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Interspecific Hybridization Yields Strategy for South Pacific Filariasis Vector Elimination

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Abstract

Background: Lymphatic filariasis (LF) is a leading cause of disability in South Pacific regions, where >96% of the 1.7 million population are at risk of LF infection. As part of current global campaign, mass drug administration (MDA) has effectively reduced lymphatic filariasis prevalence, but mosquito vector biology can complicate the MDA strategy. In some regions, there is evidence that the goal of LF elimination cannot be attained via MDA alone. Obligate vector mosquitoes provide additional targets for breaking the LF transmission cycle, but existing methods are ineffective for controlling the primary vector throughout much of the South Pacific, Aedes polynesiensis.

Methodology/Principal Findings: Here we demonstrate that interspecific hybridization and introgression results in an A. polynesiensis strain (‘CP’ strain) that is stably infected with the endosymbiotic Wolbachia bacteria from Aedes riversi. The CP strain is bi-directionally incompatible with naturally infected mosquitoes, resulting in female sterility. Laboratory assays demonstrate that CP males are equally competitive, resulting in population elimination when CP males are introduced into wild type A. polynesiensis populations.

Conclusions/Significance: The findings demonstrate strategy feasibility and encourage field tests of the vector elimination strategy as a supplement to ongoing MDA efforts.

Introduction

Lymphatic filariasis (LF) is a global health problem, with over 120 million infected individuals and an estimated one billion people at risk of infection [1]. The current LF elimination campaign is premised upon the lack of a non-human reservoir for Wuchereria bancrofti and is enabled by recent advances in diagnostic tools and treatment as well as the donation of microfilaricidal drugs [1–3]. In the absence of appropriate macrolarialid prophylactic or therapeutic treatments, the current strategy focuses on interruption of LF transmission via Mass Drug Administration (MDA); treatment of the entire ‘at risk’ population with microfilaricidal compounds to suppress microfilariae levels below that required to sustain transmission. The MDA strategy calls for drug treatment to continue annually over a period exceeding the ~5-year lifespan of adult worms, [4] with a goal of global LF elimination by 2020.

The efficacy of the MDA strategy is compromised in some regions by the biology of the insect vectors. A notable example is provided in endemic areas within the South Pacific, where the diurnal subperiodic form of W. bancrofti is transmitted by A. polynesiensis. A. polynesiensis displays a pattern of negative density dependent transmission, such that this mosquito is a more efficient vector in low-level microfilaraemics, such as that which occurs with MDA strategies [5,6]. The stabilizing impact of negative density dependent transmission is enabled by recent advances in diagnostic tools and bancrofti annually over a period exceeding the suppress microfilariae levels below that required to sustain entire ‘at risk’ population with microfilaricidal compounds to transmission via Mass Drug Administration (MDA): treatment of the primary vector throughout much of the South Pacific, Aedes polynesiensis.

Conclusions/Significance: The findings demonstrate strategy feasibility and encourage field tests of the vector elimination strategy as a supplement to ongoing MDA efforts.

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The efficacy of the MDA strategy is compromised in some regions by the biology of the insect vectors. A notable example is provided in endemic areas within the South Pacific, where the diurnal subperiodic form of W. bancrofti is transmitted by A. polynesiensis. A. polynesiensis displays a pattern of negative density dependent transmission, such that this mosquito is a more efficient vector in low-level microfilaraemics, such as that which occurs with MDA strategies [5,6]. The stabilizing impact of negative density dependent transmission is hypothesized as a contributor to an inability to eliminate LF in French Polynesia despite decades of ongoing MDA [5,7]. Since mosquitoes are an obligate host for W. bancrofti, anti-vector interventions are recognized as a supplemental method to break the LF transmission cycle, leading to recommendations for the integration of vector control with MDA in areas where A. polynesiensis is the primary vector [3]. Unfortunately, effective control of A. polynesiensis has never been accomplished, due to problems including the inaccessibility of A. polynesiensis breeding sites and geography of Pacific island nations, which complicate ongoing vector control efforts [3,8]. In contrast, Pacific island geography can simplify and facilitate an area-wide elimination approach, by subdividing vector mosquitoes into discrete populations with limited immigration.

