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Wolbachia Infection Alters Olfactory-Cued Locomotion in *Drosophila* spp.[▽]

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***Wolbachia pipientis* is an endosymbiotic bacterium present in diverse insect species. Although it is well studied for its dramatic effects on host reproductive biology, little is known about its effects on other aspects of host biology, despite its presence in a wide array of host tissues. This study examined the effects of three *Wolbachia* strains on two different *Drosophila* species, using a laboratory performance assay for insect locomotion in response to olfactory cues. The results demonstrate that *Wolbachia* infection can have significant effects on host responsiveness that vary with respect to the *Wolbachia* strain-host species combination. The *w*Ri strain, native to *Drosophila simulans*, increases the basal activity level of the host insect as well as its responsiveness to food cues. In contrast, the *w*Mel strain and the virulent *w*MelPop strain, native to *Drosophila melanogaster*, cause slight decreases in responsiveness to food cues but do not alter basal activity levels in the host. Surprisingly, the virulent *w*MelPop strain has very little impact on host responsiveness in *D. simulans*. This novel strain-host relationship was artificially created previously by transinfection. These findings have implications for understanding the evolution and spread of *Wolbachia* infections in wild populations and for *Wolbachia*-based vector-borne disease control strategies currently being developed.**

Infections with the endosymbiotic bacterium *Wolbachia pipientis* are best known for their capacity to cause reproductive manipulations in their insect hosts, including male killing, feminization, parthenogenesis, and most commonly, cytoplasmic incompatibility (CI). Since the microbe is maternally transmitted via the egg, each of these manipulations has the effect of assisting *Wolbachia* spread through host populations (28). The nature of the symbiotic association has justifiably led to the generation of a large body of knowledge that is gonad centric. *Wolbachia* bacteria, however, are not only present in the gonads but can be found throughout a diverse array of host organs and tissues, including nerve and muscle (7, 17, 24). The consequences of somatic tissue infection for hosts have not been explored fully (9, 11).

The impact of *Wolbachia* infection on host longevity represents one of the few nonreproductive phenotypes in the primary model for insects, i.e., *Drosophila* that have been well studied. In particular, the virulent *Wolbachia* strain *w*MelPop has been shown to reduce the life span of both its native host, *Drosophila melanogaster*, and artificially infected *Drosophila simulans* (21, 24, 30). This strain is presumed to shorten host life span by overreplicating to the point of host cell rupture in older adult flies. Other strains infecting *D. melanogaster* have shown milder effects, both positive and negative, on host longevity, but these have tended to be highly dependent on the host genetic background (10). Anecdotally, we have observed in the laboratory that old adults who are consequently heavily infected with *w*MelPop appear to be more sedentary and erratic in their motion. Reduced locomotory activity as the result of *Wolbachia* infection has previously been documented for the *Drosophila* parasitoid *Leptopilina heterotoma* (9).

The need to understand *Wolbachia* effects on host locomotion is twofold. First, documented effects of *Wolbachia* infection on *Drosophila* fitness are rare (12). The expression of CI is usually relied upon to explain the high infection prevalence of *Wolbachia* in wild populations (34, 35). This cannot explain the distribution of all strains, though. In species like *D. melanogaster*, the expression of CI is often weak and decreases with male age (29). The *w*Au strain present in Australian populations of *D. simulans*, for example, appears to cause no CI at all (13). With few exceptions (5, 37), standard laboratory and semifield measures of reproductive fitness (productivity, fecundity, etc.), as well as nontraditional measures such as responses to environmental stresses, have not revealed evidence of strong *Wolbachia*-conferred benefits for hosts (12, 14, 15, 27, 30). It is possible, however, that *Wolbachia* may alter more complex aspects of host biology in the field, which are not captured by traditional laboratory assays but could benefit infected hosts. Second, *w*MelPop infection is currently being developed for biological control. The virulence of this strain has placed it at the center of strategies aimed at shortening the life span of insect vectors of human diseases (2, 33). A clear understanding of the complete suite of effects that *Wolbachia* may have on hosts is necessary to determine if and how the microbe could be utilized for vector control in the field.

