Impacts of *Wolbachia* Infection on Predator Prey Relationships: Evaluating Survival and Horizontal Transfer Between *w*MelPop Infected *Aedes aegypti* and Its Predators

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ABSTRACT The *w*MelPop strain of *Wolbachia* is currently being investigated for its potential use as a biological control agent to reduce the ability of *Aedes aegypti* (L.) mosquitoes to transmit dengue viruses. The survival of a potential *w*MelPop infected *Ae. aegypti* strain for field release is important as a higher susceptibility to predation in the *w*MelPop strain could result in difficulties in achieving fixation. We investigated immature and adult survival as a function of susceptibility to predation by six naturally occurring predator species; cyclopoid copepods, fish, predatory *Toxorhynchites* mosquito larvae and a salticid jumping spider. The trials indicated that *w*MelPop infected and uninfected *Ae. aegypti* larvae and adults were equally susceptible to predation to all six tested predators. In addition to evaluating any potential fitness costs to the infected host, we were unable to demonstrate horizontal transfer of *w*MelPop via consumption of infected *Ae. aegypti* larvae to the above predators. That susceptibility to predation was consistent across mosquito life stage, predator species and experimental venue is strong evidence that despite the neurotrophic and extensive nature of *w*MelPop infection, behavioral changes are not occurring, or at least not a determining factor in survival when exposed to a predator. Based on our results and the ecology of *Wolbachia* and mosquito predators, horizontal transfer of *w*MelPop from *Ae. aegypti* into naturally occurring predators is not cause for concern.

KEY WORDS *Wolbachia*, fitness, *Aedes aegypti*, transfer, predation

Wolbachia are intracellular bacteria that infect a wide range of arthropods, including insects, arachnids, and crustaceans (O'Neill et al. 1992; Werren et al. 1995a,b; Bouchon et al. 1998; Werren and Windsor 2000). Recently, it has been demonstrated that *w*MelPop infection inhibits the ability of a range of pathogens to infect *Aedes aegypti* (L.) (Kambris et al. 2009, Moreira et al. 2009a) and as such the *w*MelPop strain of *Wolbachia* is currently being investigated for its potential to reduce the capacity for transmission of dengue viruses, with planned field releases in North Queensland, Australia, and Vietnam. While the release of the *w*Mel strain of *Wolbachia* in North Queensland was extremely successful (Hoffman et al. 2011), there are demonstrated fitness costs associated with the *w*MelPop strain that will strongly influence the success of this control strategy. These include impacts on the lifespan of adult mosquitoes (McMeniman et al. 2009) feeding success in older mosquitoes (Moreira et al. 2009b, Turley et al. 2009), locomotor activity (Evans et al. 2009), egg desiccation (McMeniman and O'Neill 2010), hatch rate, and fecundity (McMeniman et al. 2011). Currently, there is little information on the cost of *w*MelPop infection on larval survival, despite the obvious impact on the success of field releases. Harcombe and Hoffman (2004) demonstrated that *w*MelPop infection of *Wolbachia* had no effect on larval development time of *Drosophila melanogaster* Meigen but more recent work in mosquitoes has demonstrated minor costs to *Wolbachia* infected male *Aedes albopictus* (Skuse) larvae reared under food-limited conditions (Islam and Dobson 2006, Gavotte et al. 2009) and to male *Ae. aegypti* (McMeniman and O'Neill 2010). Thus, there is the need to investigate a wide range of host fitness parameters, not only in assessing the potential success of the strategy but to aid in modeling required release size and frequency (Brelsfoard and Dobson 2009).

In addition to the traits listed above which contribute to species fitness, antipredator behavior is important to species survival under certain circumstances. In the presence of chemical predation cues larvae adopt 'low risk' behaviors by moving less and by mod-

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ifying their behavior to safer microhabitats (Ferrari, 2008, Kesavaraju and Juliano, 2005, Juliano and Gravel, 2002, Grill and Juliano, 1996, Sih 1986). This was recently demonstrated by Van Uitregt et al. (2011) using *Aedes notoscriptus* (Skuse) and predatory fish species *Hypseleotris galii* (Ogilby). Thus, predators may be useful tools to explore changes in behavior of *w*MelPop infected individuals that will ultimately result in reduced survival.

