Wolbachia Influences the Production of Octopamine and Affects *Drosophila* Male Aggression

Chelsie E. Rohrscheib,^{a,b,c} Elizabeth Bondy,^{a,d} Peter Josh,^c Markus Riegler,^e Darryl Eyles,^c Bruno van Swinderen,^c Michael W. Weible II,^{a,b} Jeremy C. Brownlie^{a,f}

School of Natural Sciences, Griffith University, Nathan, Queensland, Australia^a; Eskitis Institute for Cell and Molecular Therapies, Griffith University, Nathan, Queensland, Australia^b; Queensland Brain Institute, University of Queensland, St. Lucia, Queensland, Australia^c; Department of Evolutionary Biology, University of Arizona, Tucson, Arizona, USA^d; Hawkesbury Institute for the Environment, University of Western Sydney, Penrith, New South Wales, Australia^e; Environmental Futures Research Institute, Griffith University, Nathan, Queensland, Australia^f

***Wolbachia* bacteria are endosymbionts that infect approximately 40% of all insect species and are best known for their ability to manipulate host reproductive systems. Though the effect *Wolbachia* infection has on somatic tissues is less well understood, when present in cells of the adult *Drosophila melanogaster* brain, *Wolbachia* exerts an influence over behaviors related to olfaction. Here, we show that a strain of *Wolbachia* influences male aggression in flies, which is critically important in mate competition. A specific strain of *Wolbachia* was observed to reduce the initiation of aggressive encounters in *Drosophila* males compared to the behavior of their uninfected controls. To determine how *Wolbachia* was able to alter aggressive behavior, we investigated the role of octopamine, a neurotransmitter known to influence male aggressive behavior in many insect species. Transcriptional analysis of the octopamine biosynthesis pathway revealed that two essential genes, the tyrosine decarboxylase and tyramine β -hydroxylase genes, were significantly downregulated in *Wolbachia*-infected flies. Quantitative chemical analysis also showed that total octopamine levels were significantly reduced in the adult heads.**

Male aggression is an important social behavior common throughout the animal kingdom. Aggressive behaviors are utilized to secure food, mates, and territory; these, in turn, ensure reproductive success and species propagation (1). An interplay between environmental influences, together with epigenetic and genetic factors (acting through neurotransmitters, hormones, and pheromones), shapes and influences behaviors in animals (2–6). Recently, evidence of the importance of microbial symbionts in directly manipulating host behavior, often by altering neurotransmitter levels in the brain (7–11), has changed the manner in which behavior is viewed. Inherited microbes exert such a strong effect that they, in addition to genetics/epigenetics and the surrounding environment, have been described as a third major determinant of behaviors (12).

Wolbachia bacteria are reproductive endosymbiotic alphaproteobacteria and are the most common intracellular bacteria on Earth, infecting filarial nematodes, arachnids, and at least 40% of all insect species, including the model insect, *Drosophila melanogaster* (13–16). *Wolbachia* bacteria are maternally transmitted and are best known for their ability to manipulate host sex determination or reproductive systems in order to promote germ line transmission (17, 18). *Wolbachia* bacteria have also been shown to influence host metabolic pathways (19–21), provide protection from pathogens (22, 23), influence host life span (24), and play a role in host speciation events (25).

Wolbachia infections have been correlated to changes in behaviors, including male mating frequencies (11), mate discrimination (26), and responses to olfactory cues (27–29). *Wolbachia* can also induce host selective mate avoidance, i.e., causing the host to avoid mates harboring another, incompatible symbiont variant, which could contribute to driving host speciation events (25). Taken together, these studies suggest that *Wolbachia* acts as a reproductive parasite of insects by subtly altering mating behaviors

that may in turn affect both the host and *Wolbachia*'s ability to flourish together in the wild.

A behavior that influences reproductive fitness is male aggression. Male *Drosophila* flies compete with one another to secure resources and territory as well as to acquire mates; thus, male aggression behavior is considered to be of ecological significance and influences male reproductive fitness (30, 31). While *Wolbachia* bacteria are transmitted through maternal and not paternal lines, male reproductive success is critical for the spread of *Wolbachia* into naive insect populations. The most commonly employed manipulation of reproductive systems is cytoplasmic incompatibility, which imposes a reproductive cost to *Wolbachia*-free females after they mate with a *Wolbachia*-infected male (32). Thus, male reproductive success is a key component of how *Wolbachia* invades insect populations. Evolutionary theory predicts that *Wolbachia* should impose relatively few, if any, detrimental effects upon male mating behaviors, including male aggression; yet all strains that infect *D. melanogaster* are known to infect neural tissue (33).

Here, we set out to determine if three different *Wolbachia* strains that infect *D. melanogaster* affect male aggression. We pre-

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Address correspondence to Jeremy C. Brownlie, j.brownlie@griffith.edu.au.

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dicted that *Wolbachia* strains commonly found in wild populations of *D. melanogaster* (34) would have no effect on male aggression, while a strain observed in only a limited number of lab stocks and known to reduce adult life span at restrictive temperatures (24) would modify male aggression. As the wMelPop-CLA strain has been shown to modulate biogenic amine neurotransmitter biosynthetic pathways (35), the production of the neurotransmitter octopamine (OCT), which is known to regulate male aggression in *Drosophila* (36), was analyzed using quantitative methods.

