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Increased locomotor activity and metabolism of *Aedes aegypti* infected with a life-shortening strain of *Wolbachia pipientis*

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SUMMARY

A virulent strain of the obligate intracellular bacterium *Wolbachia pipientis* that shortens insect lifespan has recently been transinfected into the primary mosquito vector of dengue virus, *Aedes aegypti* L. The microbe's ability to shorten lifespan and spread through host populations under the action of cytoplasmic incompatibility means it has the potential to be used as a biocontrol agent to reduce dengue virus transmission. *Wolbachia* is present in many host tissues and may have local effects on diverse biological processes. In other insects, *Wolbachia* infections have been shown to alter locomotor activity and response time to food cues. In mosquitoes, locomotor performance relates to the location of mates, human hosts, resting sites and oviposition sites. We have therefore examined the effect of the virulent, life-shortening *Wolbachia* strain *w*MelPop on the locomotion of *Ae. aegypti* as they age and as the pathogenicity of the infection increases. In parallel experiments we also examined CO_2 production as a proxy for metabolic rate, to investigate a potential mechanistic explanation for any changes in locomotion. Contrary to expectation, we found that the infection increased activity and metabolic rate and that these effects were relatively consistent over the insect's lifespan. The results do not fit a standard model of bacterial pathogenesis in insects, and instead may reveal additional physiological changes induced by infection, such as either increased hunger or defects in the nervous system.

Key words: *Aedes aegypti*, *Wolbachia pipientis*, locomotor activity, metabolic rate, insect.

INTRODUCTION

Wolbachia is a common bacterial endosymbiont of insects that infects both germ line and somatic tissues (Dobson et al., 1999; Ijichi et al., 2002). Because the microbe is transmitted maternally through the egg, both somatic tissue infection in females and the infection of males are effectively a dead end for the microbe. While most research has understandably focused on the ability of *Wolbachia* to manipulate host reproduction, there is growing evidence that the infection may have additional consequences for hosts. For example, in the parasitoid wasp *Leptopilina heterotoma* (Fleury et al., 2000), *Wolbachia* decreases locomotor activity, whereas in *Drosophila* it can either increase or decrease activity, depending on the host species and the infecting *Wolbachia* strain (Peng et al., 2008). The underlying causes of these infection-induced effects are not known, but may include *Wolbachia*-induced pathogenesis, effects on local cellular and tissue function, and/or changes in energetic demands.

The *Wolbachia* strain *w*MelPop, first identified in *Drosophila melanogaster*, shortens adult lifespan (Min and Benzer, 1997). Unlike most other *Wolbachia* infections, *w*MelPop acts more like a traditional bacterial pathogen than an intracellular symbiont. Insects infected with *w*MelPop survive roughly half as long as uninfected counterparts and premature death is thought to be caused by bacterial over-replication leading to local cell rupture and tissue damage (McGraw et al., 2002; McMeniman et al., 2008; Min and Benzer, 1997; Reynolds et al., 2003). Recently, the mosquito disease vector *Aedes aegypti* was artificially infected with this strain of *Wolbachia* as the first step in developing a biocontrol strategy. The goal of the strategy is to shift mosquito population age structure such that it leads to a reduction in human pathogen transmission (Brownstein et al., 2003; McMeniman et al., 2009). This approach takes advantage of the extrinsic incubation period (EIP) or the delay in time between when an insect consumes a pathogen-infected blood meal and when it can actively transmit the agent in subsequent feeding. This time window varies depending on the host–pathogen association as dictated by developmental constraints of the pathogen and/or the process of pathogen migration from the gut to the salivary glands (Brownstein et al., 2003). The result of this EIP is that only older individuals in vector populations transmit disease. As such, premature insect death caused by *Wolbachia* infection has the potential to significantly reduce the transmission of insect-transmitted pathogens like dengue viruses.