We investigate larval survival as a function of predation by two copepod species *Mesocyclops darwini* Dussart and Fernando and *Mesocyclops aspericornis* (Daday), two fish species *Pseudomugil signifer* (Kner) and *Poecilia reticulata* Peters and one predatory mosquito species *Toxorhynchites speciosus* (Skuse). All five species readily consume *Ae. aegypti* and other larvae, and have been applied in biological control programs against *Ae. aegypti* in Vietnam (Nam et al. 2000) and Queensland, Australia (Brown et al. 1991, 1992, 1996). We also investigated the ability of adult *Ae. aegypti* to avoid predation by jumping spiders *Hasarius adansoni* (Audouin). The jumping spiders (Salticidae) are a ubiquitous group and known predators of mosquitoes (Popovici et al. 2010).

An additional impediment to the planned release strategy is the concern by the public associated with the risk of unintentional *Wolbachia* spread into nontarget populations (Popovici et al. 2010), despite *Wolbachia* having rarely spread by horizontal transfer (de Barro et al. 2011) to other organisms in the wild. Popovici et al. (2010) investigated the possibility of human infection historically and experimentally by bite and also failed to infect jumping spiders, *Menemerus bivittatus* (Dufour) and Daddy Long Legs, *Pholcus phalangioides* (Fuesslin) by feeding. Despite this early evidence, this possibility would provide a major impediment to the use of any *Wolbachia*-based disease reduction strategy (Brelsfoard and Dobson 2009) and requires further evaluation.

The occurrence of closely related strains of *Wolbachia* in distantly related insect species indicates that these infections can colonize new host species by horizontal transfer (Werren et al., 1995b, van Meer et al. 1999, Cordeaux et al. 2001, Russell et al. 2009), although the natural mechanisms by which this occurs are unknown. To date, the only experimental studies to demonstrate interspecific transmission of *Wolbachia* have focused on parasitic wasps that can pick up infections either from their insect host or from other parasitic wasp species sharing the same host (Heath et al. 1999, Huigens et al. 2004). However, most strains that appear closely related in *Wolbachia* phylogenies occur in arthropods not linked by this interaction and other means of horizontal transfer seem likely (Sintupachee et al. 2006). Some studies suggest that *Wolbachia* could spread through consumption of infected or contaminated diets (Kittayapong et al. 2003, Sintupachee et al. 2006). In this article, we examine the issues of 1) survival in terms of predator avoidance and 2) any horizontal transfer that may have occurred in the predators after being fed a diet of *w*Melpop infected *Ae. aegypti.*

Materials and Methods

Mosquito Rearing. Four *Ae. aegypti* lines were used in these experiments. A genetically diverse line derived from PGYP1 (McMeniman et al., 2009), named PGYP1.out (Moreira et al. 2009), was generated by backcrossing PGYP1 for three generations to F1 males of 52 independent field-collected isofemale lines from Cairns, Australia. Two further generations of backcrossing were conducted with F2 field-collected material (wild-type from Cairns, Australia). A tetracycline-cured counterpart (PGYP1.out.tet, -Wolb) was generated by antibiotic treatment of back-crossed adults, followed by two generations of recovery and recolonization with gut bacteria as previously described by McMeniman et al. (2009).

Mosquitoes were kept in an insectary at 26°C, 70% RH, 12 h light regime. *Ae. aegypti* larvae were maintained with TetraMin (Tetra, Melle, Germany) tablets and adults were offered 10% sucrose solution, ad libitum. Adult females were bloodfed on human volunteers (UQ human ethics approval 2007001379; QIMR approval P361) for egg production. *Tx. speciosus* larvae were maintained with larvae of colony reared *Ae. notoscriptus* and adults were offered 20% sucrose solution, ad libitum.