This study aimed to determine whether avirulent *Wolbachia* strains and the virulent, life-shortening *w*MelPop strain may affect complex behaviors in *D. melanogaster* and *D. simulans*. We compared the performance of *Wolbachia*-infected hosts to that of uninfected controls in a laboratory-based measure of

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olfactory-cued locomotion for a range of adult ages. Both olfaction and locomotion are critically important for finding food and mates and are therefore of likely ecological significance (38). We predicted that the benign *Wolbachia* strains native to *D. melanogaster* and *D. simulans* might negatively affect host capture performance, given previous evidence from *Leptopilina heterotoma* (9). We expected that the virulent *w*MelPop strain would have very detrimental consequences for its hosts, particularly as the insects aged and bacterial densities increased (24). In keeping with novel host theory (1), we also hypothesized that the effects of *w*MelPop might be more severe in *D. simulans*, given that this species was artificially infected (21).

MATERIALS AND METHODS

Fly strains and rearing. The following three *Wolbachia* strains were compared in this study: *w*Mel, *w*MelPop, and *w*Ri. Both *w*Mel (from yw^{67c23}) and *w*MelPop (from w^{1118}) were backcrossed into the common host genetic background Canton-S for 5 generations prior to experimentation. These host-*Wolbachia* combinations are denoted Dmel *w*Mel and Dmel *w*MelPop, respectively. Dsim *w*Ri represents a laboratory stock from the original Riverside, CA, population (16). Dsim *w*MelPop was generated ~100 generations previously via transinfection of *w*MelPop into Dsim *w*Ri hosts that had been tetracycline treated to remove the *w*Ri infection (21, 22). All stocks have been in the laboratory for years and hence were highly inbred and homogeneous in nature. Uninfected controls were created for each of the above lines through standard procedures for tetracycline treatment (16). Large population sizes (>100 females) were treated to reduce drift effects during tetracycline treatment, and lines were reared for >3 generations posttreatment prior to experimentation (10). Introgression of infected and uninfected lines after tetracycline treatment could also have been employed but was not, for two reasons. First, the independently generated uninfected controls for each species did not perform differently from one another. Second, such a regimen dictated by *Wolbachia* crossing limitations would not homogenize the mitochondrial genome, which is likely to play a role in the phenotypes of interest here (18).

Flies were reared at 25°C with a 12-h light-dark regimen in large bottles containing 100 ml of standard cornmeal diet with active yeast sprinkled on the surface. Bottle populations were maintained at low densities (~50 females per bottle, with one 8-h day of egg laying) to minimize any possible negative physiological consequences due to crowding. Newly eclosed flies were collected daily and reared to 5, 15, and 35 days of age for testing in capture arenas. Females were isolated from aged bottle populations and placed onto vials 2 days prior to behavioral assessment. Flies were starved for 19 h prior to assessment in 15-ml vials, which contained a layer of cotton wool soaked with distilled water to prevent desiccation.