Area-wide elimination programs targeting mosquito populations have been attempted previously, with mixed results [9]. A notable success was reported in a field trial in which a Culex quinquefasciatus population was eliminated via repeated, inundative releases of male mosquitoes infected with an incompatible Wolbachia pipientis bacteria [10]. Wolbachia are obligate intracellular bacteria that are maternally inherited in insects and other invertebrates [11]. In mosquitoes, Wolbachia cause a form of sterility known as cytoplasmic incompatibility (CI), which results in karyogamy failure and arrested embryonic development. In populations where individuals are infected with different Wolbachia types, bi-directional CI can occur: sterility results in both cross directions between mates infected with different Wolbachia types. Models
Author Summary

Lymphatic filariasis (LF) is a global health problem, with over 120 million people affected annually. The current LF elimination program is focused on administering anti-filarial drugs to the entire at-risk population via annual mass drug administration (MDA). While the MDA program is proving effective in many areas, other areas may require augmentative measures such as vector control. An example of the latter is provided by some regions of the South Pacific where Aedes polynesiensis is the primary vector. Here, we describe a novel vector control approach based upon naturally occurring Wolbachia bacterial infections. Wolbachia are endosymbiotic intracellular bacteria that cause a form of sterility known as cytoplasmic incompatibility. We show that introgression crosses with mosquitoes that are infected with a different Wolbachia type results in an A. polynesiensis strain (designated “CP”) that is incompatible with naturally infected mosquitoes. No difference in mating competitiveness is observed between CP males and wild type males in laboratory assays. The results support continued development of the strategy as a tool to improve public health.

predict that in natural populations, sterility resulting from bi-directional CI is a transient event, since one infection will predominate and replace the other cytotype [12]. In the Wolbachia-based vector control strategy however, female sterility is artificially sustained by repeated, inundative releases of incompatible males analogous to traditional sterile insect technique (SIT) [13], resulting in mosquito population decrease and elimination. It is emphasized that the released male mosquitoes do not blood feed, vector disease or transmit Wolbachia. Further development and expansion of the Wolbachia-based suppression approach was not subsequently pursued due to strategy complications including immigration of mated females and variable CI patterns observed in Culex populations in different geographic areas. Furthermore, the application was viewed as specialized to Culex, since bi-directional CI was not observed in additional vector species. The latter problem has recently been addressed with a demonstrated ability to artificially generate new Wolbachia infection types in mosquitoes [14].

Problems with insecticidal approaches (e.g., resistance and non-target effects) have led to a renaissance of interest in genetic control of disease vectors, using newly-developed transgenic approaches such as the Repressive Dominant Lethal (RIDL) technology [15]. With the new reality of potential transgenic insect releases, considerable thought is being devoted to addressing the requirements for field implementation of an approach employing transgenic mosquitoes. It is recognized that experience is lacking in infection that naturally occurs in Aedes polynesiensis infection from A. riversi; the black-filled symbol represents the A type Wolbachia infection from A. polynesiensis (AP strain); unshaded symbols represent aposymbiotic individuals (APT strain). The theoretical percentage of A. polynesiensis genotype is shown as a percentage below symbols. The Wolbachia infection is maternally inherited, while the genotype is inherited from both parents. Repeated introgression of hybrid females with APT males results in the CP strain. (B) Wolbachia type-specific primers demonstrate that the CP strain results from a different infection type in A. polynesiensis (AP), Wolbachia type-specific primers used in amplification reactions are indicated above the horizontal lines. STD is the molecular weight marker: 1kb DNA Ladder Plus (Fermentas Inc., Hanover, MD).

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Methods

Insect rearing and crosses

Mosquito strains and details of maintenance and experimental crosses are as previously described [16]: AR = Aedes riversi; ART = Aposymbiotic AR strain (Wolbachia removed via tetracycline treatment); AP = Aedes polynesiensis; APT = Aposymbiotic AP.

