, *KR* Kent Ridge Park, *KJ* Kranji Marshes, *M* Mandai Track 15, *RR* Rifle Range Road, *SBG* Singapore Botanic Garden, *SBL* Sungei-Buloh, *T* Tampines Eco-Green, *U* Ubi Avenue 1

[a] Species in which, according to our knowledge, *Wolbachia* infection has not been previously reported

[b] *Wolbachia* infections that were unclassified (*UC*) with respect to supergroup [60] because their DNA sequences were either too short (< 400 base pairs), or there were alignment issues during the phylogenetic analyses

[c] *Culex* (*Lophoceramyia*) comprises seven unique species, which were not identified here

percentage infection across the three dissected tissues was statistically significant (Cochran's $Q$-test: $Q = 109.5$, $df = 2$, $P < 0.0001$). The percentage of infection in the reproductive tissues was significantly higher than in the gut (McNemar's post hoc test: $P < 0.0001$) and leg tissues (McNemar's post hoc test: $P < 0.0001$), but the difference in percentage of infection between the gut and leg tissues was not significant (McNemar's post hoc test: $P = 1.0$). Notably, the amplicon size of *wsp* in the gut and leg tissues tended to be shorter than 400 base pairs.

### *Wolbachia* diversity among mosquito fauna from Singapore

Following Zhou et al. [60], all *wsp* sequences obtained in this study can be broadly classified into A and B *Wolbachia* supergroups. Out of 21 infected species, six were infected with supergroup A, ten with supergroup B, and one species, *Ae. albopictus*, was infected with both supergroups (Fig. 2). Infection of the remaining four species (*Culex tritaeniorhynchus*, *Tripteroides* sp., *Verrallina butleri*, and *Verrallina* sp.) was unclassified due to short sequences (< 400 base pairs) or sequence alignment issues during sequences analyses. The analysed *wsp* sequences were also clustered into 12 putative strains: 'Wol 1' to 'Wol 12'. Four (Wol 1, Wol 2, Wol 3, and Wol 8) out of the 12 putative strains could be matched to previously typed strains [60, 80]. *Wolbachia* strains from this study are also closely related to those isolated from other insect groups (Fig. 2). For instance, Wol 9 and Wol 10 are closely related to the *Wolbachia* strains harboured by *Drosophila* spp. (bootstrap value > 99%).

### Host specificity of *Wolbachia* strains

The degree of host specificity varied across the 12 putative strains. Seven out of the 12 strains (Wol 2, Wol 4, Wol 5, Wol 6, Wol 8, Wol 10, and Wol 12) were considered as specialists. These strains were host specific and were only detected in one host species each (Fig. 3). The remaining five strains were considered as generalists as they were found in more than one host. Amongst the generalists, Wol 3 was found in the highest number of host species, i.e. three, *Coquillettidia crassipes*, *Mansonia indiana*, and *Culex sitiens*. The SPS scores revealed that Wol 1 had the lowest degree of host flexibility (SPS test: $Z = -1.41$, $P = 0.049$). Wol 7 had the highest degree of host flexibility, but this was not statistically significant (SPS test: $Z = 0.07$, $P = 0.779$) (Table 2).

### Evolutionary relationship between mosquitoes and *Wolbachia*

We recorded 18 counts of mosquito-*Wolbachia* associations in wild-caught mosquitoes from Singapore. A visualisation of these associations using a tanglegram showed patterns of broad associations (Fig. 3). For instance, the clade which consists of *Aedes* species was observed to be mostly associated with *Wolbachia* supergroup A. In contrast, other species, especially the clade representing various *Culex* species, had numerous associations with *Wolbachia* supergroup B.

The distance-based quantitative test showed that mosquito and *Wolbachia* phylogenies were weakly congruent at the global level (ParaFit Global test: ParaFit Global = 0.006, $P = 0.048$). Among the numerous

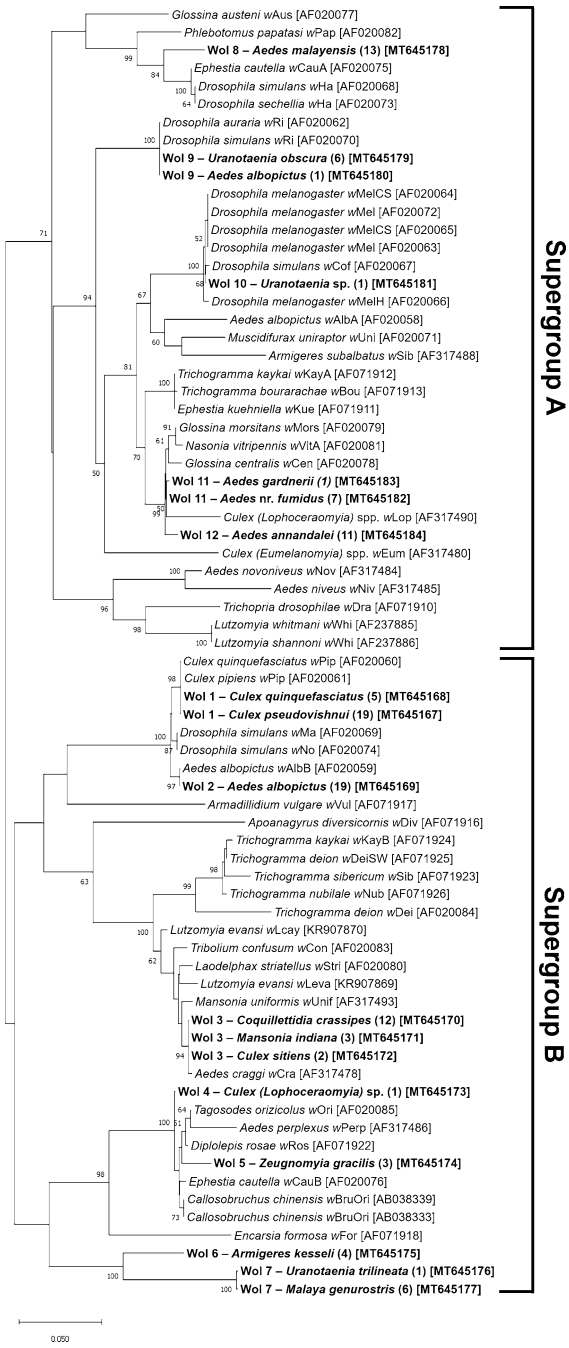


**Fig. 2** *Wolbachia* neighbour-joining (NJ) tree constructed with the *Wolbachia* surface protein gene (*wsp*). All analysed sequences generated from this study (*bold*) were broadly classified into *Wolbachia* supergroups A or B and clustered into 12 putative strains ('Wol 1'–'Wol 12'). The number of sequences of each putative strain is indicated *within parentheses*. Also included are 54 sequences obtained from GenBank. Taxa are labelled as the host from which the *Wolbachia* strain was isolated, followed by the strain name. The NJ tree was mid rooted due to a lack of appropriate outgroups [45]. Bootstrap probability (generated with 1000 replicates) higher than 50% is indicated on the tree. Genbank accession number of each sequence is indicated *within brackets*

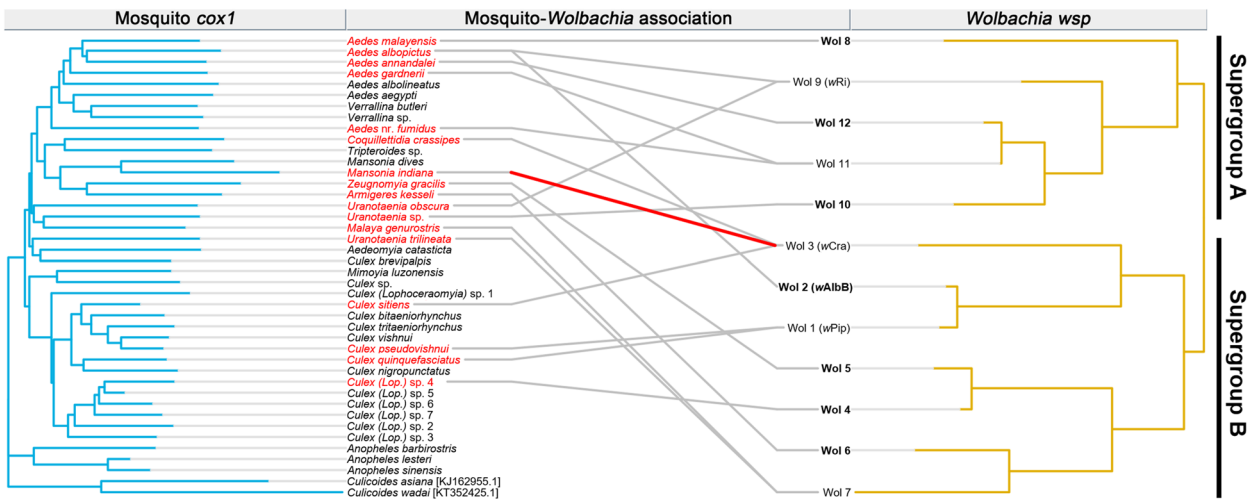


**Fig. 3** Tanglegram of mosquito *cox*1 NJ tree compared to the *Wolbachia* endosymbiont NJ tree. Mosquito host species that harboured *Wolbachia* infection are indicated in *red*. Specialist *Wolbachia* strains are in *bold*. *Grey lines* represent the associations between hosts and endosymbionts. A *red line* indicates the host-endosymbiont association that was significant in the Global ParaFit test of congruence between host and endosymbiont phylogenies (Parafit Link test: ParaFit Link = 0.045, $P = 0.029$)

**Table 2** Standardised phylogenetic host-specificity (*SPS*) scores of putative *Wolbachia* generalists

| Putative *Wolbachia* strain | No. of infected hosts | Phylogenetic host-specificity score | SPS score | *P*-value |
|---|---|---|---|---|
| Wol 1 | 2 | 0.281 | − 1.41 | 0.049* |
| Wol 3 | 3 | 0.391 | − 0.162 | 0.421 |
| Wol 7 | 2 | 0.281 | 0.068 | 0.779 |
| Wol 9 | 2 | 0.281 | − 0.234 | 0.249 |
| Wol 11 | 2 | 0.281 | − 0.817 | 0.157 |

* $P < 0.05$

host-endosymbiont links, only the association between *Mansonia indiana* and Wol 3 was statistically significant (ParaFit Link test: ParaFit Link = 0.045, $P = 0.029$) (Fig. 3).