Olfactory response assays. We used a modified version of a behavioral response assay, the olfactory trap (6, 39), to score insect responses to an attractive olfactory cue. Traps consisted of a 2-ml glass vial into which we inserted a 0.2-ml Eppendorf pipette tip, with the narrow end trimmed off (Fig. 1). The narrow opening prevented flies from easily exiting the traps once they were captured. Traps were baited with 0.2 ml of *Drosophila* diet sprinkled with active dry yeast and were placed horizontally in 60-ml plastic wide-mouth jars covered with a square piece of muslin (held in place by a rubber band), which acted as testing arenas. Beginning 1 h after photophase commenced, starved flies were placed individually into arenas via aspiration and assessed for capture every 20 min for a period of 220 min. A total of 150 flies were assessed for each adult age-strain combination. Testing was carried out on three separate days for each treatment (50 flies each). Each fly was used only once to avoid any effects of learning (6, 19). To prevent odor contamination from previously used traps or *Drosophila* aggregation pheromones (38), arenas and muslin squares were washed and allowed to dry between subsequent experiments, and olfactory traps were discarded after each experiment. A parallel set of experiments (three replicates, with 50 flies each) using unbaited traps was carried out for all strains at 15 days of age to assess baseline activity. Log-rank tests (survival analysis) were used to compare capture performances of infected and uninfected controls for each adult age with the statistics software JMP 7.0 (SAS Institute, Inc.). The strength of this approach was in coding uncaptured flies in both baited and unbaited assays as censored. Individual log-rank comparisons were made between infected and uninfected lines for each host-strain-age combination for diet-baited capture assays. The degrees of freedom are therefore always equal to 1.0 (number of groups − 1). We employed a conservative critical rejection value (α) of 0.01.

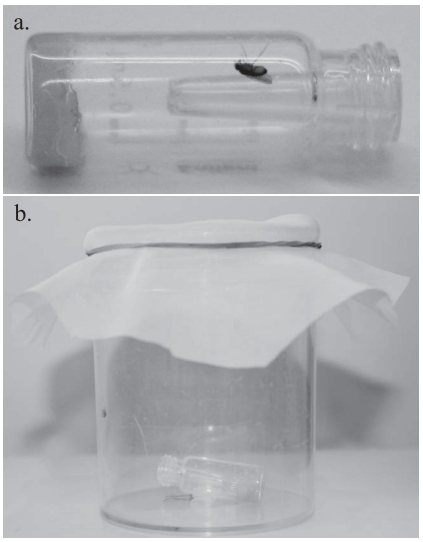

FIG. 1. *Drosophila* diet-baited trap (a) and capture arena containing a trap (b). After overnight starvation, individual flies representing the different host-strain combinations were placed inside capture arenas with either baited or unbaited traps. The time that individual flies took to enter the trap was recorded during a 220-min assay period.

RESULTS

Capture performance. (i) Baited traps. *D. melanogaster* flies infected with *w*Mel performed slightly worse than uninfected controls at 15 and 35 days of age (for day 5, $\chi^2 = 2.6$ and $P = 0.10$; for day 15, $\chi^2 = 9.6$ and $P = 0.0019$*; and for day 35, $\chi^2 = 12.2$ and $P = 0.0005$*) (asterisks indicate significant differences) (Fig. 2a). The *w*MelPop strain decreased host performance at all three ages (for day 5, $\chi^2 = 24.1$ and $P < 0.0001$*; for day 15, $\chi^2 = 7.8$ and $P = 0.0052$*; and for day 35, $\chi^2 = 8.0$ and $P = 0.0045$*) (Fig. 2b). The largely parallel slopes of the cumulative capture curves also indicate that most of the differences in host performance can be attributed to the earliest time points (20 and 40 min). Additional direct comparisons between *w*Mel- and *w*MelPop-infected flies for each age (for day 5, $\chi^2 = 5.08$ and $P = 0.024$; for day 15, $\chi^2 = 0.017$ and $P = 0.89$; and for day 35, $\chi^2 = 0.61$ and $P = 0.43$) indicated that the two strains did not differ with respect to their effects on the host. Comparisons between the two uninfected lines at each age also showed no differences (for day 5, $\chi^2 = 0.91$ and $P = 0.33$; for day 15, $\chi^2 = 0.25$ and $P = 0.61$; and for day 35, $\chi^2 = 0.33$ and $P = 0.56$), which was not surprising given the homogenization of host genetic background via backcrossing just prior to tetracycline treatment.