For the CP male competitiveness assay, 25 cages were established, each with ten virgin A. polynesiensis females (<2 days post eclosion) and 20 males (<3 days post eclosion). Five days after introduction, females were blood fed, isolated and allowed to oviposit. The egg hatch assay consisted of allowing one week for egg maturation, submerging eggs for three days, and then

Figure 1. Introgression Strategy and PCR confirmation of Wolbachia infection type. (A) Wolbachia infection type is indicated by symbol shading: gray-filled symbols represent the B type Wolbachia infection from A. riversi; the black-filled symbol represents the A type Wolbachia infection from A. polynesiensis (AP strain); unshaded symbols represent aposymbiotic individuals (APT strain). The theoretical percentage of A. polynesiensis genotype is shown as a percentage below symbols. The Wolbachia infection is maternally inherited, while the genotype is inherited from both parents. Repeated introgression of hybrid females with APT males results in the CP strain. (B) Wolbachia type-specific primers demonstrate that the CP strain results from a different infection type in A. polynesiensis (AP), Wolbachia type-specific primers used in amplification reactions are indicated above the horizontal lines. STD is the molecular weight marker: 1kb DNA Ladder Plus (Fermentas Inc., Hanover, MD).

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observing eggs from individual females using a dissection scope. Females producing fewer than ten eggs were excluded from the data set. To confirm female insemination, spermathecae were checked for females producing broods with low egg hatch.

Microsatellite analysis
DNA was extracted from individual mosquitoes using DNAeasy kits (Qiagen, Valencia, CA) following manufacturer’s instructions. Six-microsatellite primer pairs were used to amplify loci using PCR conditions as previously described [17]. Left primers were fluorescent labelled with different WellRED dye colors (Integrated DNA technologies, Coralville, IA). Fragments sizes were measured using a CEQ 2000 sequencer (Beckman Coulter, Fullerton, CA) according to manufacturer’s instructions. Allele frequencies and genotypic dis-equilibrium were calculated, and Fisher’s exact tests [18] were performed using GENEPOP version 3.4. All calculations were performed using the Markov chain method with demORIZATION set to 1000, 100 batches, and 1000 iterations per batch.

DNA extraction, PCR, and maternal inheritance assay
Adults were homogenized in 100 μl of buffer containing 10 mM Tris-HCL, 1 mM EDTA, and 50 mM NaCl, at pH 8.2 using a Mini-beadbeater (BioSpec Products, Inc., Bartlesville, OK). After homogenization, samples were boiled for 5 min and centrifuged at 16,000g for 5 min. One μl of supernatant was used for each PCR reaction. PCR conditions were as described previously [19]. Infection type of CP, AP, and AR was determined using PCR primers specific for A type Wolbachia (136F and 691R) or B type Wolbachia (81F and 522R) [20]. To assess maternal inheritance rates, CP females were mated with CP males, blood fed, isolated, and allowed to oviposit. CP females and their progeny were examined via PCR using the 81F and 691R primers [20] using the above-described methods.

Statistical analysis
To analyze male mating competitiveness a Chi-square goodness of fit test was performed to compare observed and expected numbers of hatching broods for the replicate cages of the varying ratios of CP:AP males. To analyze population suppression in numbers of hatching broods for the replicate cages of the varying numbers of hatching broods for the replicate cages of the varying ratios of CP:AP males. To analyze population suppression in numbers of hatching broods for the replicate cages of the varying ratios of CP:AP males.

Results
Prior studies demonstrate that A. polynesiensis and A. riviensis, two closely related members of the Aedes (Stegomyia) scutellaris complex are naturally infected with differing Wolbachia infection types (A and B clades, respectively) [16], and that removal of the Wolbachia infection results in egg hatch, which does not occur in interspecific crosses between these closely related Aedes species. [17]. Here, we report that hybrids resulting from crosses of uninfected (aposymbiotic) A. polynesiensis and A. riviensis are viable and fertile (Table 1). Hybrid fertility allows a strategy in which the A. riviensis Wolbachia type is introgressed into the A. polynesiensis genotype, resulting in the ‘CP’ strain (Figure 1A). As shown in Figure 1B, PCR confirms that Wolbachia in the CP strain is B-type Wolbachia, as predicted.

