Published in final edited form as:
J Invertebr Pathol. 2013 October ; 114(2): 173–177. doi:10.1016/j.jip.2013.08.001.

Reduced competitiveness of *Wolbachia* infected *Aedes aegypti* larvae in intra- and inter-specific immature interactions

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Abstract

Wolbachia are maternally inherited intracellular bacteria that frequently infect a diverse range of arthropod species. Empirical and theoretical studies examining *Wolbachia* invasiveness have emphasized *Wolbachia* effects on adult hosts, but recent studies show that *Wolbachia* impacts on immature hosts can be important also. Here, we have examined for effects of *Wolbachia* infection in *Aedes aegypti*. Specifically, differential survivorship is observed when young larvae (1st instar) are exposed to older *Aedes albopictus* larvae (4th instar) or con-specific larvae. In an additional experiment, we have examined for differential behavior and observed that *Wolbachia*-infected larvae differ from uninfected larvae in their reaction to light stimulation. Our results support a hypothesized effect of *Wolbachia* on *A. aegypti* larval behavior. The results are discussed in relation to the ability of *Wolbachia* to invade natural populations and recently applied public health strategies that target the replacement or suppression of this important disease vector.

Keywords

Wolbachia; Dengue; insect behavior; fitness cost; population replacement; predation

1. Introduction

Wolbachia pipientis bacteria are maternally inherited endosymbionts commonly detected in a wide array of invertebrate species (Hilgenboecker et al., 2008). *Wolbachia* infections are responsible for different types of host reproductive manipulations, including feminization, parthenogenesis, male killing and cytoplasmic incompatibility (CI) (Werren et al., 2008). CI and other reproductive manipulations can provide *Wolbachia* infected females with an advantage, promoting the spread of the maternally- inherited *Wolbachia* in natural populations. In brief, the crossing pattern that can result in populations that include both infected and uninfected insects can favor infected females, which can mate successfully with all males in the population. In contrast, CI can cause reduced egg hatch when uninfected females are mated to infected males.

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Cytoplasmic incompatibility has received considerable attention, both naturally- occurring *Wolbachia* invasions, and as a method to control insects and insect vectored disease (Iturbe-Ormaetxe et al., 2011). The latter include strategies for both the suppression and replacement of medically important mosquito populations. With population replacement strategies, *Wolbachia* infections are intended to drive desired traits into a mosquito population. Previous studies show *Wolbachia* associated pathogen inhibition to occur in insects, including *Aedes aegypti* (Bian et al., 2010; Moreira et al., 2009a; Walker et al., 2011; Xi et al., 2008). Recently, Australian researchers have openly released *Wolbachia* infected *A. aegypti* in two residential areas of northern Australia, with the goal of replacing the naturally uninfected population with the *Wolbachia* infected cytotype (Hoffmann et al., 2011). Repeated releases of female mosquitoes infected with the *w*Mel *Wolbachia* resulted in high infection frequencies among the field populations. Additional releases are currently underway with the *w*MelPop-CLA *Wolbachia* infection type (Cyranoski, 2012).

The ability of *Wolbachia* to invade and persist within populations has been a focus of prior empirical and theoretical examinations, with key parameters that include CI-levels, maternal inheritance rates of the *Wolbachia* infection and *Wolbachia* effects on host fitness. More recently, the impacts of *Wolbachia* infection on the immature fitness have been shown also to be important in determining the invasion success of *Wolbachia* (Gavotte et al., 2010; Gavotte et al., 2009). A recent theoretical examination implicates *Wolbachia* reduction of immature fitness to be among the strongest impediment to population replacement (Crain et al., 2011). Thus, it is important to understand the potential for *Wolbachia* effects on immature host fitness.