In contrast, the *w*Ri strain in *D. simulans* dramatically increased the performance of flies at all adult ages (for day 5, $\chi^2 = 177.1$ and $P < 0.0001$*; for day 15, $\chi^2 = 135.9$ and $P < 0.0001$*; and for day 35, $\chi^2 = 212.4$ and $P < 0.0001$*) (Fig. 3a). The *w*MelPop strain did not alter the behavior of 5- and 15-day-old flies relative to that of controls (for day 5, $\chi^2 = 5.2$ and $P = 0.021$; and for day 15, $\chi^2 = 4.06$ and $P = 0.043$), but it did marginally improve the performance of 35-day-old flies ($\chi^2 =$

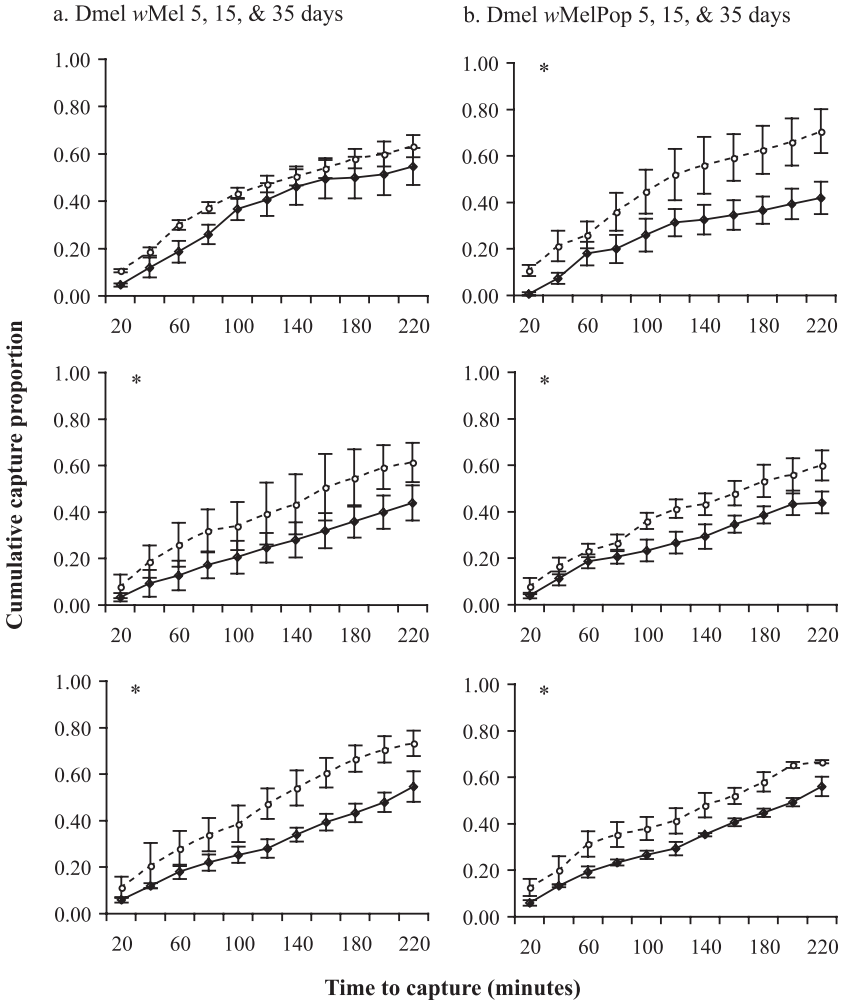

FIG. 2. Cumulative mean proportion of *D. melanogaster* flies captured ± standard error of the mean (SEM) per 20-min period in baited traps. Means represent three replicate assay dates each, based on the performance of 50 individual flies. Infected (solid lines) and uninfected (dashed lines) flies were tested at each of three adult ages (5, 15, and 35 days). Significant differences between the performances of infected and uninfected flies for each adult age were determined by the log-rank test. *, $P \leq 0.01$.