The potential success of a *w*MelPop-based biocontrol strategy hinges upon a range of other factors in the insect–microbe association. Firstly, as for all infectious agent or genetic modification strategies, the altered insects must be competitive in the field when compared with wild counterparts. This requirement is buffered somewhat by the action of cytoplasmic incompatibility (CI), a reproductive manipulation caused by *Wolbachia*. The expression of CI is predicted to aid in the spread of *Wolbachia* even when the infection confers moderate reductions in host fitness (O'Neill et al., 1997; Turelli, 1994). Secondly, the introduced symbiont must not inadvertently enhance the transmission efficiency of vectored disease agents. Simply reducing the number of old age individuals

in the population is not sufficient if the infection simultaneously improves mosquito vector competence. In *Ae. aegypti*, *w*MelPop has already been shown to cause life shortening and strong CI (McMeniman et al., 2009). The further progression of this biocontrol strategy, however, requires a broader understanding of the microbe's effects on host biology that might impact on fitness and vectorial capacity.

In mosquitoes, locomotor activity underpins the activities of locating mates, suitable hosts for feeding, resting places for blood meal digestion and finally oviposition sites. Changes in these behaviours could substantially affect both the transinfected mosquito's competitiveness in the field and its capacity to transmit disease. Here we report the results of a study aimed at determining whether *w*MelPop alters the locomotor activity of *Ae. aegypti*. Behavioural observations were made over three adult mosquito ages in an attempt to capture the progression of *w*MelPop pathogenesis in the host. We also measured, in parallel experiments, the carbon dioxide production of the mosquitoes to examine the effect of *Wolbachia* infection on metabolic rate. Our expectation was that the infected mosquitoes would demonstrate decreased activity and a corresponding decrease in metabolic rate, due to energetic drain or bacterial pathogenicity. Both trends were expected to intensify as the insect aged and *Wolbachia* pathogenesis advanced.

MATERIALS AND METHODS

Experimental organisms

The *w*MelPop-infected *Ae. aegypti* line (PGYP1) used in this study was generated as previously described (McMeniman et al., 2009). In brief, the *Wolbachia* strain *w*MelPop, native to *Drosophila melanogaster* (Min and Benzer, 1997), was transferred into *Ae. aegypti* by embryonic microinjection. Descendants of this isofemale line were outcrossed for several generations to the original recipient line of mosquitoes and selected for stable infection before closing the colony. At generations 8 and 9 post-transinfection, an aposymbiotic control line was created by antibiotic treatment of the *Wolbachia*-infected line (McMeniman et al., 2009). All experiments reported here were carried out on mosquitoes at generations 14–16 post-transinfection (i.e. 4–6 generations post-treatment), with replicates representing different generations. Mosquitoes were reared under standard conditions (25°C, 12 h:12 h L:D, 80% relative humidity, RH) (Gerberg et al., 1994). Larvae were reared in plastic trays at a density of 150 per 3 l of water and supplied with a daily dose of 0.15 g TetraMin aquarium fish food (Tetra, Melle, Germany). Adults were separated by sex and maintained as virgins in cages (30 cm×30 cm×30 cm) of ~150 individuals. Adults were supplied with a basic diet of 10% sucrose solution administered through cotton pledgets. The adult ages of 3, 15 and 25 days were selected to represent the periods when 100%, ~90% and ~20% of the *w*MelPop-infected population were still surviving, respectively (McMeniman et al., 2009).

Video recording of mosquito locomotion

Our locomotor assay was based on several previously published models (Allemand et al., 1994; Bonatz et al., 1987; Grobbelaar et al., 1967; Kawada and Takagi, 2004; Liseichikov and Zakharevskii, 1978; Mankin, 1994; Reynolds and Riley, 2002; Rowley et al., 1987; Sbalzarini and Koumoutsakos, 2005), but was most heavily influenced by Williams and Kokkinn (Williams and Kokkinn, 2005). Mosquitoes were placed in an observation chamber (Fig. 1) during experiments and their motion captured *via* a video camera. The observation chamber (Fig. 1) was constructed using white (sides and back) and transparent Perspex (front pane) and contained distinct cells that allowed for the simultaneous observation of 10 individual mosquitoes, one per cell. Mosquitoes were provided with 10% sucrose solution *ad libitum* during observation periods, dispensed through dental cotton wicks (1×Ø0.5 cm). The wicks placed in each observation cell also provided constant humidity (80–85% RH). Mosquitoes were transferred from rearing cages to observation chambers 20 min prior to recording of activity to allow them to adapt to the new environment. Recording began daily at 14:30 h, was paused during the hours of darkness (21:00–07:00 h) and was completed at 12:30 h the following day to allow time to transfer in the next set of mosquitoes. After each observation period mosquitoes were aspirated out of the chamber and killed. The chambers were cleaned with ethanol (80%) and the food supply replaced prior to subsequent observation periods. No mosquito mortality was observed during the observations. A total of three replicates each of 10 mosquitoes were studied per sex×strain×age per study chamber.