Conspecific and heterospecific competition affect population dynamics, which can affect the community structure. Mosquitoes including *A. aegypti* and *A. albopictus* tend to oviposit on multiple habitats (Colton et al., 2003; Corbet and Chadee, 1993), and gravid mosquitoes are more attracted to habitats that have been accupied with conspecific immature individuals (Heard, 1994; Mokany and Shine, 2003; Sherratt and Church, 1994; Wong et al., 2011). The larval community in natural habitats are thus often age structured with mixture of sibling families and tend to increase intraspecific competition involving various interactions between immature individuals of multiple larval developmental stages (Focks et al., 1981; Gomes et al., 1995; Southwood et al., 1972). Mosquito species such as *A. aegypti*, *A. albopictus* and *A. triseriatus* often share their breeding sites and increased competition for resources promoted exclusion of cohabiting heterospecific competitors (Braks et al., 2004; Juliano, 1998; Livdahl and Willey, 1991).

In this report, we describe experiments conducted to examine a previously demonstrated impact of *Wolbachia* infection on the larvae of *Aedes* mosquitoes. In an initial experiment, we examine the relative competitiveness of *Wolbachia* infected and uninfected first instar *A. aegypti*, when interacting with fourth instar conspecifics or *A. albopictus* under laboratory conditions. In a second set of experiments, we compare the behavior of infected and uninfected larvae in response to light. The results are discussed in relation to infection dynamics in natural populations and applied strategies targeting public health outcomes.

2. Materials and methods

2.1. Insect strains

Experiments used wild type *A. albopictus* (Lexington, KY) (MID) naturally infected with two *Wolbachia* strains, *A. aegypti* (JCU) naturally uninfected (McMeniman et al., 2009), the *w*MelPop-CLA infected colony of *A. aegypti* (PGYP1) generated by introducing *w*MelPop-CLA in JCU, and *w*MelPop-CLA removed *A. aegypti* (PGYP1.tet) by treating tetracycline on PGYP1 strain (McMeniman et al., 2009). Maintenance of *A. aegypti* mosquitoes was as

previously described (McMeniman et al., 2009). In brief, all maintenance and experiments were conducted at 28 ± 2°C, 75 ± 10% RH, and a photoperiod of 18:6 h (L:D). Eggs were submerged in a mixture of fish food (TetraMin Tropical Tablets, Tetra, Germany) in 400 ml of water. Larvae were given fish food ad libitum and adults were transferred into 30 × 30 × 30 cm cages with constant access to a 10% sucrose solution. The females were blood fed with an artificial feeder using human blood collected at a blood bank (Kentucky Blood Center, Lexington, KY) or an anesthetized mouse (A3336-01; PHS Assurance). MID strain was maintained as previously described (Dobson et al., 2001).

2.2. Immature competition assay

Two experiments were conducted. In the first experiment, 30 1st instar (L1) of PGYP1 and PGYP1.tet strain (< 2 hours post hatch) were transferred into separate petridishes (60 × 15 mm) (BD BioSciences, Franklin Lakes, NJ) with 10 ml fish food solution (0.1%). The experimental density and food amount was similar to previous studies where competitive interactions between L1 and L4 were examined to exihibt cannibalism or predation in Aedes species (Edgerly et al., 1999; Koenekoop and Livdahl, 1986). The experiment consisted of three treatments to test the effect of 4th instars (L4) of different mosquito strains on L1 survival. In the control treatment, no L4 were introduced. The other two treatments received six L4 (4 days old) of PGYP1 or PGYP1.tet. Surviving L1 were counted after 48 hours. Any pupated L4 were replaced with the cohort of L4 at 24 and 36 hours after the experiment was initiated.

The second experiment used same protocol as described above, but with different strains of mosquito larva. PGYP1 and JCU strains were used as L1, and JCU and MID strains were used as L4. Both experiments were replicated six times. Generalized linear models were used to examine for an effect of L4 and/or L1 type on the survival of L1 using binomial distribution with Logit link (JMP 8.0.1; SAS Institute, Cary, NC), and post-hoc contrasts were specified to compare the survival of L1 within each treatment.

2.3. Light response assays

Two experiments were performed to estimate larval behavior in reaction to light stimulation. In the first experiment, L4 of PGYP1, PGYP1.tet and JCU strains were tested using a rectangular container with a darkened area at one side, simulating a refuge (Fig. 1). The container was filled with 300 ml deionized (DI) water and larvae were introduced into the center of the lighted area. Thirty L4 (4 days old) were introduced within a darkened tube fabricated from a conical 50 ml tube (BD Biosciences, Franklin Lakes, NJ). Larvae were released by lifting the tube, exposing larvae to a fluorescent light from above (Helical 20W; General Electric, Milwaukee, WI). Larval movement was video recorded until all larvae reached the refuge. Larvae were scored upon reaching the darkened area. Larvae were not observed to exit the darkened area. A complete block design was used, with 20 replicates per strain.