The event-based analysis between mosquito and *Wolbachia* phylogenies resulted in a reconstructed output of one co-speciation event, three counts of duplication, seven counts of duplication with host shift, 29 losses, and six counts of failure to diverge, amounting to a total cost of 52 (Fig. 4). Interestingly, the number of duplications with a host shift and losses was much greater than co-speciation events. Notably, multiple host shift events tend to follow after loss events occurring earlier in the evolutionary history of the endosymbiont. For example, we see instances of consecutive host shifts to new hosts that were not previously infected (Fig. 4, red arrows). Additionally, based on random tip mapping, 14% of the random solutions had lower costs than the reconstructed output. Overall, there was support for multiple host shift events and losses of *Wolbachia* among the mosquitoes, and no clear signal for mosquito-*Wolbachia* cophylogeny.

## Discussion

### Detection of *Wolbachia* infection and distribution in wild mosquitoes

In this study, the PCR-based *Wolbachia* screening method had a high positive detection rate with 86.3% of all sequenced amplicons having successful BLAST matches to *Wolbachia*. This suggests that the conventional PCR method used is adequate for *Wolbachia* detection. Even if the study had been carried out without the additional DNA sequencing step, observed amplicon bands would likely have indicated true positives.

Our results indicate that *Wolbachia* are widespread across members of the family Culicidae. To our knowledge, *Wolbachia* infections have not been previously reported in seven of the mosquito species collected in this study. Overall, the percentage infection of screened individuals was 43.9%, which was largely congruent with percentages reported in past studies from the Oriental region, i.e. 31% infection in Malaysia [81], 26.4% in Sri Lanka [39], and 61.6% in Thailand [82]. At the species level, previous studies reported *Wolbachia* infection in 40% of all tested mosquito species in India [83], 18.2% in Sri Lanka [39], 51.7% in Taiwan [84], and between 28.1% and 37.8% in Thailand [82, 85]. Our study showed that 51.2% of all tested species were infected with *Wolbachia*,


**Fig. 4** Least-cost evolutionary reconstruction between mosquito (*black*) and *Wolbachia* (*blue*) phylogenies achieved using Jane 4.0. In total, one co-speciation event (*open circle*), three counts of duplication (*grey dot*), seven counts of duplication with host shift (*black dot* with an *arrow* pointing outwards), 29 losses (*dotted line*), and six counts of failure to diverge (*squiggly line*) were mapped out. *Red arrows* indicate periods where multiple host shifts occurred in succession

which is generally higher than the percentage reported in most studies. This was likely due to the broad range of species tested, including those from the genera *Malaya*, *Verrallina*, and *Zeugnomyia* [85]. It is also possible that infection prevalence may vary across geographical regions.

*Wolbachia* detection in three medically important mosquito genera, *Culex*, *Anopheles*, and *Aedes*, was highly consistent with that of past studies. These genera are responsible for the transmission of vector-borne diseases such as filariasis, malaria and arboviral diseases [86]. Within the genus *Culex*, *Wolbachia* infection has been reported to be variable across its member species [39, 46, 82, 84]. In this study, infections were observed only in five out of 16 *Culex* species. We noticed moderately high *Wolbachia* infection in *Cx. quinquefasciatus* (62.5%), which is a member of the *Culex pipiens* complex responsible for the transmission of filariasis in Singapore [86, 87]. Surprisingly, no *Wolbachia* infection was observed in *Cx. vishnui*–which has been found to harbour Japanese encephalitis virus in Southeast Asia [89]–although it is closely related to *Cx. pseudovishnui* [88] in which the rate of *Wolbachia* infection was high. However, studies in India and Thailand showed a reverse pattern, with *Wolbachia* infection present in *Cx. vishnui* but not in *Cx. pseudovishnui* [39, 85]. As the two species are morphologically similar [53], DNA barcoding was conducted to aid morphological identification, and thus avoid any misidentification. The results lend further support to possible variation in infection prevalence between geographically distant populations.

We did not detect *Wolbachia* in any of the wild-caught *Anopheles* species (18 individuals representing three species), many of which are potential malaria vectors [86]. This is largely consistent with previous reports from different countries [39, 90, 91]. The absence of *Wolbachia* in *Anopheles* mosquitoes is thought to be due to the unsuitability of *Anopheles* reproductive tissues for *Wolbachia* establishment [84, 85]. However, there have been recent reports of *Wolbachia* detected in wild *Anopheles* mosquitoes from West Africa [42, 92, 93] and Malaysia [94]. Knowledge of natural *Wolbachia* infections in *Anopheles* mosquitoes is important for malaria control strategies [93], hence more wild-caught *Anopheles* samples should be screened in Singapore to determine more accurately their infection status.

*Wolbachia* were not detected in *Ae. aegypti*, the primary vector of dengue in Southeast Asia [87]. Conversely, *Wolbachia* infection was moderately high in the secondary vector *Ae. albopictus*. These results are highly consistent with those of past studies, which reported an absence of infection in wild *Ae. aegypti* [21, 95], but found stable infection in wild *Ae. albopictus* [96]. Although *Ae. aegypti* and *Ae. albopictus* belong to the same subgenus, *Stegomyia*, and occupy similar ecological niches [97], they are rarely found in the same locality, [43, 98, 99], as also observed in this study. This could imply a certain degree of competitive exclusion between the two species, preventing them from occupying the same space. There is evidence that symbionts may influence a host's resource acquisition and specificity, which may ultimately lead to competitive exclusion between closely related host species with differing symbiont infections [100, 101]. However, research on *Wolbachia*-induced competitive exclusion is scarce except for a few studies on heterogonic gall wasps [102], grasshoppers [103], and gall-inducing aphids [104]. Given the widespread influence of *Wolbachia*, future research should explore potential cases of *Wolbachia*-induced competitive exclusion between closely related species of mosquitoes as this may have major implications for an understanding of their symbioses and speciation.

Additionally, although *Ae. aegypti* is frequently artificially infected with *Wolbachia* for biocontrol purposes [105–109], our findings suggest that infected *Ae. aegypti* might not be stably maintained in the wild. This may be advantageous for vector population suppression as the cytoplasmic-incompatibility effect of any artificially introduced *Wolbachia* strain will likely be fully manifested in the uninfected native population [21]. However, this also implies that this type of biocontrol method may have low long-term effectiveness if the infection cannot be naturally sustained in the wild population. The detection of natural *Wolbachia* infection in wild *Ae. aegypti*, therefore, has huge implications for vector control programmes [21]. Not only does it inform the selection of a suitable *Wolbachia* strain prior to its field release, but it can also be used to gauge the long-term effectiveness of a specific vector control programme.

Interestingly, the sex of the mosquitoes had an effect on their *Wolbachia* infection status. This could be an artefact of various *Wolbachia*-induced reproductive phenotypes, such as parthenogenetic and male-killing ones, resulting in offspring that are largely female [15]. If this were true, over multiple generations with vertical *Wolbachia* transmission, one should observe an increasing proportion of females that are infected. Hence, the phenomenon observed here could be a consequence of reproductive manipulation by *Wolbachia* and vertical transmission.

While we were unable to statistically test for the effects of locality on infection status due to uneven and small sample sizes of the respective species across different localities, our results suggest that mosquitoes found in localities across Singapore have roughly equal chances of harbouring *Wolbachia*. This also suggests that underlying physiological factors and phylogenetic relatedness in mosquitoes contribute more to their infection by *Wolbachia* than the habitat in which they are found.

The reproductive effect of *Wolbachia* can be masked or enhanced by other reproductive endosymbionts such as *Cardinium*, *Rickettsia*, and *Spiroplasma* [7, 26–29]. Unfortunately, we were unable to detect these

endosymbionts due to a high degree of false positives with the PCR-based screening methods used here (Additional file 1). This was likely due to using primers that are not optimised for screening mosquito-specific endosymbionts [110–112]. As a result, co-infections with various reproductive endosymbionts, which would have provided greater insights into the synergistic effects of co-infections on mosquito evolution, could not be identified among the wild mosquitoes examined here. There is, hence, a need to develop and optimise alternative screening methods, such as multilocus sequence typing (MLST) techniques, especially for the detection of *Cardinium*, *Rickettsia*, and *Spiroplasma* in mosquitoes.