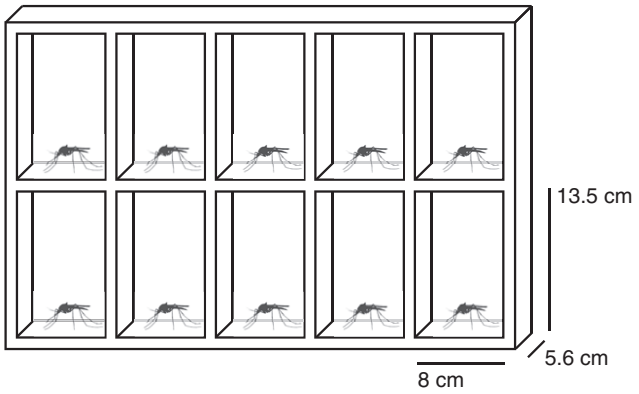

Fig. 1. Mosquito observation chamber with 10 individual cells constructed with white opaque plastic. Cells are covered with a sliding door of transparent plastic for videography.

A two-colour camera (DR2-13S2m/C-CS, Point Grey Research, Vancouver, BC, Canada) was fitted with a CCTV lens (12VM412ASIR, Tamron, Commack, NY, USA) and fixed on a mounting bracket 110 cm from the chamber. The distance of the camera to the object, the zoom, and the focus and iris aperture was optimized to reduce barrelling and distortion of images. A flat source light was placed 10 cm behind the chamber, which provided sufficient lighting for the camera sensor to capture high quality images but did not increase ambient temperatures. The light source power switch was synchronized with the room lights using a timer. The entire experimental setup was enclosed in cardboard to minimize intrusion of additional stimuli.

The file format used for recording, Audio Video Interleave (AVI), is limited to a maximum size of 2 GB, which amounted to approximately 8 min of video footage. To obtain a continuous video recording, we developed a program called Mossiecap in Matlab (Mathworks, Inc., Natick, MA, USA) that recorded multiple sequential 1.5 GB AVI files. This file size captured 6 min of video (i.e. 10 files=60 min) at 12 frames s^{-1}. Each day's footage (~420 GB) was recorded onto an external hard drive connected to a desktop computer. The contents of each hard drive were then transferred to the hierarchical storage management (HSM) system at The University of Queensland. Video files stored on the HSM were then evenly distributed to local disks on 20 workstations located in the Visualization and Advanced Computing (ViSAC) laboratories at The

University of Queensland. Mossiefly, a custom-designed program developed in Matlab was used to process videos for motion detection and tracking. This program detected and tracked movement (walking and flying separately) of individual mosquitoes and digitized the coordinates and time for each movement. The files containing data from movement detection were then analysed using Mossiestat, a program developed in Matlab that summarized the movement data captured with Mossiefly into numerical values used for statistical analysis. A total measure of activity (summation of time spent flying and walking) reported per hour was used for all subsequent statistical analysis as it was more informative than examining the variables independently.

Metabolic rate

Closed-system respirometry was used to measure CO_2 production ($\dot{V}_{CO_2}$) in the mosquitoes. CO_2 production has been shown extensively to be an accurate measure of metabolism for small and highly aerobic organisms such as insects (Lighton, 1991; Lighton and Duncan, 2002; Van Voorhies et al., 2004). Our experiment was designed to determine whether metabolic rate was significantly different between *w*MelPop-infected and uninfected mosquitoes in each of two daytime intervals lasting 4 h. Fifteen individual mosquitoes were measured for each sex×strain×age×interval combination. These measurements were replicated 3 times. Mosquitoes were discarded after the recording interval and replaced with fresh mosquitoes from the same rearing cage.