In a second behavioral assay, L1 (< 2 hours post hatch) of PGYP1, PGYP1.tet and JCU strains were tested following the similar protocol described above but with a different arena design. Due to the smaller larval size, a petridish (BD BioSciences, Franklin Lakes, NJ) was used instead of the larger container (Fig. 2). The petri dish contained 20 ml water with a covered edge to create a darkened area. The petri dish was placed on fluorescent light table (Porta-Trace Light table; Gagne, INC, Johnson City, NY). A darkened tube was used for larval release, and larval movement was recorded for two minutes or until all larvae entered the darkened area. A randomized design was used, with > 8 replicates per strain.

For statistical analysis, mean time required for median proportion of larvae to reach the darkened area (i.e. mean of median time) was compared using a one-way ANOVA test (JMP 8.0.1; SAS Institute, Cary, NC) and Tukey-Kramer Honestly Significant Difference (HSD) test for multiple comparison among strains. The effect of strain and infection status was examined using a student s t-test.

3. Results

3.1. Immature competition assay

In an initial experiment, we examined for an effect of L4 (i.e., none, infected, and uninfected) and L1 type (i.e., infected and uninfected) on the survivorship of L1. A generalized linear model with L1 and L4 type as factors and survivorship of L1 as the variable was significant (GLM: $\chi^2 = 16.7$, Df = 5, $p = 0.005$) (Table S1), and there was a significant interactive effect of L1 and L4 type (LR-$\chi^2 = 9.1$, Df = 2, $p = 0.011$). A significant difference was observed in survivorship (Fig. 3A). Post-hoc analysis for each treatment showed that the survival of infected L1 was reduced relative to that of uninfected L1, when in the presence of uninfected L4 (LR-$\chi^2 = 0$, Df = 1, $p = 0.00078$) while no difference was observed in L1 survival when in the absence of L4 (LR-$\chi^2 = 0.85$, Df = 1, $p = 0.36$) or the presence of infected L4 (LR-$\chi^2 = 0$, Df = 1, $p = 1$).

To examine whether the observed differential survival was specific to the tested strains, the experiment was repeated using alternative mosquito strains. Specifically, survival of naturally-uninfected and artificially-infected L1 *A. aegypti* were either not exposed or exposed to L4 that were either wild type *A. albopictus* (MID) or naturally uninfected *A. aegypti* (JCU). A generalized linear model with L1 and L4 type as factors and survivorship of L1 as the variable was significant (GLM: $\chi^2 = 60.4$, Df = 5, $p < 0.0001$) (Table S2), and there was significant effect of L1 (LR-$\chi^2 = 8.6$, Df = 1, $p = 0.0034$) and L4 type (LR-$\chi^2 = 40.7$, Df = 2, $p < 0.0001$) on survivorship of L1. Similar to the first experiment, significant differences were observed in the L1 survivorship (Fig. 3B). Post-hoc analysis showed that the survival of infected L1 was significantly reduced with the presence of wild type *A. albopictus* (LR-$\chi^2 = 6.0$, Df = 1, $p = 0.014$) as well as with the wild type *A. aegypti* (LR-$\chi^2 = 8.6$, Df = 1, $p = 0.0033$), while no difference was observed in L1 survival in the absence of L4 (LR-$\chi^2 = 1.3$, Df = 1, $p = 0.24$).