To examine introgression of the CP strain, allelic distributions of six-microsatellite loci were compared between the AP, AR and CP strains. All loci investigated are polymorphic between strains, and four loci are polymorphic within strains. The test for genotypic dis-equilibrium across all pairs was not significant (g>0.2), suggesting loci are not linked. The CP and AP strains were observed to share a similar distribution of alleles across all loci (Figure 1A). In contrast, allele distributions of CP and AP are significantly different from AP (Fisher’s exact test, P<0.0001), with only two alleles commonly shared by all three strains at two loci (Loci 1 and 3) (Figure 2). Thus, the results are consistent with the hypothesized CP introgression with the AP genotype.

CROSSES AND PATTERN OF CYTOPLASMIC INCOMPATIBILITY

| Female x Male | Percent Egg Hatch ± s.e.m; number of cross replicates |
|---------------|------------------------------------------------------|
| AR x APT a    | 13.6±17%; n=14                                        |
| ART x APT b    | 27.8±26.6%; n=14                                      |
| AP x ART       | 0.2±0.5%; n=7                                         |
| APT x ART      | 0.6±1.8%; n=21                                        |
| APT x CPT      | 59.1±43.5%; n=7                                       |
| CPT x APT      | 73.7±23.4%; n=7                                       |
| CP x AP        | 0.23±0.11%; n=18                                      |
| AP x AP        | 87.8±9.7%; n=8                                        |
| AP x CP        | 0.0±0.9%; n=28                                        |
| CP x CP        | 62.1±4.01%; n=18                                      |

AR = Aedes riversii; ART = Aposymbiotic AR strain; AP = Aedes polynesiensis; APT = Aposymbiotic AP
*Hybrid progeny designated as “CP line” (see Figure 1A)
**Hybrid progeny designated as “CPT line” doi:10.1371/journal.pntd.0000129.e129

Strong bi-directional CI and high maternal transmission support investigation of a vector control strategy in which CP male releases are used to suppress A. polynesiensis populations. To examine the strategy: virgin AP females were introduced into cages with varying ratios of CP:AP males. Following mating, females were isolated and the egg hatch rate was examined. As shown in Figure 3, the egg hatch was observed to significantly decrease from 75% to 0% egg hatch, inversely related to the frequency of incompatible CP males. The experimental design also permits an assessment of CP male competitiveness relative to AP males. As illustrated in Figure 3, the number of observed compatibly mated females (i.e., producing hatching broods) did not differ from predictions that assume equal male competitiveness (Chi Square; P>0.1). The data shown in Figure 3 also support the hypothesis that females utilize sperm from one male. A comparison of egg hatch rates resulting from compatibility mating females did not differ significantly between treatments (Kruskal-Wallis, P>0.3). If females were to utilize sperm from
multiple males, then a lower egg hatch rate would be expected in treatments with a mixture of CP and AP males. The results support the feasibility of a CI-based *A. polynesiensis* suppression strategy and encourage additional experiments to assess the strategy under more natural conditions (e.g., field cages).

**Discussion**

The inability of prior MDA efforts to eliminate LF transmission from some Pacific regions represents a potential weakness in the current global campaign. Therefore, an ability to reduce or eliminate the required mosquito vector populations would provide a useful augmentative tool for blocking LF transmission. *A. polynesiensis* populations provide a logical target, given their broad geographic range in the South Pacific and ability to vector filariasis in low-level microfilaraemics (i.e., ‘limitation’ LF transmission) [5,7]. Unfortunately, existing vector control tools have proven unsuccessful against this mosquito species.

The results presented here support the feasibility of a species-specific approach in which inundative releases of bi-directionally incompatible males induce sterility in *A. polynesiensis* females, resulting in vector population elimination. The geography of the South Pacific is ideal for the proposed *A. polynesiensis* suppression strategy, since the *A. polynesiensis* population is subdivided into islands with limited immigration [22,23]. The natural subdivision of *A. polynesiensis* into isolated populations will facilitate a sequential elimination approach, in which transient entomological teams focus effort on one island and then progress to a subsequent island. Following elimination, a reporting system would be deployed, monitoring for *A. polynesiensis* reintroduction and reestablishment.