#### Tissue tropism of *Wolbachia* infection in mosquitoes

*Wolbachia* were detected mainly in the reproductive tissues, which agrees with results from studies across multiple insect groups [15, 84, 113], and suggests that *Wolbachia* are mainly vertically transmitted. Interestingly, through the course of this study, there was significant variation in reproductive traits (testis and ovary length) across and within species. These reproductive traits did not vary significantly with *Wolbachia* infection status, even after accounting for phylogenetic relatedness (see Additional file 2).

Infection in the gut and leg tissues was detected, albeit infrequently. This is not surprising, as previous studies have also detected *Wolbachia* in those tissues [34–36, 114]. Interestingly, the nucleotide sequences from gut and leg infections tend to be shorter in length. Considering that *Wolbachia* are unlikely to survive extracellularly for a long period of time [35], the small amplicon size suggests potential horizontal integration of the *Wolbachia* genome into the host genome for a few species. This phenomenon has been observed in several *Wolbachia* hosts [115, 116], and mosquito species such as *Ae. aegypti* and *Cx. quinquefasciatus* [117, 118]. A recent study showed that horizontal integration of the *Wolbachia* genome into the host genome can have implications for sex determination and evolution. This is evident in the common pillbug *Armadillidium vulgare*, and results in the formation of a new sex chromosome [119]. Researchers have also proposed that horizontal gene transfer between an endosymbiont and host can result in evolutionary innovation where new functional genes arise in both host and bacteria [117, 118].

Future research should explore the relative importance of each transmission method with relation to host-endosymbiont ecology and evolution. Tissue-specific screening methods such as those used here can be used in other arthropods, especially when the mode of transmission is not clear. Currently, most *Wolbachia* screening is conducted on ground specimens or specimens in their entirety [39–41]. In these cases, researchers are unable to determine tissue tropism of *Wolbachia* infection, which could provide clues to its mode of transmission. Thus, adopting tissue-specific screening methods would enable researchers to verify or refute the commonly reported assumption that *Wolbachia* is transmitted vertically [15, 30].

#### Diversity and host-specificity of *Wolbachia* strains

Not only does the *wsp* molecular marker allow successful detection of *Wolbachia* infection across numerous taxa, it also enables strain genotyping and evolutionary comparison between detected *Wolbachia* strains [60]. In this study, *Wolbachia wsp* sequences were clustered into 12 putative *Wolbachia* strains falling within supergroup A or B. This is consistent with the results of previous studies that looked at *Wolbachia* infections in mosquitoes [39, 80, 85]. Each mosquito host species was only infected by strains belonging to supergroups A or B, with the exception of *Ae. albopictus*, which harboured both. Infection with more than one strain (superinfection of wild *Ae. albopictus* with *Wolbachia* supergroups A and B) has been previously reported, and this phenomenon was commonly observed to be fixed in the examined populations due to strong cytoplasmic incompatibility effects [120, 121]. This suggests stable vertical transmission of both strains in *Ae. albopictus*. Additionally, only four out of 12 putative strains were identified as previously typed *Wolbachia* strains reported by Zhou et al. [60] and Ruang-Areerate et al. [80]—Wol 1, Wol 2, Wol 3, and Wol 8 were identified as *w*Pip, *w*AlbB, *w*Cra, and *w*Ri strain, respectively.

Host specificity is thought to be a characteristic of the ancestral *Wolbachia* strain, with host flexibility reported mainly in *Wolbachia* supergroups A and B [122]. In our study, we found a combination of specialists and generalists, with more of the former. A study of mosquitoes from Taiwan showed a similar pattern [84]. In beetles, a mixture of *Wolbachia* supergroup A host-specific and host-flexible strains within a population has also been reported [49]. While our estimates of specialists and generalists might vary with greater sampling effort, the higher numbers of specialists observed can be explained by the process of reciprocal selection between host and endosymbiont over evolutionary time [123]. This is also known as Red Queen dynamics, where the endosymbiont constantly adapts to its host to ensure continued establishment in the same host [124]. An alternative, generalist strategy can also be maintained in a population. It ensures survival in an environment where resources (i.e. hosts) are rarely found [123]. However, there are generally more instances of

host specialists than generalists across numerous parasitic and endosymbiotic taxa [125–127].

The SPS scores revealed that host flexibility among the generalists varied greatly. Understanding *Wolbachia* host specificity has huge implications, especially for the optimisation of *Wolbachia* biocontrol strategies. Not only should researchers select strains that can effectively limit pathogen replication [128], they should also select strains for their host specificity. This is not possible without the screening of a wide variety of species or closely related species, which was achieved in this study. Using a host-specific strain will decrease the likelihood of host shift to non-target species, and thereby minimise the overall ecological risk of a strategy.

### Evolutionary relationships between mosquitoes and *Wolbachia*

Host-*Wolbachia* relationships are often understudied and limited to a few taxa [52]. Studies have shown that the evolutionary associations between *Wolbachia* and their insect hosts do vary across taxa [49–52, 129]. Likewise, our exploratory analyses of mosquito hosts and their *Wolbachia* infection support such a complex relationship, with neither co-speciation nor host shifting fully accounting for evolutionary association in these lineages.

Based on the tanglegram, a broad association pattern between mosquitoes and *Wolbachia* strains was observed (Fig. 3). *Aedes* mosquitoes tended to be associated with *Wolbachia* supergroup A, while other mosquito species, particularly of the genus *Culex*, were largely associated with *Wolbachia* supergroup B. This showed that closely related *Wolbachia* strains are likely to establish themselves in related hosts. There might have been radiation of *Wolbachia* in these clades after their respective initial establishment. Nevertheless, the observed variations in host-endosymbiont associations make us question the mosquito-*Wolbachia* association pattern.

The ParaFit analysis showed weak support for congruency between host and endosymbiont phylogenies. Among the 18 host-*Wolbachia* associations, only the link between *Mansonia indiana* and Wol 3 showed a significant association (Fig. 3). This was interesting considering that Wol 3 was largely host flexible. Given that this was the only significant association, it is worth carrying out further genus-specific study on *Mansonia* spp. to elucidate coevolutionary patterns within a group of closely related mosquito species. It is possible that the degree to which *Wolbachia* co-evolve with their mosquito hosts varies across different taxonomic levels [74]. The analyses carried out thus far suggest that mosquito-*Wolbachia* associations are likely random at higher taxonomic levels, and that mosquito-*Wolbachia* co-speciation occurs at finer phylogenetic resolution (i.e. similar to patterns seen in diffuse coevolution).

The event-based analysis performed in Jane 4.0 (Fig. 4) indicated that co-speciation events were infrequent as compared to other evolutionary events. We noticed a greater proportion of host shifts and numerous losses. Interestingly, the least cost coevolutionary reconstruction indicated multiple consecutive host shifts occurring near the tips of the cladogram. This suggests that co-speciation does not fully explain the evolutionary associations between mosquito hosts and *Wolbachia*. Instead, recent host shifting through horizontal transmission seems to promote *Wolbachia* diversification. This lends greater support to the idea that horizontal transmission between distantly related species is possible [32, 33, 130].

Furthermore, losses, which represent endosymbiont extinction events that occurred upon host speciation, seem to dominate the evolutionary history of *Wolbachia*. Extinction events are believed to be frequent in host-endosymbiont systems [123], due to either evolution of resistance in the host or declining host population size, which result in the inability of highly specialised endosymbionts to establish themselves [131, 132]. Additionally, losses could potentially influence endosymbiont evolution through the creation of vacant niches [131]. The observed losses followed by host shifts in the mosquito-*Wolbachia* relationship are possible consequences of vacant niche exploitation by generalists. Perhaps this enabled successful endosymbiont invasion due to minimal intra-strain competition. If this were true, horizontal *Wolbachia* transmission and losses may play a bigger role in accounting for *Wolbachia* diversity than previously thought.

As this was an exploratory study, we were unable to determine the exact mechanism behind the diversity and evolutionary associations of *Wolbachia*. The presence of numerous specialists could be a sign of mosquito-*Wolbachia* coevolution since coevolution is fundamentally reciprocal selection between host and endosymbiont which gives rise to micro-evolutionary changes [133]. The numerous host shifts and losses might have, however, blurred the effects of vertical transmission over a long evolutionary period [52]. Thus, co-speciation might have occurred within smaller clades of *Wolbachia* and mosquitoes, but at higher taxa levels, horizontal transmission and loss events are more likely the prominent force driving *Wolbachia* evolution.

### Strengths, limitations, and future directions

The three distinct methods employed here to explore evolutionary associations have both strengths and limitations. The tanglegram allows for clear visualisation of host-endosymbiont association without taking into

account any evolutionary relationships, but there have been calls for careful interpretation of the results generated using this method as the degree of entanglement may not necessarily represent phylogenetic congruence [134]. The Global ParaFit test seeks to address this limitation by testing for global congruency with an unbiased, statistical approach [74]. The event-based method enables the evaluation of potential evolutionary events that might have occurred throughout an endosymbiont's evolutionary history such as co-speciation, duplication, and host shifting. This last method, however, cannot fully differentiate a topological congruence from an evolutionary event [135]. Without knowledge of the time of divergence for both symbiont and host, a co-phylogenetic pattern may be better explained by ecological factors (as compared to co-speciation) given that bacterial lineages often evolve faster than their hosts [136, 137], and the high likelihood of host shifts among closely related species [133].