3.2. Light response assay

The differences in L1 survival observed in the preceding experiments could result in part from differential larval motility. As a motility test, an experiment was conducted in which *Wolbachia* infected and uninfected larvae were exposed to light, and their response time in seeking a dark refuge was examined. A significant difference was observed in the time for median proportion of 4th instar to reach refuge in the comparison of three mosquito strains (one way-ANOVA; $F_{2,57} = 5.13$, $p = 0.009$) (Fig. 4A). Post-hoc Tukey-Kramer HSD test showed the PGYP1 strain was the slowest to find the refuge (12.4 ± 0.7 sec; $N = 20$), JCU intermediate (11.5 ± 0.6 sec; $N = 20$) and PGYP1.tet the fastest (10.4 ± 0.5 sec; $N = 20$). Additional statistical analysis showed the *Wolbachia* infected larvae (PGYP1 strain) to be significantly slower to reach the refuge relative to the uninfected strains (PGYP1.tet and JCU strains) (10.9 ± 0.5 sec; $N = 40$) (student s t-test; Df = 1, $p = 0.0095$).

A similar pattern was observed in a second experiment testing L1. A significant difference was observed in the time for median proportion of 1st instar to reach refuge in the comparison of three strains (Fig. 4B). Post-hoc Tukey-Kramer HSD test showed the PGYP1 was slowest to find the refuge (16.3 ± 1.2 sec; $N = 8$), JCU intermediate (14.8 ± 0.9 sec; $N = 8$) and PGYP1.tet fastest (12.2 ± 1 sec; $N = 11$) (one-way ANOVA; $F_{2,24} = 4.4$, $p < 0.024$). The infected larvae of PGYP1 strain were significantly slower to reach the refuge than the

uninfected larvae of PGYP1.tet and JCU strains (13.3 ± 0.7 sec; $N = 19$), when compared by infection status of larvae (student's t-test; Df = 25, $p = 0.035$).

4. Discussion

In the absence of L4, similar survivorship was observed for both *Wolbachia*-infected and uninfected L1. In contrast, the mortality of *Wolbachia* infected L1 was significantly higher when exposed to L4. This outcome was repeatable and occurred regardless of whether the L4 were *A. albopictus* or *A. aegypti*. An exception was when the L4 *A. aegypti* were artificially infected with *Wolbachia*. In the latter test, the survival of infected L1 was indistinguishable from the test in which no L4 were present.

Dead L1 were not located after 48 hour of interactions between L1 and L4, suggesting that the L4 consumed L1. Cannibalism in *A. aegypti* was initially reported by MacGregor (MacGregor, 1915) and subsequently, cannibalistic and intraguild predatory behaviors have been reported between larger and smaller larvae in additional *Aedes*, *Anopheles* and *Culex species* (Edgerly et al., 1999; Koenekoop and Livdahl, 1986; Koenraadt and Takken, 2003; Muturi et al., 2010). This is believed to constitute little of immature *A. albopictus* and *A. aegypti* diet, since their primary food consists of microorganisms and organismal detritus (Merritt et al., 1992). Particularly relevant to observations reported here, L4 caused increased L1 mortality in a previous study using *Aedes* species (Edgerly et al., 1999).

L1 mortality can be explained by predation if it involved attacking behavior of L4 as defined by "an interaction in which one free-living individual kills and derives resources from another organism" (Abrams, 2001). In the experimental design used here, we did not directly observe for predation events. Thus, an alternate explanation for the disappearance of L1 is that it results from the scavenging of L1 carcasses by L4. This could be resolved in future studies through continuous visual observation to detect behaviors of L4 including attacking (i.e., chewing with mandibles) or killing L1.

Regardless of whether due to increased L1 mortality or predation, significant differences were associated with the L1 *Wolbachia* infection type. These results are consistent with a cost to the immature host, caused by artificial *Wolbachia* infection. A difference is not observed in the absence of L4 or when the L4 is artificially infected with *Wolbachia*. Thus, we hypothesize that artificially infected L1 are more susceptible to predation, relative to naturally uninfected L1. Artificially infected L4 *A. aegypti* are affected by *Wolbachia* also and are less likely to predate L1.