An ADInstruments (Sydney, Australia) gas analyzer (ML205) and a PowerLab (85P) analog-to-digital converter connected to a computer running data acquisition software (ADInstruments, Chart 5) were used to measure CO_2 production from mosquitoes. Before each experiment, the gas analyser was calibrated with gas of a known CO_2 content. Individual mosquitoes were loaded into 25 ml syringes, mounted with a three-way valve stopcock. Before the three-way valve was closed the syringe was carefully flushed with room air to remove possible CO_2 traces. Immediately after the 15 syringes were closed, a separate syringe was filled with air and kept as a control sample for initial room air CO_2 concentration. After the 4 h interval, the syringes were injected into the gas analyser at 2 ml s^{-1} until 5 ml of air remained. The gas concentrations for each mosquito were used to calculate mosquito metabolic rate. The dry mass of each mosquito was obtained after freezing them for 48 h at –20°C and desiccating the tissue in a dry vacuum pump. Dry mass was measured with an electronic balance (Sartorius bp211D; Goettingen, Germany) to the nearest 0.01 mg. Mosquitoes were not weighed before metabolic rate experiments because immobilization methods (i.e. CO_2 asphyxiation) may alter metabolic rate.

The following formulas based on those of Bartholomew and colleagues (Bartholomew et al., 1985) were used for calculation of metabolic rate (ml CO_2 h^{-1}):

$$\dot{V}_{CO_2} = V_a V_b t^{-1}\,, \quad (1)$$

where V_a is the increase in the volume of carbon dioxide in the samples (calculated from the difference between final and initial CO_2 fractional concentration), V_b is the effective volume in the syringe (25 ml minus the mosquito volume, estimated as 1.01×body mass), and t is the elapsed time in hours. Due to variation in mass between males and females, mosquito metabolic rate (MR; ml CO_2 h^{-1}) was allometrically scaled using the following formula based on Fuery et al. (Fuery et al., 1998):

$$\text{Scaled MR} = [(\overline{M}/M)^{0.75}]\dot{V}_{CO_2}\,, \quad (2)$$

where $\overline{M}$ is the mean mass of male and female mosquitoes used for each of the metabolic experiments, and M is the mass of individual mosquitoes. This formula assumes that CO_2 production is proportional to mass$^{0.75}$ (West et al., 2002).

Statistical analysis

Transformations (square root) of the activity measures and the scaled metabolic rate were necessary to generate normal distributions. General linear models were then constructed in Statistica Release 8 (StatSoft; www.statsoft.com) for each of the sexes separately to explore the effects of age, infection status, time of day and replicate on each of the activity and metabolic rate datasets separately. Student's *t*-tests were then employed to specifically test for differences in metabolic rate between infected and uninfected mosquitoes at each of the three ages.

RESULTS

Mosquito activity

On average, *Wolbachia*-infected individuals were more active during the day than their uninfected counterparts at each of the three adult ages examined (Fig. 2). Increases in activity were significant for both females (d.f.=1, F=54.8, P<0.0001) and males (d.f.=1, F=33.3, P<0.0001). Median increases in activity over the daytime period ranged from 1.0- to 2.5-fold higher for infected mosquitoes depending on the adult age. Age itself also played a role in mosquito activity (females: d.f.=2, F=20.7, P<0.0001; males: d.f.=2, F=13.1, P<0.0001). In general, both infected and uninfected, male and female, mosquitoes showed decreasing activity with age (Fig. 2). Only males, however, demonstrated a significant interaction between age and infection status (d.f.=2, F=5.1, P<0.01), where the increase in activity due to infection was enhanced with age (Fig. 2B,D,F).

Mosquito metabolic rate

Metabolic rate was measured for separate sets of mosquitoes during two daytime windows, 07:30–11:30 h and 11:30–15:30 h. The data from the two windows were combined after they were shown not to differ from one another using a general linear model (data not shown). In females (Fig. 3A), both infection status (d.f.=1, F=9.7, P=0.002) and age (d.f.=2, F=15.7, P<0.0001) were significant predictors of metabolic rate. On average infected females had higher metabolic rates than uninfected females, with young mosquitoes showing no difference and 15 day old mosquitoes showing the greatest increase (d.f.=58, t=2.6, P<0.01). Female mosquitoes, both infected and uninfected, were most active at 15 days of age (Fig. 3A). In males, infection played a much less consistent role in determining metabolic rate (Fig. 3B). Infection alone was not a factor (d.f.=1, F=0.81, P=0.36) in determining metabolic rate, while age was statistically significant (d.f.=2, F=15.7, P<0.0001). There was, however, a significant interaction between age and infection (d.f.=2, F=16.7, P<0.0001). This interaction can be seen between 15 and 25 day old males (Fig. 3B), where at 15 days of age infected males have higher metabolic rates (d.f.=55, t=4.1, P<0.001) and at 25 days of age they have lower rates (d.f.=58, t=–2.40, P<0.05).