The *Wolbachia wsp* gene has been shown to provide well-resolved phylogenies [60], and this study provides an exploratory snapshot of the evolutionary associations between mosquito hosts and their *Wolbachia* endosymbionts. There is, of course, a potential caveat, since only a single gene was used to construct the respective phylogenetic trees. To obtain a more accurate phylogeny, future studies could adopt MLST [17, 51], or whole-genome shotgun sequencing [52]. The former could potentially characterise putative *Wolbachia* strains that cannot be distinguished with *wsp* gene primers.

Notwithstanding their limitations, the employment of various analytical methods allows for a comprehensive examination of the evolutionary associations between *Wolbachia* and mosquito hosts which are presently lacking in the literature. The scope of future studies that examine the evolution of medically important vector species could be narrowed to the Aedini tribe, as this would provide greater statistical power for the examination of mosquito-endosymbiont associations.

## Conclusion

To our knowledge, this is the first study to examine *Wolbachia* infections in wild mosquitoes in Singapore. We detected 12 putative strains of *Wolbachia* among 40 mosquito species, and recorded infections in seven species for which, to our knowledge, *Wolbachia* infections have not been previously reported. By employing a tissue-specific PCR screening method, we were able to observe that the *Wolbachia* infections were preferentially located in the reproductive tissues, which provides support for vertical transmission as the main mode of infection transmission. However, even if *Wolbachia* infection is mainly transmitted vertically, this is unlikely to fully explain the observed diversity of *Wolbachia* and why closely related *Wolbachia* lineages were found in distantly related mosquito species. Hence, this study also served as an exploratory study which examined mosquito-*Wolbachia* evolutionary associations across a wide range of host mosquito species through three evolutionary analyses. Overall, we propose that the evolutionary associations between mosquito hosts and *Wolbachia* are consequences of both vertical and horizontal transmission and various evolutionary events.

## Supplementary information

**Supplementary information** accompanies this paper at https://doi.org/10.1186/s13071-020-04466-8.

**Additional file 1: Table S1.** Polymerase chain reaction (PCR) screening of *Cardinium*, *Rickettsia*, and *Spiroplasma* in wild mosquitoes from Singapore.

**Additional file 2: Figure S1.** Weighted reproductive tissue length across various mosquito species.

### Abbreviations

BLAST: Basic Local Alignment Search Tool; *cox*1: Cytochrome c oxidase subunit I gene; MLST: Multilocus sequence typing; mtDNA: Mitochondrial DNA; NJ: Neighbour joining; PCR: Polymerase chain reaction; SPS: Standardised phylogenetic host specificity; *wsp*: *Wolbachia* surface protein gene.

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### Authors' contributions

HY and NP designed the research. HD and HY collected the mosquitoes from the field. HY identified the mosquito samples. HD performed the DNA extraction and PCR. HD and HY carried out the sequence analyses. HD, HY, and NP interpreted the results and wrote the manuscript. All the authors read and approved the final draft of the manuscript.

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### Availability of data and materials

The datasets generated and/or analysed during this study are available in the Dryad repository, https://doi.org/10.5061/dryad.zs7h44j63. Sequence data that support the findings of this study have been deposited in Genbank with the accession codes MT645167–MT645184.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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## Publisher's Note

**From:** Gail Kelly
**To:** DLNR.BLNR.Testimony
**Subject:** [EXTERNAL] Oppose C6 Regarding Contract For Mosquitoes Release On Kaua'i
**Date:** Thursday, May 9, 2024 9:02:42 AM

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Aloha,

This is scary stuff we are considering. I hate that I am sending a copy and paste response below, yet my husband's father is on his death bed and I do not have the time nor energy to act on this case as I would like.

The bottom line is this: Saving bird species, also bees, butterflies and other endangered animals, is an effort I can easily get behind. For years, the Circle of Life has always been at risk. Normally, we change our human habits, decreasing over fishing, protecting reef and birthing areas. However, in this case, we are introducing an "unnatural" component to the world. It has not been fully studied and the impact can be more hazardous than the original problem. My family lives on Hawaii Island, and as we know, the wind blows and mosquitoes travel. Show me an Environmental Impact Study and I may change my mind. But right now, this is no better than Big pharma playing God and we are the Guinea Pigs. Please ponder further comments below for which we firmly agree. Let us all learn from this.

Gail and Rich Kelly
HC 1 Box 5383
Keaau, HI 96749
808.785.8443

Copy and Paste:

I'm opposed to the BLNR 5/10/24 agenda item C6. These bacteria-infected mosquito releases are a dangerous experiment on our islands. Serious concerns about this plan have not been adequately addressed. The agencies involved in this project have lied about the introduction of foreign organisms into the islands and about the release of female mosquitoes that bite, breed, and spread disease. On Maui, these agencies are also flagrantly deviating from the approved plan, increasing the risks of helicopter fire and accident incidents.

Southern house mosquitoes transmit diseases to people and animals, and pathogen screenings are not being disclosed. Wolbachia bacteria can cause mosquitoes to become more capable of spreading diseases. The agencies releasing these lab-altered mosquitoes have admitted that the plan does not include monitoring the effects of the experimental mosquitoes on forest birds. This project has the potential to cause the extinction of the native birds it is meant to protect, and it could impact the health of the people. I demand an environmental impact statement.

Gail and Rich Kelly
HC 1 Box 5383
Keaau, HI 96749
808.785.8443

**From:** Lisa Kerman
**To:** DLNR.BLNR.Testimony
**Subject:** [EXTERNAL] Agenda C6
**Date:** Wednesday, May 8, 2024 8:31:56 PM

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To Anyone Who Truly Cares,

I'm absolutely opposed to the BLNR 5/10/24 agenda item C6 because it is absurd to pass a bill that will more than likely have disastrous outcomes. And, we must ask the question…who and what is driving this agenda?

Please do the right thing and kill this bill!
Lisa on Kuaui

Sent from my iPad

**From:** Elizabeth Kibble
**To:** DLNR.BLNR.Testimony
**Subject:** [EXTERNAL] Mosquito release on Kauai
**Date:** Wednesday, May 8, 2024 11:21:09 PM

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Our community is opposed to the release of these lab-altered mosquitoes on the island of Kauai.

Release of any non indigenous species has proven to be a disaster for our ecosystem and our environment.

Do not allow these lab-altered mosquitoes to be released on Kauai.
The impact will be devastating for future generations and your decision will most certainly hold you responsible and accountable.

Elizabeth Kibble

**From:** Kim
**To:** DLNR.BLNR.Testimony
**Subject:** [EXTERNAL] BLNR 5/10/24 Agenda Item C6
**Date:** Wednesday, May 8, 2024 9:06:23 PM

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aloha,

I am writing to share my opposition to the BLNR 5/10/24 agenda item C6.

These bacteria-infected mosquito releases are a dangerous experiment on our islands. Serious concerns about this plan have not been adequately addressed. The agencies involved in this project have lied about the introduction of foreign organisms into the islands and about the release of female mosquitoes that bite, breed, and spread disease. On Maui, these agencies are also flagrantly deviating from the approved plan, increasing the risks of helicopter fire and accident incidents.

Southern house mosquitoes transmit diseases to people and animals, and pathogen screenings are not being disclosed. *Wolbachia* bacteria can cause mosquitoes to become more capable of spreading diseases. The agencies releasing these lab-altered mosquitoes have admitted that the plan does not include monitoring the effects of the experimental mosquitoes on forest birds. This project has the potential to cause the extinction of the native birds it is meant to protect, and it could impact the health of the people. I demand an environmental impact statement.

thank you,

Kimberly Hughes

**From:** L L
**To:** DLNR.BLNR.Testimony
**Subject:** [EXTERNAL] Kauai mosquito bacteria C6
**Date:** Thursday, May 9, 2024 12:13:57 AM

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Aloha ,
Please do not disperse mosquito injected with a poisonous bacteria into the Aina of Kauai without doing a previous environmental impact study!The detrimental effects of such bacteria can seriously disturb our very precious and natural ecosystems in one of the last places on this earth to be free from such toxic unnecessary pollutants!Take care of this world and it will take care of you!Do you know the serious harm that this mosquito can do to the innocent lives of humanity and creatures and future of our planet?Obviously you don't or this would not even be allowed.

Mahalo,
Lisa Lucas

Sent from my iPhone

**From:** rich1@startmail.com
**To:** DLNR.BLNR.Testimony
**Subject:** [EXTERNAL] BLNR 5/10/24 Agenda Item C6
**Date:** Thursday, May 9, 2024 3:00:10 AM

I'm opposed to the BLNR 5/10/24 agenda item C6. These bacteria-infected mosquito releases are a dangerous experiment on our islands. Serious concerns about this plan have not been adequately addressed. The agencies involved in this project have lied about the introduction of foreign organisms into the islands and about the release of female mosquitoes that bite, breed, and spread disease. On Maui, these agencies are also flagrantly deviating from the approved plan, increasing the risks of helicopter fire and accident incidents.

Southern house mosquitoes transmit diseases to people and animals, and pathogen screenings are not being disclosed. *Wolbachia* bacteria can cause mosquitoes to become more capable of spreading diseases. The agencies releasing these lab-altered mosquitoes have admitted that the plan does not include monitoring the effects of the experimental mosquitoes on forest birds. This project has the potential to cause the extinction of the native birds it is meant to protect, and it could impact the health of the people. I demand an environmental impact statement.