A prior study showed no effect of *Wolbachia* infection on the survival of immature *A. aegypti* mosquitoes exposed to natural predators (Hurst et al., 2012). The authors of the prior work emphasize that the absence of observed survival effects does not necessarily exclude possible changes in predator avoidance between the infected and uninfected strains. As an example, they point to prior studies in which *A. aegypti* larvae made more sedentary by nematode infection did not lead to reduced predation of infected individuals (de Valdez, 2006; de Valdez, 2007) and emphasize the need for direct behavior observation. Here, we have directly observed for behavioral differences in one of our exerpiments, the assay to compare the larval response to light. A sudden change in light condition was considered to be a predation risk (Folger, 1946; Omardeen, 1957; Thomas, 1950). Larvae recognize visual change, thus they present avoidance behavior responding to dark subject approaching (potential predators) or brighter condition which stimulates them to find darker condition. The darker condition is considered as a refuge where predation risk is limited. The assay results show that artificially infected L1 *A. aegypti* require significantly more time to reach refuge, compared to uninfected L1. This is consistent with the hypothesized increased susceptibility to predation associated with *Wolbachia* infection in *A. aegypti*. However, the

difference in escape behavior reported here was apparently insufficient to cause observable predation differences in the earlier assays (Hurst et al., 2012). Thus, additional work is required to better understand and predict the impact in nature of a *Wolbachia* effect on larvae, which will be determined largely by the relative impacts of predators versus cannibalistic and intraguild larval predation.

Potential mechanisms for the *Wolbachia*-associated differences observed in this study include previously described *Wolbachia* effects on host metabolism rates and the potential for *Wolbachia* effect on behavior caused by infection of host tissues. First, a genomic study of *w*Mel strain of *Wolbachia* indicated the lack of complete metabolic pathways and limited ability to synthesize metabolic intermediates (Wu et al., 2004), suggesting that a genetically similar strain of *w*MelPop-CLA may require energy resources from the host. If hosts require more energy due to *Wolbachia* infection, hungry larvae can increase browsing behavior for food as observed in other *Aedes* species, and foraging larvae are more likely to be predated (Juliano et al., 1993). Second, if the *Wolbachia* infection enhanced immune response in immature stage as previously shown in adult stage (Moreira et al., 2009a; Moreira et al., 2009b), the infection could be associated with trade-off between survival of infected individual and potential predation risk as revealed in a recent study of other insect species (Otti et al., 2012). Third, *Wolbachia* infection could directly affect host behavior as *w*MelPop-CLA infection has been shown to be widespread in *A. aegypti* tissues, including ommatidia cells in eyes, brain neuronal cells and muscle tissues (Moreira et al., 2009a). Similar examples are reported in the previous studies that *w*Mel and *w*MelPop infections were associated with decreased responsiveness to food cues in its native host *Drosophila melanogaster* (Peng et al., 2008), as well as a modification of tissue characteristics, known as bendy proboscis (Moreira et al., 2009b; Turley et al., 2009).

The effects of *Wolbachia* on immature *A. aegypti* are directly relevant to recent field trials of *Wolbachia* based public health strategies, particularly for the releases with *w*MelPop-CLA infections (Cyranoski, 2012). For example, there is substantial effort to prevent an infestation of *A. albopictus* in Northern Australia from spreading southward (Ritchie et al., 2006). In regions of overlap, the competition between *A. aegypti* and *A. albopictus* is an important determinant for the range of the two species (Juliano, 1998; Juliano and Lounibos, 2005). Thus, a reduced competitiveness of an artificially infected *A. aegypti* population could increase the risk of *A. albopictus* spread.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank S. O Neill for providing mosquito strains (PGYP1, PGYP1.tet and JCU). This research was supported by grants from the National Institutes of Health [AI-067434] and the Bill and Melinda Gates Foundation [#44190]. The information reported in this paper (No. 13-08-010) is part of a project of the Kentucky Agricultural Experiment Station and is published with the approval of the Director.

Abbreviations

CI	Cytoplasmic incompatibility
MID	Wild type *A. albopictus* naturally infected with two *Wolbachia* strains
JCU	*A. aegypti* naturally uninfected
PGYP1	*A. aegypti* infected with *w*MelPop-CLA

PGYP1.tet	*w*MelPop-CLA removed *A. aegypti* by treating tetracycline on PGYP1 strain
L1	1st instar
L4	4th instar

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Research Highlights

- We examine effect of *Wolbachia* on larval competition in *A. aegypti*
- We examine effect of *Wolbachia* on larval behavior in *A. aegypti*
- *Wolbachia* infected *A. aegypti* larvae experience reduced survival when competing
- *Wolbachia* infected *A. aegypti* larvae react slower than uninfected larvae