14.5 and $P = 0.0001$*) (Fig. 3b). Total capture of *w*Ri-infected *D. simulans* ranged from 90 to 96% for the three adult ages, which was substantially greater than that for uninfected control flies, which ranged from 28 to 48%. Direct comparisons, as described above, between *w*Ri- and *w*MelPop-infected *D. simulans* flies not surprisingly revealed significant differences at all adult ages (for day 5, $\chi^2 = 167.7$ and $P < 0.0001$*; for day 15, $\chi^2 = 110.9$ and $P < 0.0001$*: and for day 35, $\chi^2 = 115.2$ and $P < 0.0001$*). The two uninfected control *D. simulans* lines differed from one another only at 5 days of age (for day 5, $\chi^2 = 15.6$ and $P < 0.0001$*; for day 15, $\chi^2 = 0.0064$ and $P = 0.93$; and for day 35, $\chi^2 = 4.8$ and $P = 0.027$). These two host lines have a less recent shared origin (~100 generations previous) than that of the *D. melanogaster* uninfected controls.

(ii) Unbaited traps. A set of assays (three replicates, with 50 flies each) were carried out at 15 days of age with unbaited traps to assess how much of the capture success was due to baseline activity of the flies rather than to olfactory-cued locomotion. Capture percentages were generally very low for all host-strain combinations (Fig. 4). *D. melanogaster* flies infected with *w*Mel showed no difference in activity ($\chi^2 = 0.55$; $P = 0.45$) relative to controls (Fig. 4a). The activity levels of *D. melanogaster* ($\chi^2 = 1.03$; $P = 0.30$) and *D. simulans* ($\chi^2 = 1.60$; $P = 0.20$) were not altered by the *w*MelPop strain (Fig. 4b and d). Most surprisingly, the *w*Ri strain significantly increased the *D. simulans* activity level ($\chi^2 = 212.4$; $P < 0.0001$*), with up to 48% of flies being captured, compared to only 2.7% of uninfected controls (Fig. 4c). This represents roughly half of the capture percentage obtained when traps were baited (Fig. 3a).

DISCUSSION

Our results show that *Wolbachia* infection may induce strain- and host species-specific changes in olfactory-cued locomotion in adult *Drosophila* flies that could influence the host's behavior in nature. The patterns of insect performance across our study system revealed several key points. In *D. melanogaster*, both *w*Mel and *w*MelPop decrease host performance. More surprisingly, *w*MelPop-induced changes are not more severe than those induced by *w*Mel, nor do they worsen