The proposed strategy would be integrated with the existing MDA strategy, to be deployed in areas where LF elimination is complicated by *A. polynesiensis* biology. It is emphasized that the primary goal of breaking the LF transmission cycle does not require the permanent eradication of *A. polynesiensis*. Instead, a transient elimination of *A. polynesiensis* will suffice, as long as the period of vector elimination extends beyond the lifespan of adult *W. bancrofti* in the reservoir human population. However, *A. polynesiensis* eradication would be desirable from the broader public health perspective that *A. polynesiensis* is a biting nuisance and serves as a vector during periodic dengue epidemics. The successful demonstration that releases of bi-directionally incompatible males can impact LF transmission will encourage an extension of the strategy to a broader geographic range and to additional vector species (e.g., *Culex* spp. or other aedine LF vectors in the South Pacific) using the previously demonstrated ability to artificially generate novel *Wolbachia* infections in medically important mosquitoes [14].

A concern relates to downstream logistical aspects associated with the subsequent ‘scale up’ required for suppression of larger populations. Due to bi-directional CI, females that are unintentionally released are incompatible with wild type males. With the reduction of the population size due to CI-induced sterility, there is an increasing probability that accidental female releases will permit the establishment of the new infection type, resulting in population replacement instead of population elimination [12]. Thus, strategy success requires releases to consist of males only. While a variety of mechanical sex separation tools for mosquitoes have been developed, available devices are not sufficiently accurate. Therefore, to ensure male-only releases, early field trials will be on a relatively small scale.

![Figure 2. Microsatellite allelic frequencies](https://example.com/figure2.png)
that allows visual verification of mechanically-separated males, similar to prior trials [10]. In the event that visual inspection is not cost effective for subsequent large-scale releases, deployment of the proposed approach in larger areas may require additional technology to improve cost efficacy, such as genetic sexing [24]. An additional possibility is premised upon the observation that female mosquitoes are typically more susceptible to radiation relative to males [25] and would treat release individuals with low levels of radiation to render unintentionally released females impotent (i.e., sterile or of negligible fitness). The risk of compatible matings between released CP males and \textit{A. riversi} females in the field is not a concern, since populations of \textit{A. riversi} occur in Japan, remote from South Pacific islands that are proposed for field releases.

The non-transgenic, species-specific, \textit{Wolbachia}-based elimination strategy proposed here provides a logical segue toward transgenic approaches (i.e., RIDL [15]), which may yield improved efficacy and/or cost. Furthermore, the social palatability of transgenic mosquito releases can be increased via an approach that is integrated with \textit{Wolbachia}-induced CI. Specifically, if released transgenic males are cytoplasmically incompatible with the targeted mosquito population, the released transgene has a reduced probability of establishing in the field.

Future efforts must define the vectorial competency of CP females relative to wild-type females. In the event that the CP strain is observed to be refractory to LF transmission, replacement of the naturally-susceptible wild-type population with a refractory CP population may be a desirable outcome. Based upon the results of prior vector competency studies examining hybridizations between members of the \textit{Aedes scutellaris} complex, the prospect of CP displaying reduced vectorial competency is not a remote possibility. Notably: prior cross experiments demonstrate that the \textit{Wuchereria} refractoriness phenotype is dominant, [27,28] prior hybridization experiments provide evidence for cytoplasmic inheritance of susceptibility [29] and AR is not recognized as a disease vector. Since periodic dengue epidemics occur within the \textit{A. polynesiensis} range, the competency of CP to transmit dengue and additional pathogens (e.g., chikungunya) would need to be assessed prior to implementing a population replacement program. The latter complication would be less of a concern with an elimination strategy, since following successful intervention, neither CP or \textit{A. polynesiensis} would occur in the field.

**Supporting Information**

**Alternative Language Abstract S1** Abstract translated into French.

Found at: doi:10.1371/journal.pntd.0000129.s001 (0.02 MB DOC)

**Alternative Language Abstract S2** Abstract translated into Spanish.

Found at: doi:10.1371/journal.pntd.0000129.s002 (0.02 MB DOC)
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