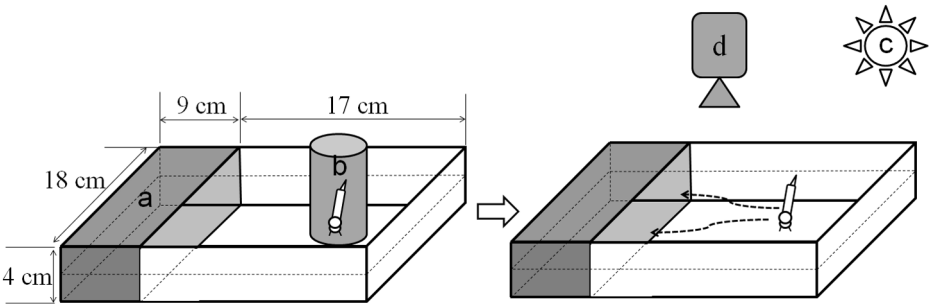

Figure 1.

Schematic diagram of light response assay using 4th instar larvae (L4) of PGYP1 (*A. aegypti* strain infected with *w*MelPop-CLA), PGYP1.tet (tetracycline treated strain of PGYP1) and JCU (wild type strain of *A. aegypti*). A container with a dark area at one side was filled with 300 ml water (a). An opaque tube was used to acclimate 30 larvae within darkened conditions for two minutes (b). The larvae were released from the tube, and the larval avoidance rate to light (c) was recorded using a camera (d).