Richard McIntyre
Princeville, Hawaii

| | |
|---|---|
| **From:** | Michelle Melendez |
| **To:** | DLNR.BLNR.Testimony |
| **Subject:** | [EXTERNAL] OPPOSE C6 regarding the contract for mosquitoes to be released on Kauaʻi. |
| **Date:** | Thursday, May 9, 2024 7:04:31 AM |

I'm opposed to the BLNR 5/10/24 agenda item C6. These bacteria-infected mosquito releases are a dangerous experiment on our islands. Serious concerns about this plan have not been adequately addressed. The agencies involved in this project have lied about the introduction of foreign organisms into the islands and about the release of female mosquitoes that bite, breed, and spread disease. On Maui, these agencies are also flagrantly deviating from the approved plan, increasing the risks of helicopter fire and accident incidents.

Southern house mosquitoes transmit diseases to people and animals, and pathogen screenings are not being disclosed. *Wolbachia* bacteria can cause mosquitoes to become more capable of spreading diseases. The agencies releasing these lab-altered mosquitoes have admitted that the plan does not include monitoring the effects of the experimental mosquitoes on forest birds. This project has the potential to cause the extinction of the native birds it is meant to protect, and it could impact the health of the people. I demand an environmental impact statement.

Michelle Melendez-Freedom Activist
Fitness and Wellness Expert Since 1996
Author Of The Best Selling and 4x Award Winning Book,
*End Dieting Hell: How to find peace in your body and release the weight*
https://blossominnerwellness.com/
**Order your copy** of End Dieting Hell Click Here

**From:** A. Russell
**To:** DLNR.BLNR.Testimony
**Subject:** [EXTERNAL] STOP NOW
**Date:** Thursday, May 9, 2024 7:40:52 AM

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THINK FOR A MOMENT - YOU & YOUR LOVED ONES LIVE WHERE THESE MOSQUITOS ARE PROPOSED TO BE RELEASED.

DO YOU REALY WANT TO LIVE WITH THESES SWARMING, BITING, INFECTING YOU ?

REMEMBER YOUR OATH TO SERVE OUR COMMUNITY.

YOUR & OUR LIVES, HEALTH ARE A STAKE.

STOP NOW !

Department of Land and Natural Resources 9 May 2024

**Testimony:** Oppose Funding Request

I'm strongly opposed to the release of lab altered mosquitoes in the State of Hawai'i. These mosquitoes are an experiment with unknown outcome that could harm the health of the people, wildlife, and ecosystems of Hawai'i.

Since spring 2022, as a veteran in National Security and Investigations for over 32 years, I have personally studied the science in depth behind the use of Wolbachia for mosquito control. After reviewing thousands of pages of scientific papers, environmental assessments, government documents, videos, interviews, and grants related to Wolbachia; as well as consulting with experts regularly; what stands out from all this research is that Wolbachia bacterium strains are still being discovered and **its impacts are yet to be fully understood**. Its influence on other life forms; including humans, native birds, arthropods and filarial worms' reproductive cycle and pathogen infection (either to block or promote) is still in process of being vetted.

The state claims that the mosquito control strategy being implemented has decades of research behind it and is therefore safe. The reality is that the Sterile Insect Technique (SIT) that these decades of research are based on irradiation-induced sterility, and this is not the technique planned for use in this project. The Incompatible Insect Technique (IIT), based on Wolbachia-induced cytoplasmic incompatibility (a kind of male sterility), planned for use on the Hawaiian Islands is something entirely different with a strong potential for contamination of surface water or ground water, leaching, runoff, and spray drift have also not been evaluated.

The IIT method has never been used for conservation purposes or with the species Culex *quinquefasciatus* (southern house mosquito) anywhere worldwide. Federal documents admit the outcome is unknown and the public has already voiced numerous concerns. The IIT method proposed for Maui and Kaua'i "relies on the continuous production and release of male mosquitoes and is, therefore, more expensive than the World Mosquito Program's method. "There is no field evidence that it can reduce the risk of mosquito borne diseases."
https://www.worldmosquitoprogram.org/en/learn/how-our-method-compares

40-year tropical disease expert Dr. Lorrin Pang provided testimony in court in August 2023 about serious concerns about horizontal transmission of introduced bacteria, biopesticide wind drift of lab-altered mosquitoes into unintended areas, superinfection of mosquitoes with multiple bacteria strains, increased pathogen infection and disease-spreading capability in mosquitoes, and the experimental nature of the release plan.

Science is still grasping the mechanisms of Wolbachia as documented on page 32 of Evaluation of Existing EFSA Guidelines for their Adequacy for the Molecular Characterization and Environmental Risk Assessment of Genetically Modified Insects with Synthetically Engineered Gene Drives. "The mechanism of Wolbachia-induced

pathogen-blocking is not well understood (Marshall et al., 2019). Yet, this feature, along with the gene drive-like inheritance pattern of Wolbachia, has been harnessed in replacement strategies to limit disease transmission by mosquito populations." http://www.ask-force.org/web/EFSA/EFSA-GMO-Panel-Gene-drive-document-for-consultation-20200129.pdf

We are awaiting results of grants researched out of Penn State University thru NIH including WOLBACHIA-INDUCED ENHANCEMENT OF HUMAN ARBOVIRAL PATHOGENS. "A SOBERING REMINDER THAT THE PATHOGEN INHIBITORY EFFECTS RESULTING FROM WOLBACHIA INFECTION IN SOME INSECTS CANNOT AND SHOULD NOT BE GENERALIZED ACROSS VECTOR-PATHOGEN SYSTEMS. UNDERSTANDING THE GENERAL ARE CRITICAL FOR ESTIMATING HOW LIKELY WOLBACHIA-BASED CONTROL STRATEGIES ARE TO FAIL OR **MAKE THINGS WORSE**, FOR IDENTIFYING POTENTIAL POINTS WHERE WOLBACHIA-BASED CONTROL IS LIKELY TO BREAK DOWN IN THE FIELD, AND FOR PLANNING RISK MITIGATION STRATEGIES IN HE CASE OF UNFORESEEN HARMFUL OUTCOMES. IN THIS RESEARCH, WE WILL INVESTIGATE THE HYPOTHESIS THAT WOLBACHIA-INDUCED MODULATION OF THE MOSQUITO HOLOGENOME CAN LEAD TO INCREASED ARBOVIRUS INFECTION/TRANSMISSION IN SOME VECTOR-PATHOGEN SYSTEMS OF HUMAN IMPORTANCE."
https://govtribe.com/award/federal-grant-award/project-grant-r01ai116636

**Wolbachia Potential to Increase Pathogen Infection**
The Southern House Mosquito can transmit Avian Malaria, Avian Pox, Western Equine Encephalitis, West Nile Virus, Canine Heartworm, Lymphatic Filariasis/Elephantiasis, St. Louis Encephalitis and is a potential vector of Zika virus. There are Wolbachia studies that have shown it to increase pathogen infection.
.
"Mosquitoes infected with the bacteria Wolbachia are more likely to become infected with West Nile virus and more likely to transmit the virus to humans, according to a team of researchers." "The results suggest that caution should be used when releasing Wolbachia-infected mosquitoes into nature to control vector-borne diseases of humans." https://www.sciencedaily.com/releases/2014/07/140710141628.htm

Wolbachia Enhances West Nile Virus (WNV) Infection in the Mosquito Culex tarsalis https://journals.plos.org/plosntds/article?id=10.1371/journal.pntd.0002965

Wolbachia Can Enhance Plasmodium Infection in Mosquitoes: Implications for Malaria Control? https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4154766/

Peer-reviewed studies have shown Wolbachia to cause mosquitoes to become more capable of transmitting avian malaria and West Nile virus (bird and human). Pathogen screenings for these lab-altered mosquitoes are unknown, and that information is being withheld from the public upon FOIA request. EPA guidelines allow for the release of thousands of female mosquitoes that bite, breed, and spread disease. Just 3 females

that escaped in Singapore were able to start a new lab strain colony in the wild since lab strain females and males can mate. There are no known biosecurity protocols for the imported mosquitoes and no mitigation measures in place. Population replacement of wild mosquitoes with the lab-altered mosquitoes could cause significant impacts.

**Antibiotic Resistance**
Page 12 of Kauai EA states: “To produce the incompatible male southern house mosquitoes for this project, a laboratory line of Hawai‘i mosquitoes was generated with the wAlbB strain of Wolbachia. This was accomplished through a multi-step process that involved rearing Hawai‘i mosquitoes in the lab and removing the wPip Wolbachia from their bodies with **common antibiotics**. The wAlbB strain of Wolbachia was then transferred into the eggs of these Wolbachia-free Hawai‘i mosquitoes.”

Use of this method over time with constant releases can lead to antibiotic resistance with unknown effects on the environment and can cancel out effectiveness of treatment for diseases in which Wolbachia is implicated in humans which is highly concerning.

The endosymbiont Wolbachia rebounds following antibiotic treatment
https://pubmed.ncbi.nlm.nih.gov/32639986/

Previous mosquito control projects in California and Cayman Islands using Genetically Modified (GM) mosquitoes (which also uses antibiotics during lab rearing) have not renewed contracts. “Cayman Island officials were set to renew their contract. But data from the trials indicated serious problems, leading the territory’s environmental health minister to tell the Edmonton Journal, the scheme was not getting the results we were looking for. There was further concern that the released mosquitoes could be spreading antibiotic resistance or make mosquito-borne diseases worse by lowering individual immunity.”