DISCUSSION

This study has revealed that the *w*MelPop infection increases daytime activity of both female and male *Ae. aegypti* adults from 3 to 26 days of age. The work has also demonstrated *w*MelPop-associated increases in CO_2 production in females. Infected males shared this increase in metabolic rate at 15 days of age, but by 25 days exhibited declining rates of CO_2 production relative to uninfected controls. Here we examine four distinct mechanistic explanations

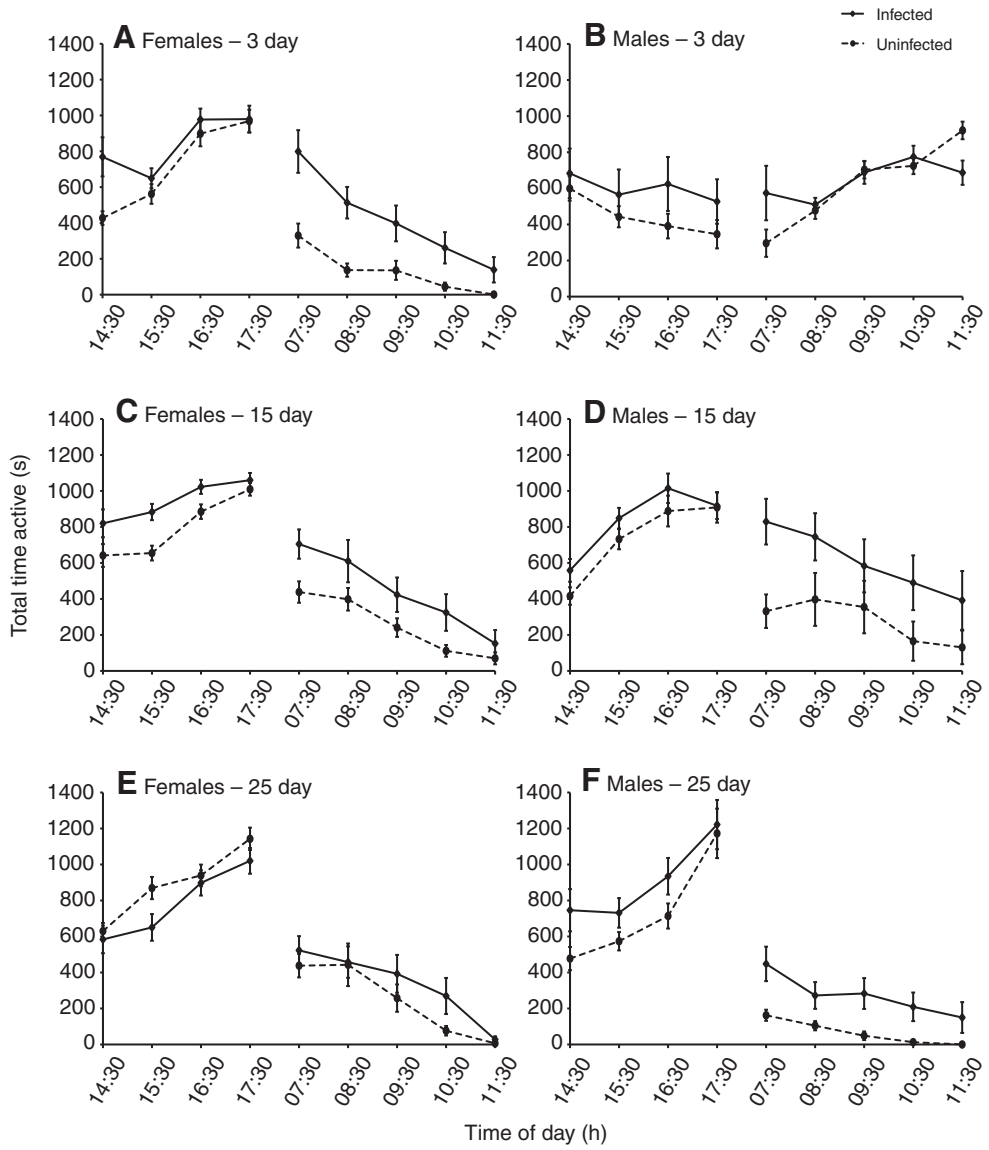

Fig. 2. Mean total time active (±s.e.m.) per 1 h window for infected and uninfected males and females at three adult ages. Times on *x*-axis denote the beginning of the hour-long session. Lights were turned on daily at 07:00 h and off at 19:00 h. Each point represents 10 mosquitoes×3 replicate recording days.