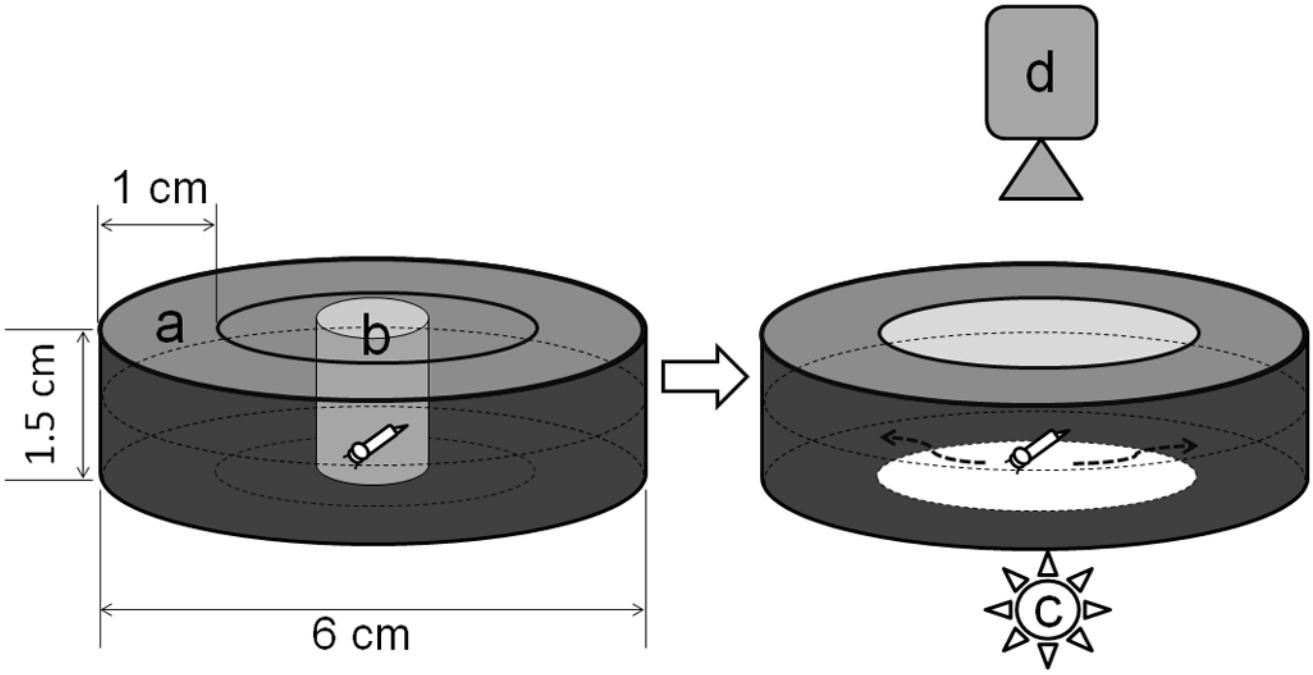

Figure 2.
Schematic diagram of light response assay using 1st instar (L1) of PGYP1 (*A. aegypti* strain infected with *w*MelPop-CLA), PGYP1.tet (tetracycline treated strain of PGYP1) and JCU (wild type strain of *A. aegypti*). A petridish with a dark area around the edge was filled with 20 ml water (a). An opaque tube was used to acclimate 30 larvae within darkened conditions for two minutes (b). The larvae were released from the tube and exposed to light (c); their movement into the darkened area was recorded using a camera (d).

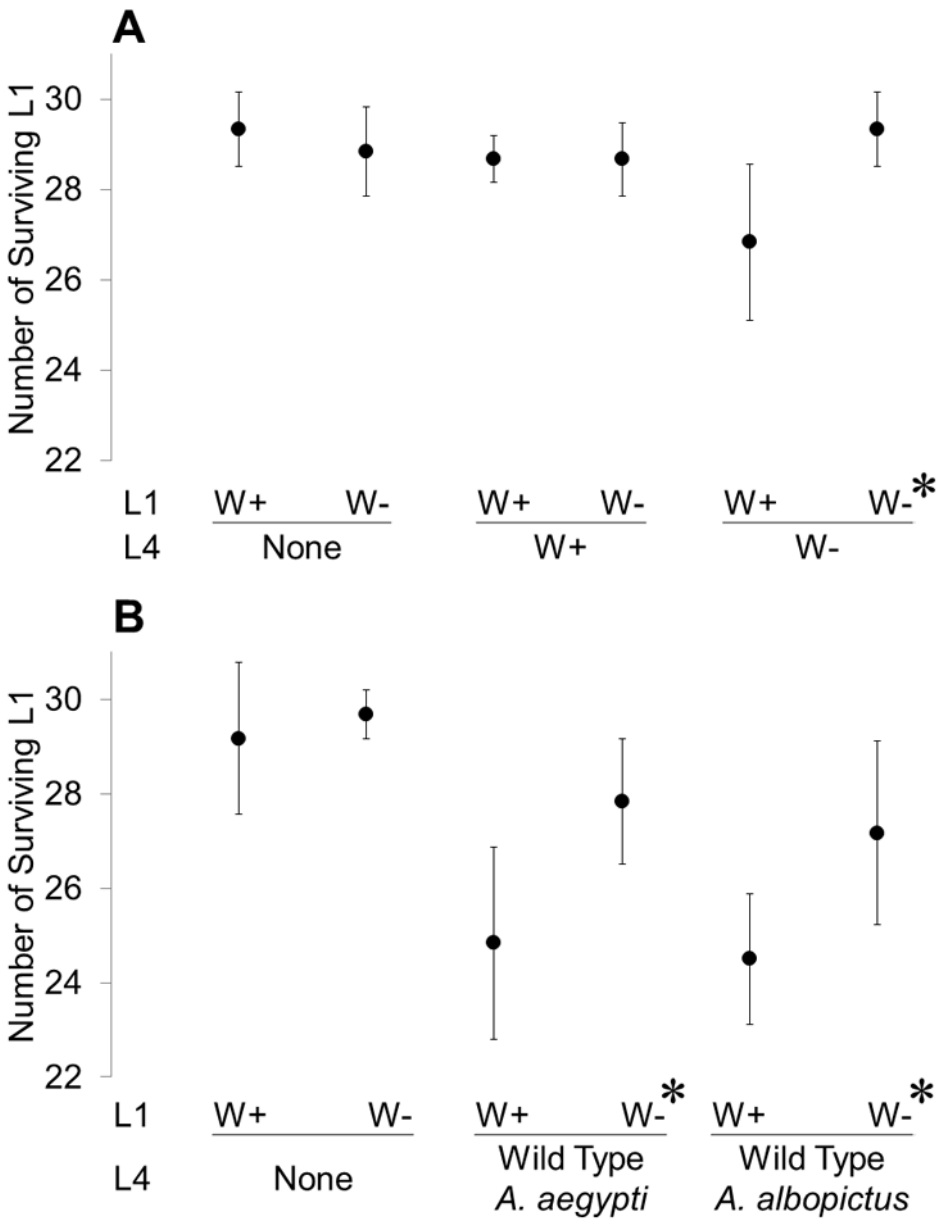

Figure 3.