Modified Mosquitoes Fail to Beat Malaria
https://www.pressreader.com/canada/edmonton-journal/20181126/281951723871847
“British biotechnology company Oxitec is withdrawing its application to release billions of genetically engineered mosquitoes in California, according to a recent update from the California Department of Pesticide Regulation.”
https://beyondpesticides.org/dailynewsblog/2023/05/efficacy-and-health-issues-stop-release-of-genetically-engineered-mosquitoes-in-california-florida-continues/

There are parallels between GM and Wolbachia techniques. Biologically Wolbachia lab infected mosquitoes are not GM mosquitoes, but the study designs, math, and adherence to protocol apply to both situations. The main biological difference is there is slower horizontal transfer of mutations of the GM mosquito than with horizontal transfer of Wolbachia. **This means Wolbachia as a natural gene drive has the potential to have greater unknown impact on the environment**.

**Horizontal Spread, Vertical Transmission, and Wolbachia as Gene Drive**
"The evidence of horizontal spread of Wolbachia shows that the bacteria go not only to sexual cells, but also to somatic cells (non-sexual cells of the body). Wolbachia can also live outside of the intra-cellular systems for several months." Wolbachia Horizontal Transmission Events in Ants: What Do We Know and What Can We Learn? https://pubmed.ncbi.nlm.nih.gov/30894837/

Horizontal Gene Transfer Between Wolbachia and the Mosquito Aedes *aegypti* https://bmcgenomics.biomedcentral.com/articles/10.1186/1471-2164-10-33

This document submitted by Oxitec to the EPA in 2015 outlines numerous legitimate and studied issues regarding the use of Wolbachia. https://downloads.regulations.gov › EPA-HQ-OPP-2015-0374-0018 › attachment_1.pdf

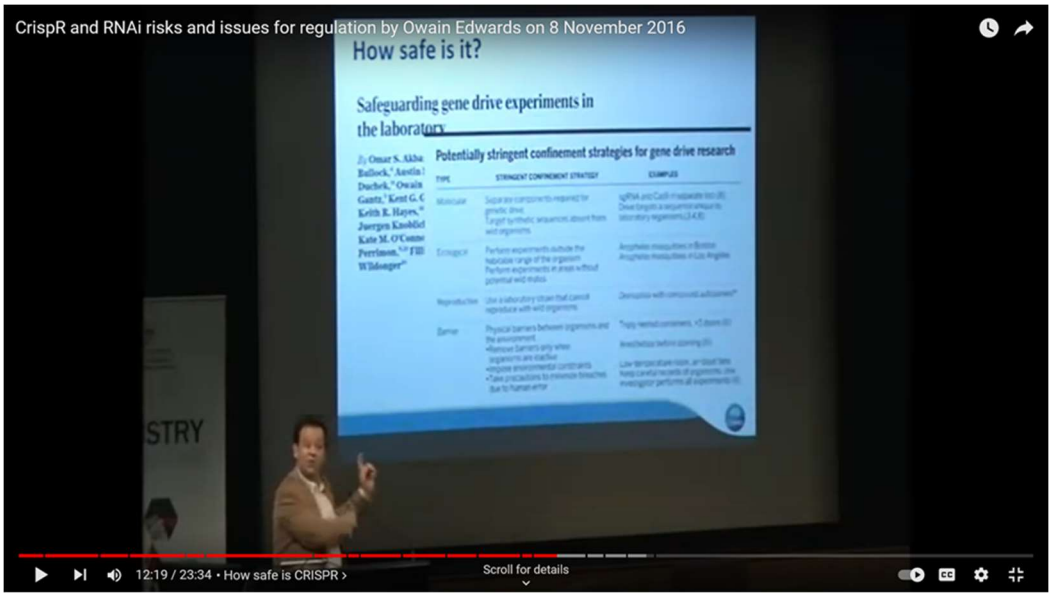
"Wolbachia is a bacterium residing within the cells of insects, and is passed through vertical transmission from mother to offspring. **Even a single Wolbachia infected female could lay hundreds of eggs that would invade the wild population, rendering the Incompatible Insect Technique ineffective** and spreading a new strain of Wolbachia into the environment. Modelling has shown that conditions of lower competition can favour infected females [6-8]. In other words, as a mosquito population is reduced, or if a population is already low, the chances of Wolbachia invading the wild population are increased."

"The Wolbachia is an endosymbiont on the cytoplasm of the cell so over a thousand new genes are introduced into the insect cells, some or all of which have the potential to randomly integrate into the insect's nuclear genome with unknown consequences. Moreover, the possible persistence of Wolbachia mosquitoes themselves is a significant concern. For the reasons set forth below, each new strain of mosquito, or indeed any artificially Wolbachia infected insect needs to be treated as a new strain and thoroughly tested in the laboratory before any field releases."

"The whole genome of Wolbachia can transfer to a host genome, meaning a host mosquito could be transformed with over one thousand new genes with unpredictable results [2-5]."

"It has already been shown that horizontal gene transfer (HGT) can transfer genes between Wolbachia and its host in Aedes aegypti [12] and several other mosquito species [13]. Therefore**, Wolbachia can genetically transform** its host with functional genes with currently unknown consequences."
"Horizontal transmission between unrelated host species is a proven phenomenon in Wolbachia [25]. Studies have demonstrated that genetic sequences, ranging in size from Horizontal transmission between unrelated host species is a proven phenomenon in Wolbachia [25]. Studies have demonstrated that genetic sequences, ranging in size from single genes to entire bacterial genomes, have been transferred from Wolbachia to many of their insect hosts [2-5], and its effect on disease transmission is variable and potentially dangerous."

Owain Edwards of CSIRO in Australia (Commonwealth Scientific and Industrial Research Organisation) was involved in the Aedes *aegypti* trial around Innisfail (Beebe et al 2021) that was funded by Verily Life Sciences. Dr. Edwards refers to Wolbachia as a type of natural gene drive during his 2016 presentation for APVMA.
https://www.youtube.com/watch?v=Lm_WS9eXYIU

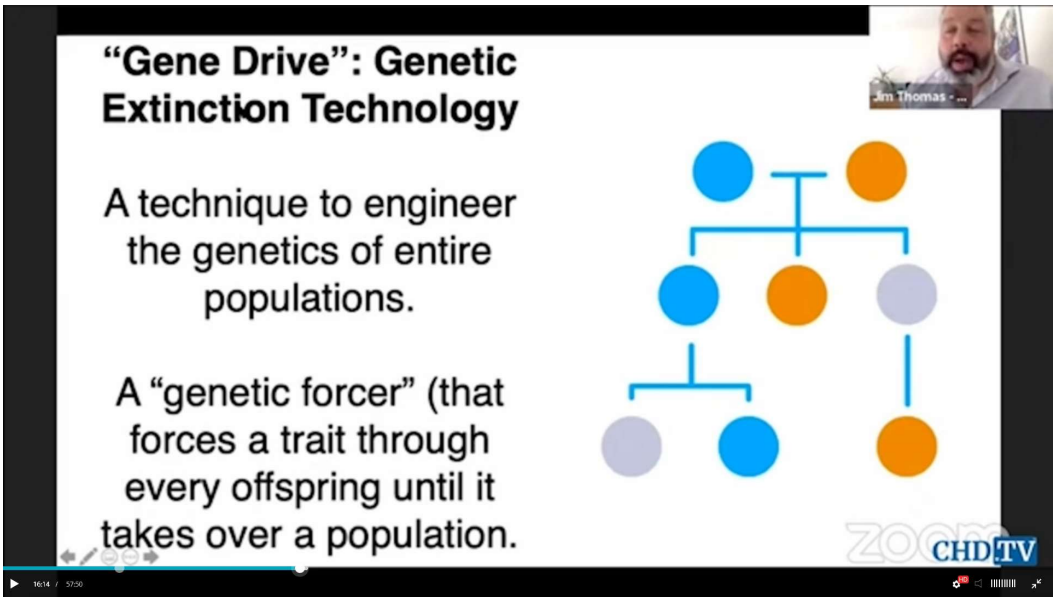



Dr. Edwards elaborates there are limitations on the use of Wolbachia application over time which can lead to limited choice of genes and for the Wolbachia technique to remain effective at suppressing mosquito population, a variety of natural strains are needed. The next step in the process is explained using CRISPR technology - synthetic gene drives. Dr. Edwards emphasizes while working on synthetic gene drives, "it requires double and triple containment to make sure these don't get out of the laboratory."