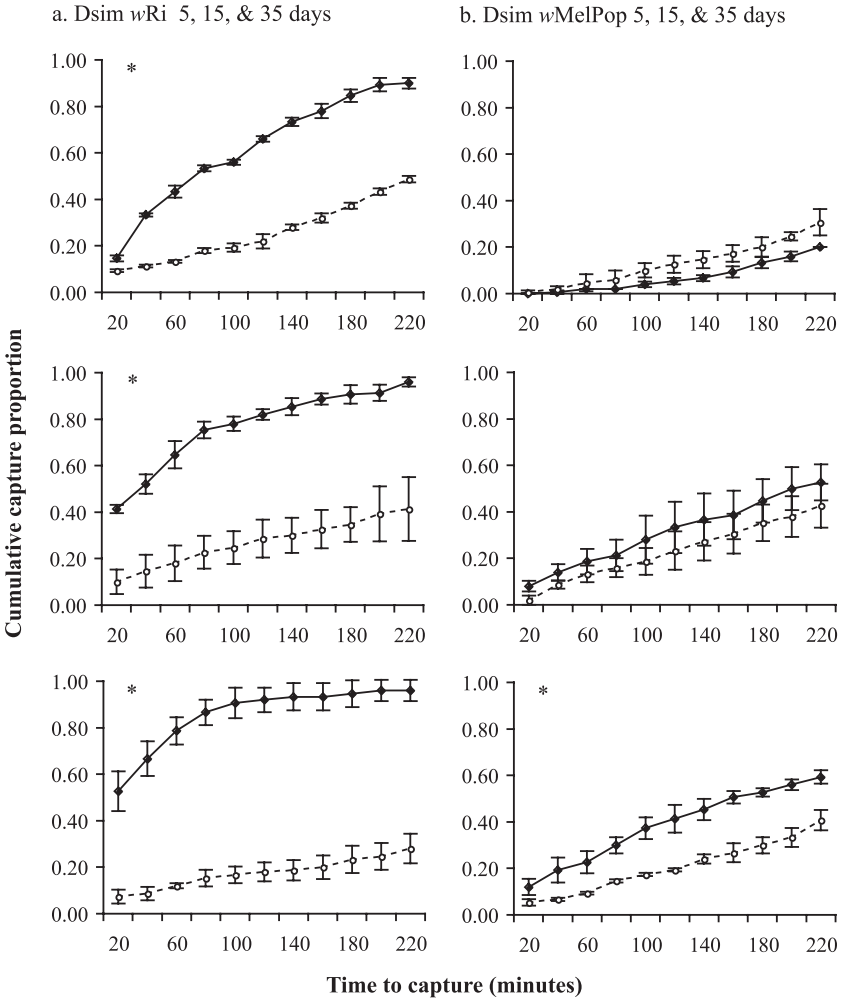

FIG. 3. Cumulative mean proportion of *D. simulans* flies captured ± SEM per 20-min period in baited traps. Means represent three replicate assay dates, each based on the performance of 50 individual flies. Infected (solid lines) and uninfected (dashed lines) flies were tested at each of three adult ages (5, 15, and 35 days). Significant differences between performances of infected and uninfected flies for each adult age were determined by the log-rank test. *, $P \leq 0.01$.

with increasing host age. In *D. simulans*, the *w*Ri strain enhances host performance. This increase includes a component of heightened baseline activity as well as greater responsiveness to food cues. The *w*MelPop strain has no effect in young flies of this species but improves host performance in old age.

While the direction and magnitude of *Wolbachia*'s effects on responsiveness vary quite substantially between the two host species, such differences are not without precedent in these associations. *D. simulans* and *D. melanogaster* have already been shown to exhibit different levels of CI expression (23) and to harbor quite specific *Wolbachia* infection densities (22). Direct relationships between whole-insect *Wolbachia* levels and various infection-induced phenotypes, while satisfying in their simplicity, seem increasingly inadequate (3, 4, 25, 26, 36). Pilot measurements of bacterial density on whole flies did not reveal clear relationships with capture performance (unpublished data) for any of the host-strain combinations. Also, the capture performance of *w*MelPop-infected *D. melanogaster* flies did not worsen with age as predicted with the overreplication model (24). The traditionally higher densities of *Wolbachia* bacteria associated with *D. simulans* infections (22), if beneficial in terms of capture performance, could explain the trends for *w*Ri and possibly for older flies infected with *w*MelPop. Targeted examination of densities in relevant tissues for the different species may reveal a more complex set of density models that are in fact predictive of host phenotypes.

The decreased responsiveness of *D. melanogaster* flies infected with *Wolbachia* could be due to greater energetic demands incurred by harboring the infection. A "cost" of *Wolbachia* has been documented for a few non-*Drosophila* species (5, 8, 9, 31). Sequencing of the *w*Mel genome revealed that the microbe is well suited to direct uptake of amino acids from its environment and is incapable of synthesizing a number of metabolic intermediates (40). Both point to specific examples where the microbe is utilizing host resources. *D. melanogaster* may cope with such demands by limiting physical activity. Alternatively, the presence of *Wolbachia* in nervous or muscle tissue could have direct effects on the functioning of olfaction or locomotion (7, 24). The latter scenario could also explain the results for *D. simulans* if the two hosts possess different