for the observed data including: possible artefacts of the study design, side effects of *w*MelPop pathogenicity, increased energetic demands due to infection, and infection-induced accelerated senescence.

The direct negative effects of antibiotic treatment on the insect and the role of genetic drift during breeding can potentially limit the capacity of uninfected lines to serve as true controls in studies like this one. Tetracycline treatment has been shown to cause reductions in the density of mitochondria in *Drosophila* that can last for several generations post-treatment (Ballard and Melvin, 2007). The mosquitoes in this study were therefore reared for 4–6 generations post-treatment prior to experimentation to minimize any such effects on the physiological phenotypes measured. The process of antibiotic treatment, aside from clearing insects of *Wolbachia*, has the added consequence of removing microbial gut flora. In mosquitoes, re-colonization of gut flora in the control line should not be a major issue given that the larval phase is subsequently reared in a microbial-rich aquatic environment parallel to that of the infected line. Lastly, experiments were conducted within 6 generations of antibiotic curing and at each generation the effective population size was kept large to minimize drift effects.

Our initial expectation was that *w*MelPop would act like a traditional bacterial pathogen. While little is known about the behavioural responses of mosquitoes with systemic bacterial infections, experiments in *Drosophila melanogaster* lend some predictions about infection and insect activity. In the case of *Streptococcus pneumoniae* and *Listeria monocytogenes*, infected flies exhibit altered circadian rhythms, but no real change in total activity in a day (Shirasu-Hiza et al., 2007). Infected flies exhibit more homogeneous patterns of activity, without pronounced peaks and troughs. Whether this change in activity is the direct result of bacterial virulence or an unintended result of the host immune response is still not known (Shirasu-Hiza and Schneider, 2007). This model, however, does not describe the behaviour of *w*MelPop-

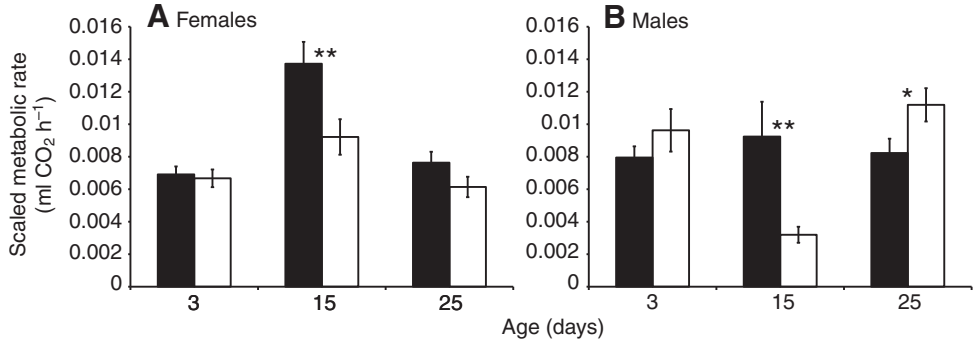

Fig. 3. Mean metabolic rate (±s.e.m.) based on two 4 h windows (07:30–11:30 h and 11:30–15:30 h) for infected (black bars) and uninfected (white bars) males and females at three adult ages. Each bar represents data from 15 mosquitoes×3 replicates×2 windows. *P<0.05, **P<0.01.

infected *Ae. aegypti*, as their activity patterns, while elevated, were largely parallel to those of uninfected mosquitoes. The *w*MelPop-infected mosquitoes did not appear to have altered circadian rhythms. Further examination of infected mosquitoes during the night-time are required to assess patterns of activity over a 24 h cycle.