Adult and Larval Fitness. *Copepods.* Experiments were conducted independently using cultures of *M. darwini* and *M. aspericornis* at the Queensland Institute of Medical Research (QIMR), Australia and the National Institute of Hygiene and Epidemiology (NIHE), Vietnam, respectively. For each experiment, 15 adult female *Mesocyclops* were placed into 10 individual 10 liter aquaria ($31 \times 20 \times 15$ cm) containing 4 liters of stock solution. The stock solution was made up of 0.5 liters of hay infusion (hay in aged tap water) and 3.5 liters of aged tap water. Aquaria were placed on a bench in the laboratory to allow partial exposure to sunlight. Based on preliminary predation trials, 100 newly hatched *w*MelPop infected and 100 newly hatched tetracycline-cured *Ae. aegypti* larvae were added to each aquaria, representing approximately twice the mean number consumed by *Mesocyclops* in a 24 h period. *Mesocyclops* were added 24 h before larvae to ensure that individuals were hungry and acclimated to the experimental conditions. At 24 h, surviving larvae and *Mesocyclops* were removed, counted, and predation rate and infection status determined.

Fish. Experiments were conducted independently using the Pacific Blue-eye *P. signifer* and the guppy *P. reticulata* at the QIMR, Australia and at NIHE, Vietnam, respectively. For both experiments, ≈100 fish were purchased from a local supplier and placed into a 100 liter stock aquarium in the laboratory. From this aquarium, 20 adult male fish of similar size were placed into individual 10 liter aquaria ($31 \times 20 \times 15$ cm) containing 9 liters aged tap water and a gravel substrate. For *P. reticulata*, 50 fourth instar *w*MelPop infected and 50 fourth instar tetracycline cured *Ae. aegypti* larvae were added to each aquaria, whereas for *P. signifer* only 25 of each was added. These numbers

were based on preliminary predation trials with each species and represented twice the average daily consumption rate. After 24 h, fish were removed and surviving larvae removed, counted, and predation rate and infection status determined.

Mosquitoes. Larvae of *Tx. speciosus* were collected from McDowall, QLD, Australia, and colonized at the QIMR insectary. Individual fourth instar *Tx. speciosus* larvae were removed from colony rearing trays and placed into 4.5 cm diameter × 9 cm deep jars containing 5 ml of algal solution (forming a mat on the bottom from which individuals could hide and ambush prey) in 100 ml water. Based on preliminary predation trials, seven fourth instar *w*MelPop infected and seven fourth instar tetracycline-cured *Ae. aegypti* were added to each container, representing approximately twice the mean number consumed by *Tx. speciosus* in a 24 h period. Forty replicate jars containing a single *Tx. speciosus* were added 24 h before *Ae. aegypti* larvae to ensure that individuals were hungry and acclimated to the experimental conditions. At 24 h, surviving larvae and *Tx. speciosus* were removed, counted, and predation rate and infection status determined.

Spiders. Adults (both sexes) of the broadly distributed jumping spider *Hasarius adansoni* were collected in buildings and glass houses at the University of Queensland, Australia. Individuals were held in plastic vials (9.5 × 3 cm diameter) with fine gauze mesh and fed on a diet of uninfected *Ae. aegypti* adults ad libitum until 4 d before the commencement of feeding trials; thereafter nothing. Four 64 cm^3 cages of fine gauze were used for the predation trials. Inside, wooden dowels were placed throughout to increase the accessibility of the mosquitoes to the spiders. For each feeding trial, two treatment cages each included 12 spiders, 100 *w*MelPop infected mosquitoes (50 male and 50 female) and 100 tetracycline cured mosquitoes (50 males and 50 females). The remaining two untreated control cages contained the same numbers of mosquitoes without spiders. These feeding trials were carried out on a weekly routine on three occasions for 1-wk old and 3-wk old mosquitoes. Surviving mosquitoes were aspirated from the cages after a 24 h period, counted and tested for *Wolbachia.*

Horizontal Transfer. *Copepods.* Experiments were conducted independently from cultures of *M. darwini* and *M. aspericornis* at the QIMR Australia and the NIHE, Vietnam, respectively. We fed 200 individual *Mesocyclops* up to 20 *w*MelPop infected *Ae. aegypti* over 4 d. Individual adult female *Mesocyclops* were placed into tissue culture plate wells (35 mm diameter, 18 mm deep) containing 10 ml of water and starved for 24 h before the trial. To each well, 10 newly hatched *w*MelPop infected *Ae. aegypti* were added. After 48 h, surviving larvae were removed, counted, and 10 additional newly hatched larvae added to each well. To ensure that any positive polymerase chain reaction (PCR) reactions were not because of undigested prey (Enigl et al. 2005), at the conclusion of the experiments, copepods were transferred to a clean well for 4 d to ensure all gut contents had been evacuated. After this time, individuals (dead or alive) were removed, counted and tested for *Wolbachia.*