**Wolbachia DNA into Host DNA** – "A team of researchers has discovered that a bacterial parasite (called Wolbachia) can insert almost its entire genome into the genomes of members of one host species (a fly called Drosophila *ananassae*), and can insert parts of its genome into the genomes of members of several other host species." https://www.nsf.gov/news/news_summ.jsp?cntn_id=109957

Federal documents state plans for future tools to include synthetic gene drives, next generation tools, synthetic biology control tools, novel technology deployment, and precision-guided Sterile Insect Technique (pgSIT) (CRISPR technology) in Hawai'i. While "technology for this approach is not available for near-term implementation," development and deployment of these tools appear to be a long-term goal at the federal level." U.S. Department of the Interior Strategy for Preventing the Extinction of Hawaiian Forest Birds –

https://www.fws.gov/sites/default/files/documents/DOI%20Strategy%20for%20Preventing%20the%20Extinction%20of%20Hawaiian%20Forest%20Birds%20%28508%29.pdf



https://live.childrenshealthdefense.org/chd-tv/shows/good-morning-chd/mosquito-bio-warfare--who-desperation-grows/

**Lack of Bio-Security**

There has been no documentation offered to the public outlining risk analysis conducted on the security vulnerabilities for lab bred mosquitoes that can be utilized as bio-weapons against a population (intended) nor details of quality control mechanisms for accidental transmission of pathogens (unintended). This includes failure to discuss how they will deal with accidental female escape, wind drift, or how male lab bred culex q. mosquitoes released into the wild can pass pathogen to biting females thru mating and shared feeding/water sources. The public has no idea how these lab mosquitoes will be quality controlled and tested. This is a major National Security liability to the state and EPA since mosquitoes are a vector of disease and have been used on multiple occasions against a population, even our own military. https://blackthen.com/operation-big-itch-operation-drop-kick-fleas-infected-mosquitoes-dropped-black-towns/

Intended entomological warfare involves infecting insects with a pathogen and then dispersing the vectors over target areas. Invasive insects can also be deployed into a country en masse to take out crops and cripple a food supply. In New York the Plum Island lab was involved in the development of offensive bioweapons that led to Lyme's disease outbreaks. Japan's biological warfare unit (Unit 731) was deployed against China during World War II. The unit deployed plague-infected fleas and cholera-infected flies to take out the Chinese. https://citizens.news/694097.html

"We recommend careful invigilation of the international borders, airports, and seaports by the trained scientists to identify any accidental and/or deliberate import of alien

arthropod vectors. Therefore, it is well advised to take seriously the possibility that arthropod could be used to attack people. Moreover, future research priorities should also includes high-throughput molecular diagnostics of diseases, identification of vectors, phylogenetic studies to understand the origin and distribution of the pathogen and vector strains. A rapid action team of trained scientist and health workers equipped with modern sophisticated diagnostic tools and suitable vector extinguishers should be appointed by the state and/or central health authorities to counter act any such emergency". Bioterrorism on Six Legs by Dr. Manas Sarkar.

A patent was developed in 2014 involving drones that transport and release mosquitoes. It mentions in the patent these drones can be co-opted for bio-weapons military programs. https://patents.google.com/patent/US8967029B1/en

Page 23 of the Kaua'i EA states "By contrast, male's proboscises are adapted to primarily feed on plant nectar and secretions, and do not feed on blood (Mullen and Durden 2009). Therefore, male mosquitoes cannot transmit disease." **This is incorrect and misleading to the public** since studies prove male lab bred mosquitoes can pass pathogens to wild biting females thru mating and shared feeding/water sources. Venereal Transmission of St. Louis Encephalitis Virus by Culex *quinquefasciatus* Males (Diptera: Culicidae) – Donald A. Shroyer (Journal of Medical Entomology, 5/1990) https://academic.oup.com/jme/article-abstract/27/3/334/2220754?login=false

The science and tech industry in the United States, to include Silicon Valley and Academia, has been heavily infiltrated by the Chinese Communist Party (CCP) and non-government organizations such as Davos and the World Economic Forum whom have been strongly pushing Agenda 2030 thru climate change initiatives. Due to the deterioration of relations between the US and China, among other adversaries, mosquito control releases should not move forward until sound security protocols are adequately implemented. https://www.justice.gov/opa/pr/harvard-university-professor-and-two-chinese-nationals-charged-three-separate-china-related

The Bill and Melinda Gates Foundation (Gates), also connected to the above-mentioned entities, are strong proponents of climate agenda and have openly discussed support of human depopulation. This is the same foundation that has been funding ongoing research of Wolbachia (World Mosquito Program and numerous grants) and GM mosquitoes including Oxitec since 2002. Gates has also funded research developing anti-malaria vaccines using mosquitoes as a delivery system which is highly concerning. https://www.npr.org/sections/goatsandsoda/2022/09/21/1112727841/a-box-of-200-mosquitoes-did-the-vaccinating-in-this-malaria-trial-thats-not-a-jo

**Wolbachia Has Been Implicated in Human Disease**

Wolbachia is NOT harmless to humans. It effects filarial worms that cause human disease such as river blindness and is implicated in Elephantiasis. These diseases effect millions of people each year. According to the CDC website, "There is a promising treatment using doxycycline that kills the adult worms by killing the Wolbachia bacteria

on which the adult worms depend in order to survive".
https://www.cdc.gov/parasites/onchocerciasis/treatment.html
.
"For decades, people have blamed a parasitic nematode worm for a disease that has blinded at least 250,000 people now living in Africa and South America. But the real culprit may be the ubiquitous Wolbachia, bacteria that colonize many hundreds of species, including the worm indicted in river blindness. Researchers now report that **Wolbachia stimulate the severe immune system response that slowly robs people of their vision**". https://www.science.org/content/article/worms-may-not-act-alone-river-blindness

Anti-Wolbachia therapy for onchocerciasis & lymphatic filariasis: Current perspectives https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6755775/

Efficacy of 2- and 4-week rifampicin treatment on the Wolbachia of Onchocerca volvulus https://pubmed.ncbi.nlm.nih.gov/18679718/

**The Maui and Kauai EA's assertion that released mosquitoes pose no risk to human health is based on unsound science.** Both EA's state "Wolbachia cannot live within vertebrate cells and cannot be transferred to humans even through the bite of a mosquito that carries it (Popovic et al. 2010). "

In contrast we know science is recently **discovering detection of Wolbachia genes in humans**: Detection of Wolbachia genes in a patient with non-Hodgkin's lymphoma https://www.clinicalmicrobiologyandinfection.com/article/S1198-743X(14)00040-8/fulltext "Wolbachia 16S rRNA and fbpA genes were twice detected over 5 days in the blood of a patient with high fever. The patient was given fluoroquinolones and the fever resolved. Four weeks later, he was diagnosed with non-Hodgkin's lymphoma and received R-CHOP (Rituximab, Cyclophosphamide, Doxorubicin, Vincristine, Prednisolone) treatment resulting in complete remission. This is the first report of detection of Wolbachia genes from the blood of human patients with non-Hodgkin's lymphoma."

The 2010 article by Popovici et al. cited in the EA has been discredited by the EPA. The EPA Human Studies Review Board met in 2018, and the following question was posed:

"Is the research described in the published article 'Assessing key safety concerns of a Wolbachia-based strategy to control dengue transmission by Aedes mosquitoes' scientifically sound, providing reliable data for the purpose of contributing to a weight of evidence determination in EPA's assessment of the risks to human health associated with releasing Wolbachia-infected mosquitoes?"

The Board's response states: "The Board concluded that the research described in the article by **Popovici et al. was not scientifically sound** and does not provide reliable data to contribute to a weight of evidence determination for assessment of human health risks due to release of Wolbachia-infected mosquitoes."

**VERILY WARRANTY STATEMENT OF USE (corporation has NO liability)**
The directions for use of this product are believed to be adequate and must be followed carefully. However, it is impossible to eliminate all risks associated with the use of this product. Ineffectiveness or other unintended consequences may result because of such factors as weather conditions, presence of other materials, or the manner of use or application, all of which are beyond the control of Verily Life Sciences. All such risks shall be assumed by the user or buyer.

**Inconsistent Climate Data and Mosquito Population Trends**
The Kaua'i EA states, "Some climate change models suggest that the mean temperatures in Hawai'i may increase by 3°– 4°C by 2100 (Hayhoe et al. 2018). The effects of climate change have been found to result in increased stress to natural systems through altered temperatures and rainfall patterns (Alexander et al. 2016). Increases in mean temperatures, for example, have facilitated the spread of mosquitoes and avian malaria into habitats where cool temperatures very recently limit mosquito presence and transmission of malaria to highly susceptible endemic forest birds (Atkinson et al. 2014)."

Contrary to the above claims, from 1978 to 2017 (0 to 1600 meters)
Kagawa and Giambelluca 2019, Spatial Patterns and Trends in Surface Air Temperatures and Implied Changes in Atmospheric Moisture Across the Hawaiian Islands, 1905–2017. Researchers summarized data from weather stations on several islands pooled together. They extended the range of observations to the year 2017. Daytime cooling was noted at upper elevation below the trade wind inversion that is consistent with observed cooling of −0.2 to −0.8°C/decade at multiple high elevation stations during 1988–2013 (960–2,990 m; Longman, Giambelluca, et al., 2015).
https://agupubs.onlinelibrary.wiley.c

In 2013 Lisa Crampton and Anouk Glad conducted a study of Plasmodium *relictum* infection in Culex *quinquefasciatus*. The rate of capture of adult mosquitoes and Plasmodium *relictum* percentage was extremely low at Alakai Plateau of Kaua'i.
https://onlinelibrary.wiley.com/doi/pdfdirect/10.1111/jvec.12157
Vol. 40, no. 2 Journal of Vector Ecology 225

"The infection rate of Plasmodium *relictum* is also essential to understanding the transmission rate to birds on the Alakai Plateau. We screened 17 mosquitoes caught at Halepa'akai and 16 mosquitoes caught at Kawaikoi in October and November for P. *relictum* infection using PCR. One mosquito from Halepa'akai tested positive for infection. We dissected 33 mosquitoes caught at Kawaikoi (winter and spring); none of them tested positive for infection by P. *relictum* (neither oocysts nor sporozoites were observed). Only three mosquitoes caught at Halepa'akai (January) were dissected, and none of them were infected (neither oocysts nor sporozoites were observed). Thus, the prevalence rate of P. *relictum* in our study is 1.45% (n=69)."