Wolbachia infections are highly dispersed throughout host tissues, with their exact tissue distribution and density dependent on both the host and *Wolbachia* strain (Dobson et al., 1999; Ijichi et al., 2002). It is possible that *Wolbachia* infections in these diverse somatic tissues underlie the changes in activity or metabolism seen here. In the original characterization of *w*MelPop in *Drosophila melanogaster*, the bacteria were thought to over-replicate, most dramatically in nervous and muscle tissues (Min and Benzer, 1997). It is conceivable that local changes or damage in the cells of these tissues could be affecting higher-level physiological functions and behaviour. The effects we see could simply be the unintended consequence of somatic tissue infection.

The genome sequence of the *w*Mel strain revealed that, as expected for an endosymbiont, *Wolbachia* does not contain the complete set of metabolic pathways possessed by free-living bacteria (Wu et al., 2004). In particular it can utilize only a limited number of substrates and is able to synthesize very few metabolic intermediates. Considered an amino acid heterotroph, the microbe probably obtains most of its energy by importing amino acids directly from the host. *Wolbachia*'s drain on host resources, especially in the case of *w*MelPop where the infection titre is high, may lead to increased energy demands. The activity seen in the infected mosquito may simply reflect more frequent trips to the sugar water source present in the cells. Increases in such food-seeking activities could also drive increases in metabolism (Delthier, 1976), although this does not seem likely as peak patterns in activity and metabolic rate do not coincide (3 days *versus* 15 days of age, respectively). If infected mosquitoes are indeed hungrier, one would predict quantifiable increases in sugar water and blood meal consumption in the presence of infection.

The last explanation that encompasses the increases in both metabolic rate and activity is that *w*MelPop-infected mosquitoes are experiencing accelerated senescence, in effect living faster and dying younger. Tradeoffs between metabolic rate and lifespan have long been proposed in insects, but most evidence from *Drosophila* suggests such relationships do not exist (Hulbert et al., 2004; Van Voorhies et al., 2003; Van Voorhies et al., 2004). The shortened lifespan of *w*MelPop-infected *D. melanogaster* has always been attributed to local tissue death and destruction caused by bacterial over-replication, but the direct relationship between bacterial density and death may not be the same in *Ae. aegypti* (A. W. C. Fong, personal communication). Until the pathology of the *w*MelPop infection is better understood in *Ae. aegypti* it will not be clear whether the phenotypes of shortened lifespan and higher metabolic rate and activity are related. One challenge to dissecting these relationships is that, as *w*MelPop-infected individuals age and become sicker, it becomes increasingly difficult to partition the direct effects of *Wolbachia* on hosts from the generalized death process. Examining the pathology of *w*MelPop in middle-aged mosquitoes, well before the onset of death, may therefore be most informative.

The effect of the *w*MelPop infection on activity has now been measured in *D. melanogaster*, *D. simulans* (Peng et al., 2008) and *Ae. aegypti*. In *D. melanogaster*, *w*MelPop behaves like another native and benign infection, *w*Mel, in reducing host activity across the insect's lifespan. This suggests the activity changes are caused simply by the presence of *Wolbachia*, rather than by any density-based effects or by increasing pathogenesis near death. *D. simulans* artificially transinfected with *w*MelPop exhibits only marginal increases in activity in very old hosts, whereas the native infection *w*Ri has the capacity to vastly increase host activity at all ages studied (Peng et al., 2008). Interestingly, the *w*Ri strain also confers greater fecundity to its host (Weeks et al., 2007) and its spreading capacity in wild populations is well documented (Turelli and Hoffmann, 1991). These varying results in *D. simulans* indicate that the *Wolbachia* strain and possibly length of association may play a substantial role in determining activity. The capacity for such different host responses from within the *Drosophila* genus does not allow for the development of broadly generalizable models of *w*MelPop effects on behavior. An understanding of the unique effects of *w*MelPop on *Ae. aegypti* will begin in the first instance by characterizing its tissue distribution and density.

While understanding the mechanism underlying *Wolbachia*-induced changes in *Ae. aegypti* is of interest for advancement of the biocontrol strategy, determining whether differences in activity and metabolism substantially change mosquito food-, human host- and mate-seeking behaviours is more important. Each of these activities critically underpins insect fitness in the field and therefore could substantially affect the competitiveness of transinfected *Ae. aegypti* in a mixed population. In addition, changes to blood feeding or biting rate of *Ae. aegypti* caused by *w*MelPop infection may further decrease or increase mosquito vectorial capacity, which this *Wolbachia*-based strategy aims to reduce.

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