Fish. Experiments were conducted independently using *P. signifer* and *P. reticulata* at QIMR, Australia, and the NIHE, Vietnam, respectively. To test whether *w*MelPop can transfer from prey to predator via ingestion we fed 37 individual *P. reticulata* a minimum of 50 *w*MelPop infected fourth instar *Ae. aegypti* over a period of 2 d. Individual adult male *P. reticulata* of similar size were placed into individual 10 liter plastic aquaria (31 × 20 × 17 cm) containing 9 liters aged tap water. Individual *P. reticulata* were starved for 24 h before the trial. To each tank, 35 *w*MelPop infected fourth instar *Ae. aegypti* from the laboratory colony were added. After 24 h, surviving larvae were removed and counted and 35 additional fourth instar larvae added to each aquarium. When each *P. reticulata* had consumed 50 or more fourth instar *w*MelPop infected *Ae. aegypti* it was transferred into a clean aquarium containing aged tap water and gut contents allowed to clear for 36 h (determined in preliminary experiments). After this time, individuals were euthanized, counted, and the head, body, fins/tail, and internal abdominal organs tested for *Wolbachia.*

In addition to *P. reticulata*, we fed a school of 40 *P. signifer* a minimum of 4,000 (100 per fish) *w*MelPop infected fourth instar *Ae. aegypti* over a period of 5 d. Forty individual adult male *P. signifer* of similar size were placed into an aquarium containing 80 liters aged tap water. The school was starved for 24 h before the trial. To the aquarium, *w*MelPop infected fourth instar *Ae. aegypti* from the laboratory colony were added in lots of 50 at 1 min intervals until fish failed to consume the full amount in that time. This was repeated every 24 h for 5 d. After this period, the school was transferred into a clean aquarium containing aged tap water and gut contents allowed to clear for 3 d (determined in preliminary experiments). Individuals were then euthanized (QIMR animal ethics protocol P1251), counted and the head, body, fins/tail, and internal abdominal organs tested for *Wolbachia.*

Mosquitoes. We fed 110 *Tx. speciosus* on a diet of *w*MelPop infected *Ae. aegypti* larvae from egg hatch to pupation. Pupae were placed into holding cages (30 × 20 × 20 cm) and emerged adults maintained on sucrose for at least 1 wk. This was to ensure the larval midgut meconium was discharged. Individuals were then anesthetized in a freezer, counted, and tested for *Wolbachia.*

Processing for *Wolbachia* infection. For all trials *Wolbachia* detection was performed by PCR targeting the specific IS5 sequence. Primer sequences: IS5-F (5′-GTATCCAACAGATCTAAGC-3′) and IS5-R (5′-ATAACCCTACTCATAGCTAG-3′). PCR conditions: 95°C for 3 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min; *b:* whenever a sample was positive for *Wolbachia* DNA, it was also screened for the presence of mosquito DNA through PCR targeting the ribosomal protein gene RpS17. Primer sequences: RpS17F (5′-CTGGAGATTTTCCGTTGTCA-3′) and RpS17R (5′-GACACTTCCGGCACGTAGTT-3′). PCR condi-

Table 1. Mean ± SD number of prey consumed and mean ± SD number *w*MelPop infected and noninfected *Ae. aegypti* remaining after 24 h exposure to six predator species