Page 19 of Kaua'i EA states "Mosquito populations and avian malaria have recently expanded into higher elevation habitat, which is the last refugia for these endangered

avian species." **I could not find a single reference study proving infected mosquitoes are invading higher elevations** in the proposed release areas in Kaua'i or recent documentation on the prevalence rate of Plasmodium *relictum* since the Crampton and Glad study in 2013.

Additional skepticism to global warming trend is gaining momentum among the scientific community. The World Climate Declaration – There is no Climate Emergency has been signed by **1,919** vetted scientists so far. https://clintel.org/

**In Summary**
I'm opposed to increased funding for the numerous reasons documented in this testimony. The Environmental Assessment provided by the state for Kaua'i lacks sufficient study, there are conflicts of interest, there are national security risks, trespasses on a non-consenting population and contains no mitigation plan or monitoring.

Respectfully,

Donna Thompson
National Security and Investigative Expert
Kamuela, HI

**From:** NANCY THORNES
**To:** DLNR.BLNR.Testimony
**Subject:** [EXTERNAL] OPPOSE C6 regarding contract for mosquitoes release on Kaua"i
**Date:** Thursday, May 9, 2024 8:42:23 AM

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**Oppose C6 Regarding Contract For Mosquitoes Release On Kaua‘i**.

> I’m opposed to the BLNR 5/10/24 agenda item C6. These bacteria-infected mosquito releases are a dangerous experiment on our islands. Serious concerns about this plan have not been adequately addressed. The agencies involved in this project have lied about the introduction of foreign organisms into the islands and about the release of female mosquitoes that bite, breed, and spread disease. On Maui, these agencies are also flagrantly deviating from the approved plan, increasing the risks of helicopter fire and accident incidents.
>
> Southern house mosquitoes transmit diseases to people and animals, and pathogen screenings are not being disclosed. *Wolbachia* bacteria can cause mosquitoes to become more capable of spreading diseases. The agencies releasing these lab-altered mosquitoes have admitted that the plan does not include monitoring the effects of the experimental mosquitoes on forest birds. This project has the potential to cause the extinction of the native birds it is meant to protect, and it could impact the health of the people. I demand an environmental impact statement.

**From:** Vinayak Vinayak
**To:** DLNR.BLNR.Testimony
**Subject:** [EXTERNAL] 100% Opposed to C6 Regarding Contract For Mosquitoes Release On Kauaʻi.
**Date:** Thursday, May 9, 2024 8:58:02 AM

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I'm opposed to the BLNR 5/10/24 agenda item C6. These bacteria-infected mosquito releases are a dangerous experiment on our islands. Serious concerns about this plan have not been adequately addressed. The agencies involved in this project have lied about the introduction of foreign organisms into the islands and about the release of female mosquitoes that bite, breed, and spread disease. On Maui, these agencies are also flagrantly deviating from the approved plan, increasing the risks of helicopter fire and accident incidents.

Southern house mosquitoes transmit diseases to people and animals, and pathogen screenings are not being disclosed. *Wolbachia* bacteria can cause mosquitoes to become more capable of spreading diseases. The agencies releasing these lab-altered mosquitoes have admitted that the plan does not include monitoring the effects of the experimental mosquitoes on forest birds. This project has the potential to cause the extinction of the native birds it is meant to protect, and it could impact the health of the people. I demand an environmental impact statement.
Dr.Vinayak

**From:** Carolina Visser
**To:** DLNR.BLNR.Testimony
**Subject:** [EXTERNAL] I'm opposed to the BLNR 5/10/24 agenda item C6.
**Date:** Thursday, May 9, 2024 8:13:31 AM

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I'm opposed to the BLNR 5/10/24 agenda item C6. These bacteria-infected mosquito releases are a dangerous experiment on our islands. Serious concerns about this plan have not been adequately addressed. The agencies involved in this project have lied about the introduction of foreign organisms into the islands and about the release of female mosquitoes that bite, breed, and spread disease. On Maui, these agencies are also flagrantly deviating from the approved plan, increasing the risks of helicopter fire and accident incidents.

Southern house mosquitoes transmit diseases to people and animals, and pathogen screenings are not being disclosed. *Wolbachia*bacteria can cause mosquitoes to become more capable of spreading diseases. The agencies releasing these lab-altered mosquitoes have admitted that the plan does not include monitoring the effects of the experimental mosquitoes on forest birds. This project has the potential to cause the extinction of the native birds it is meant to protect, and it could impact the health of the people. I demand an environmental impact statement.

Sent from my iPhone

**From:** Joanna Weber
**To:** DLNR.BLNR.Testimony
**Subject:** [EXTERNAL] Opposed to the BLNR 5/10/24 agenda item C6
**Date:** Wednesday, May 8, 2024 8:11:15 PM

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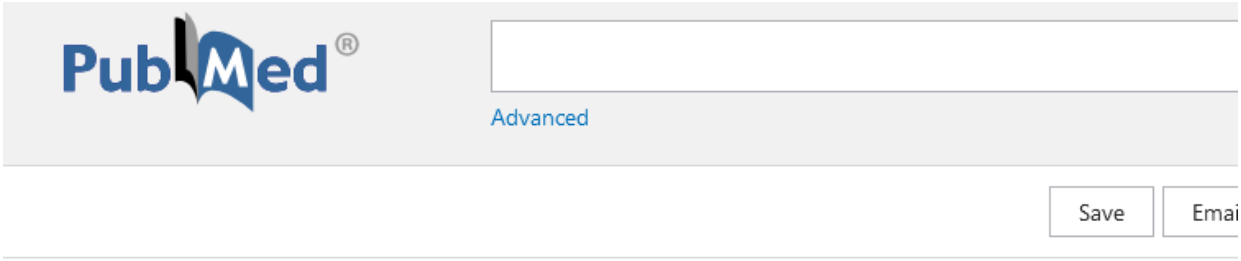
ALOHA,
I'm opposed to the BLNR 5/10/24 agenda item C6.
These bacteria-infected mosquito releases are a dangerous experiment on our islands. Serious concerns about this plan have not been adequately addressed. The agencies involved in this project have lied about the introduction of foreign organisms into the islands and about the release of female mosquitoes that bite, breed, and spread disease.
On Maui, these agencies are also flagrantly deviating from the approved plan, increasing the risks of helicopter fire and accident incidents.
Southern house mosquitoes transmit diseases to people and animals, and pathogen screenings are not being disclosed. *Wolbachia* bacteria can cause mosquitoes to become more capable of spreading diseases. The agencies releasing these lab-altered mosquitoes have admitted that the plan does not include monitoring the effects of the experimental mosquitoes on forest birds. This project has the potential to cause the extinction of the native birds it is meant to protect, and it could impact the health of the people. I demand an environmental impact statement.
ALOHA, JOANNA WEBER

| | |
|---|---|
| **From:** | Sherilyn Wells |
| **To:** | DLNR.BLNR.Testimony |
| **Subject:** | [EXTERNAL] Strongly opposed to Agenda Item C6. In addition to short comments herein, I append ALL PREVIOUS TESTIMONY from Sherilyn Wells (and from Donna Thompson and Tina Lia) regarding Item C6, as it pertained to both for Maui and for Kauai. |
| **Date:** | Thursday, May 9, 2024 7:55:05 AM |
| **Attachments:** | image.png |

I'm opposed to the BLNR 5/10/24 agenda item C6. These bacteria-infected mosquito releases are a dangerous experiment on our islands. Serious concerns about this plan have not been adequately addressed. The agencies involved in this project have lied about the introduction of foreign organisms into the islands and about the release of female mosquitoes that bite, breed, and spread disease. On Maui, these agencies are also flagrantly deviating from the approved plan, increasing the risks of helicopter fire and accident incidents.

Southern house mosquitoes transmit diseases to people and animals, and pathogen screenings are not being disclosed. *Wolbachia* bacteria can cause mosquitoes to become more capable of spreading diseases. The agencies releasing these lab-altered mosquitoes have admitted that the plan does not include monitoring the effects of the experimental mosquitoes on forest birds. This project has the potential to cause the extinction of the native birds it is meant to protect, and it could impact the health of the people. I demand an environmental impact statement.

AND, with cancers and diseases of the heart and circulatory system on the rise as a result of the experimental Covid-19 injection, the following information is directly relevant to human health in the islands and our exposure to Wolbachia:




Case Reports > Clin Microbiol Infect. 2015 Feb;21(2):182.e1-4. doi: 10.1016/j.cmi.2014.09.008. Epub 2014 Oct 29.

# Detection of Wolbachia genes in a patient with non-Hodgkin's lymphoma

X-P Chen [1], Y-J Dong [2], W-P Guo [1], W Wang [1], M-H Li [1], J Xu [1], J S Dumler [3], Y-Z Zhang [4]

Affiliations + expand

PMID: 25658559 DOI: 10.1016/j.cmi.2014.09.008

https://pubmed.ncbi.nlm.nih.gov/25658559/

Sent with Proton Mail secure email.