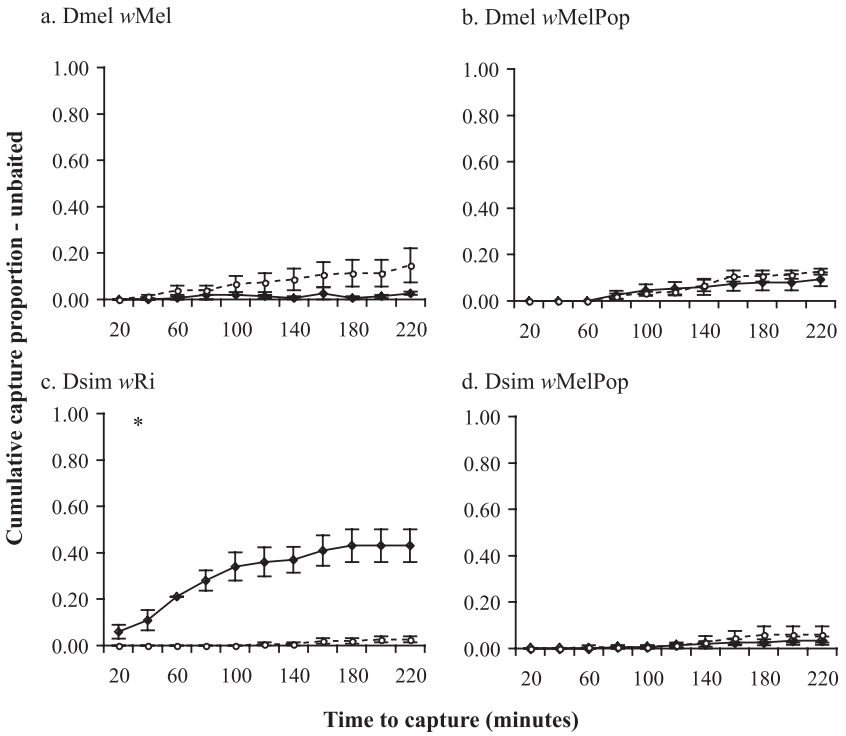

FIG. 4. Cumulative mean proportion of *D. melanogaster* and *D. simulans* flies captured ± SEM per 20-min period in unbaited traps. Means represent three replicate assay dates, each based on 50 individual flies. Infected (solid lines) and uninfected (dashed lines) flies were tested at a single adult age (15 days). Significant differences between performances of infected and uninfected flies were determined by the log-rank test. *, $P \leq 0.01$.

patterns of *Wolbachia* tissue tropism. Previous work with *Drosophila* has demonstrated that damage to the mushroom bodies can actually increase the insect's activity level (20). Heightened activity in *D. simulans* could also reflect a species-specific response to meet greater infection-associated energetic demands by seeking food more often.

The comparative effects of *w*MelPop on the different *Drosophila* species are of particular interest given current strategies being developed to use the strain to shorten the life span of the dengue fever vector *Aedes aegypti* (2, 32, 33). Surprisingly, the effects of *w*MelPop on *D. melanogaster* were no different from those of *w*Mel. Additionally, *w*MelPop-transinfected *D. simulans* flies performed no differently from uninfected counterparts until 35 days of age. The *w*MelPop strain still confers shortened life spans in both of these host species (unpublished data) and hence confers some level of virulence. Since the popcorn effect is temperature dependent (24), however, *w*Melpop-induced behavioral changes should also be examined with higher insect-rearing temperatures. For transinfected mosquitoes, understanding the effects of *w*MelPop on insect locomotion and olfaction will take on particular importance with respect to vertebrate host seeking/blood feeding.

We do not wish to overinterpret the meaning of our results for insects in the field. The magnitude and repeatability of the capture performance phenotypes, at the very least, however, provide strong evidence for *Wolbachia*'s ability to alter complex phenotypes beyond reproduction in *Drosophila*. The findings highlight a need to more closely examine *Wolbachia*'s role in an expanded set of host behaviors, under both laboratory and field conditions, and to try to develop working mechanistic models for *Wolbachia*'s local impact on host tissues and physiological processes.

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