Predatorspecies	Prey stage	No. prey offered	No. prey consumed	No. noninfected *Ae. aegypti*	No. infected *Ae. aegypti*	*P*
M. aspericornis	Newly hatched	200	110.5 ± 43.0	44.9 ± 16.7	47.1 ± 19.0	0.80
M. darwini	Newly hatched	200	101 ± 15.5	44.6 ± 12.6	48.8 ± 7.2	0.54
P. reticulata	Fourth instar	100	32.0 ± 6.7	34.4 ± 6.7	34.5 ± 8.3	0.97
P. signifer	Fourth instar	50	21.1 ± 8.6	13.3 ± 4.8	13.9 ± 4.9	0.67
T. speciosus	Fourth instar	14	5.7 ± 1.7	4.1 ± 1.2	4.1 ± 1.4	1.00
H. adansoni	1-wk-old adult[a]	100	46.2 ± 15.5	27.7 ± 9.5	25.7 ± 6.0	0.67
	3-wk-old adult[a]	100	45.7 ± 8.3	26.8 ± 4.8	27.5 ± 7.1	0.85
H. adansoni	1-wk-old adult[b]	100	49.0 ± 5.2	36.7 ± 4.7	35.2 ± 4.6	0.59
	3-wk-old adult[b]	100	48.0 ± 12.1	28.5 ± 8.6	26.0 ± 7.5	0.60

[a] Female.
[b] Male.

tions: 95°C for 3 min followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min.

For all fitness trials, five controls (without predators) were run to monitor larval survival. If mortality was below 5%, control individuals were not screened. For all horizontal transfer trials a subsample of predators were screened to ensure no natural *Wolbachia* infection existed. For all trials, a subsample of *w*MelPop and tetracycline-cured *Ae. aegypti* from the same cohort used in the trial were also screened to confirm infection status.

Statistical Analyses. Significant differences between the mean number of *w*MelPop infected and tetracycline-cured *Ae. aegypti* (larvae or adults) remaining at the end of each experiment were determined using an independent samples *t*-test. All tests were run in PASW Statistics 18.0.

Results

Adult and Larval Fitness. There were no significant differences in the mean number of infected and noninfected *Ae. aegypti* consumed by any of the six predators tested (Table 1). For *M. aspericornis*, *M. darwini*, *P. reticulata*, *P. signifier*, and *Tx. speciosus* the mean ± SD percentage of *w*MelPop infected *Ae. aegypti* larvae remaining at the end of the experiment was 50.6 ± 6.1, 52.8 ± 5.4, 49.0 ± 5.6, 51.2 ± 9.5, and 49.5 ± 0.1, respectively. The mean ± SD percentage of *w*MelPop infected 1-wk-old and 3-wk-old female and male *Ae. aegypti* remaining at the end of the *H. adonsoni* experiment was 52.3 ± 6.7 and 52.0 ± 2.7, and 52.0 ± 4.1 and 45.0 ± 5.2, respectively.

Horizontal Transfer. No *M. aspericornis*, *M. darwini*, *P. signifier*, or *Tx. speciosus* were found positive for *w*MelPop at the end of the experiment. The head of one *P. reticulata* tested positive for *w*MelPop, however, this individual also tested positive for the presence of mosquito DNA, indicating some undigested infected larvae was present.

Discussion

The fitness trials were designed to test if differential predation was occurring between *w*MelPop infected and uninfected *Ae. aegypti* larvae and adults. Based on our results, there were no significant differences in the numbers of infected and uninfected *Ae. aegypti* larvae or adults consumed by the six different predator species. That this relationship was consistent across mosquito life stage, predator species, and experimental venue suggests that behavioral change in predator avoidance is not occurring, although we know that some traits become apparent with increased age (Yeap et al. 2011). Fourth instar *Ae. aegypti* infected with mermithid nematodes *Romanomermis culicivorax* Ross and Smith and *Strelkovimermis spiculatus* Poinar & Camino were more sedentary than uninfected *Ae. aegypti* and yet, this behavioral change did not lead to a reduction in predation of infected individuals (Wise de Valdez 2006, 2007). As we did not directly observe mosquito behavior, we can only conclude that any survival effects were not important in relation to predator avoidance, not that changes in predator avoidance did not occur. Future work could focus on behavioral observation of survival effects that may be more subtle such as mobility rates in escaping predation during attack, and increased use of refuges. Based on our results, differential predation should not be impediments to a mosquito control strategy based on the release of *w*Melpop infected individuals into the wild population.