Survivorship of 1st instar (L1) from intra- (A) and inter- (B) specific interaction with six 4th instars (L4). Strains used are W+ (*Wolbachia* infected; PGYP1 = *A. aegypti* strain infected with *w*MelPop-CLA) and W- (PGYP1.tet = tetracycline treated strain of PGYP1). In (B), L4 strains are wild type strains of *A. aegypti* (JCU strain) and *A. albopictus* (MID strain). Error bar = s.d. (n=6). Asterisks represent significant difference at $p = 0.05$.

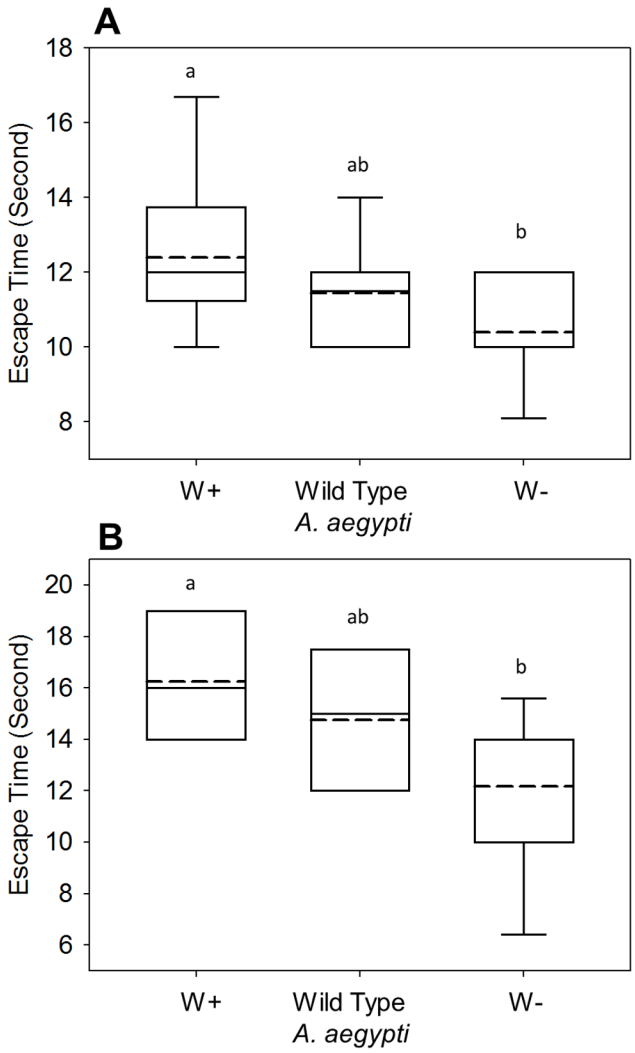

Figure 4.

Larval avoidance to light stimulation, estimated by time for median proportion of larvae to reach a darkened refuge (i.e., Escape Time); 4th instar (A) and 1st instar (B). Strains used are W+ (*Wolbachia* infected; PGYP1 = *A. aegypti* strain infected with *w*MelPop-CLA), W- (PGYP1.tet = tetracycline treated strain of PGYP1) and a wild type strains of *A. aegypti* (JCU strain). Box plots (solid line) represent 10% (bottom wisker), 25% (bottom line of box), 50% (middle line of box), 75% (upper line of box), 90% (upper wisker) quantile, and the dashed line indicates a mean value of Escape Time. Whiskers are not presented if 10% or 90% quantiles are same as 25% or 75% quantile. Different letters represent significant difference at $p = 0.05$.