We were unable to demonstrate horizontal transfer of *w*MelPop from *Ae. aegypti* larvae to any of the five species tested. That we were unable to demonstrate horizontal transfer to either of the two fish species, *P. signifer* and *P. reticulata* is not surprising given that there is no evidence for *Wolbachia* transfer from invertebrates to vertebrates in the literature. Horizontal transfer has been shown to be most successful when *Wolbachia* move between related hosts (Heath et al. 1999, Huigens et al. 2004, Haine and Cook 2005, Russell et al. 2009), likely because of the adaptation of *Wolbachia* strains to their hosts (McMeniman et al. 2008). As such, we considered exploiting the predator prey relationship between *Tx. speciosus* and *Ae. aegypti* as the most probable situation in which to demonstrate horizontal transfer of *Wolbachia* within a naturally occurring predator prey system involving *Ae. aegypti.* However, despite feeding for up to 21 d on *Wolbachia* infected *Ae. aegypti* from hatch to pupation, we were

unable to demonstrate any horizontal transfer of *Wolbachia* to *Tx. speciosus* larvae. In Australia at least, *Toxorhynchites* frequently are found cohabiting with *Culex quinquefasciatus* (Say) and *Ae. notoscriptus* (Brown et al. 1996). Both of these prey species are known to be infected with *Wolbachia* but this is not the case for the predator. One might suggest that the degree of exposure afforded in these trials is minimal compared with a natural exposure over millennia.

Horizontal transfers of *Wolbachia* have mainly been investigated in insect host-parasitoid communities, giving rise to phylogenetic evidence for horizontal transmission between parasitoids and hosts (Werren et al., 1995b, Heath et al., 1999). Cordeaux et al. (2001) also report phylogenetic evidence of horizontal transmission between isopods and ectoparasitic mites, which feed on insect haemolymph. They suggest blood to blood contact, rather than feeding, could serve as a possible mechanism of transfer, as demonstrated by Rigaud and Juchault (1995) with woodlice. Jaenike et al. (2007), however, demonstrated that mites, through feeding, can serve as interspecific vectors of *Spiroplasma poulsonii* Williamson et al. in *Drosophila.* This may have important implications, as mosquitoes are, not uncommonly parasitized by mites (Mullen 1975), principally by the aquatic genera *Thyas* (Thyasidae) and *Arrenurus* (Arrenuridae). While rates are usually higher in mosquito species that use ground pools for oviposition, there is evidence of parasitisation of container breeding species (Smith and McIver 1984, Williams and Proctor 2002, Snell and Heath 2006). Unfortunately, there is no published literature on the rates of parasitisation in *Ae. aegypti.* Given the proposed route of transfer via feeding or blood to blood contact with parasitic mites, further investigations of the association between these two groups may be warranted.

Although we did not test for horizontal transmission to *H. adonsoni*, recent evidence leads us to suggest that it is extremely unlikely. Cordeaux et al. 2001 demonstrated that the *Wolbachia* strain detected in the spider *Dysdera erythrina* (Walckenaer, 1802) was phylogenetically too dissimilar from the symbionts in its woodlouse prey to favor the hypothesis of horizontal transfers via a predator/prey system. Yun et al. (2011) assessed the horizontal transmission of *Wolbachia* between 11 spider families (predator) and six insect families (prey) based on wild caught individuals and could find no direct evidence indicating the existence of horizontal transmission of *Wolbachia* between predator and prey.

Riegler et al. (2004) generalized that *Wolbachia* must cross three filters (ecological, physiological, and population) before it can become established in a new host species. The ecological filter is defined by the interaction between the existing and potential host species, the physiological filter by the ability of *Wolbachia* to colonize the germ line of an individual and the population filter conditions the ability of *Wolbachia* to invade and maintain itself in host populations (Riegler et al. 2004). Here, we demonstrated that predation on *w*MelPop infected *Ae. aegypti* larvae could allow crossing of the ecological filter naturally (as opposed to artificially via microinjection). However, we were unable to demonstrate establishment in the germ line of any of the five predator species tested. We would concur with Popovici et al. (2010) that horizontal transfer is an extremely rare event and that what we are seeing now as a common phenomenon, that is, 65% of insect species infected, is a product of contact between them and *Wolbachia* over millions of years.

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