MATERIALS AND METHODS

Fly strains and rearing. The *Drosophila melanogaster* strain Canton-S (CS), a strain collected from Canton, OH, USA, in 1930 (37), was used for all experiments described and is naturally infected by the wMelCS strain, here referred to as the wMelCS fly strain. Two other *Wolbachia* strains, wMel, a benign strain, and wMelPop, a pathogenic strain (24, 38), were introgressed into the CS background. Briefly, virgin females from *D. melanogaster* yw^{67c23} infected with the wMel *Wolbachia* strain were mated to males of the CS line. Female offspring were then backcrossed to males of the CS line for a total of five generations, generating the wMel fly strain. Virgin females from *D. melanogaster* w¹¹¹⁸ infected with the wMelPop *Wolbachia* strain were mated to males of the CS line. Female offspring were then backcrossed to males of the CS line for a total of five generations, generating the wMelPop fly strain. Tetracycline treatments were performed as described previously (39) to generate genetically identical fly lines that lacked the *Wolbachia* infection. These lines are here referred to as wMelCS-T, wMel-T, and wMelPop-T. Gut flora was reconstituted from the *Wolbachia*-infected parental line using standardized methods (40). All experiments were conducted for a minimum of seven generations post-tetracycline treatment.

Drosophila stocks were maintained in bottles on standard food medium consisting of 1.6% yeast, 6.8% polenta, 0.8% agar, 1.5% sugar, 0.5% propionic acid, and 0.5% (wt/vol) tegosept (41). Adult flies were maintained at 24°C on a 12:12-h light-dark cycle. Bottle populations were maintained at low densities (~100 flies per bottle) to minimize the effects of overcrowding.

Male aggression. Aggression of adult *Drosophila* males was determined using a standardized aggression protocol (42). All fighting pairs had the same *Wolbachia* infection status, i.e., between infected or uninfected pairs. Studies have found that socially naive flies are more aggressive than individuals exposed to other flies (43); therefore, male pupae were isolated before eclosion. Twenty pairs of wMelCS-, wMel-, and wMelPop-infected males and their genetically paired *Wolbachia*-free lines ($n = 20$; 40 males per fly line, for a total of 240 males) were collected as pupae 1 day prior to eclosion and isolated in individual vials containing standard food medium for the remainder of their rearing period. Adult males were maintained at 24°C until 4 days of age. At day 4, individual flies were transferred to plastic Pasteur pipettes for 15 h of fasting with water. A pair of 5-day-old flies, the optimal age for observing aggression behavior (42, 44), was matched by size and placed into a fighting arena for filming.

Petri dishes (55 mm by 15 mm) were divided into top and bottom halves of the fighting arena. Small holes were drilled into the bottom to allow fly insertion, and the insides of the dishes were coated three times with clear Fluon (HerpTrader, St. Ardeer, Victoria, Australia) to prevent the flies from climbing up the sides. Eppendorf vial caps were removed, and vials were filled with standard food medium and placed in the center of the arena, acting as a food territory for competition between males. The top and bottom of the dish were attached with Parafilm. A small square of Parafilm was cut to fit over the entry hole in the bottom dish.

Flies are most active in the morning and therefore were only analyzed 1 to 2 h after first light. Flies were transferred from the starvation pipettes into the entry hole in the bottom petri dish of the fighting arena. Once all males were transferred into fighting arenas, 10 min of acclimation time

TABLE 1 Primer sequences^a

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
<i>Act88F</i>	ATCGAGCACGGCATCATCAC	CACGCGCAGCTCGTTGTA
<i>dTdc2</i>	ACGCATTGGCAGCATCCTC	TGGCAGCAAGCATCGTGAC
<i>Tβh</i>	ATACCCGAAACCGAGTTGG	GACACCAGTACGTGGTCTC

^a Primer sequences are from reference 86.

was allowed prior to analysis. The fighting chambers were filmed by a high-definition (HD) Sony Handycam (Sony Corporation) from above for 20 min in a well-lit incubator set at 24°C to reduce environmental disturbances. For each experiment both *Wolbachia*-infected and -uninfected pairs were placed randomly into the incubator to minimize position effects within the incubator.

All videos were analyzed manually with iMovie (Apple, Inc., CA, USA). An aggressive encounter was counted when the fighting pairs were at most one fly body length apart and actively engaged in aggressive, previously described offensive encounters (42). Overall male aggression was determined by three behaviors: latency to first aggressive encounter, total number of aggressive encounters, and the average length of fight bouts. For normalization of data, control values were set to 1, and the genetically paired *Wolbachia*-infected lines were then adjusted by the same ratio as that performed for the controls. Averages of aggression metrics were compared using Student's *t* test (SPSS). Locomotion behavior (fly velocity) for each fly line was determined using the Caltech Automated *Drosophila* Aggression-Courtship Behavioral Repertoire Analysis program (CADABRA) to analyze the same 20-min videos that were manually analyzed for aggressive behaviors (45). As variance was equivalent, mean velocity (mm/s) over the 20-min period was compared using Student's *t* test (SPSS).

HPLC. Total octopamine concentrations in 5-day-old male fly heads were measured using a standard high-performance liquid chromatography (HPLC) protocol (46). Five-day-old *Drosophila* males were snap frozen in liquid nitrogen, dissected on dry ice, and pooled into groups of 10 for HPLC analysis. A total of 20 flies were analyzed for each *Drosophila* strain. Heads were placed into 100 ml of deoxyepinephrine (DE) solution and homogenized via sonication (3 to 8 s), followed by centrifugation at 13,000 rpm for 5 min at 4°C. Samples were then syringe filtered, and a fixed volume of 20 µl of the resultant supernatant was injected directly into the chromatographic system. The system consisted of an autosampler and an isocratic HPLC pump (model 1100; Agilent Technologies, Inc., CA), coupled to a Sunfire C₁₈ column (4.6 mm by 150 mm; 5-µm particle size; Waters Corporation, MA) and a Coulochem III electrochemical detector (ESA Laboratories, Inc., MA), with a flow rate of 1.2 ml per min. Electrochemical detection was obtained with the working electrode set to a potential of +600 mV relative to the Ag/AgCl reference electrode. The mobile phase was a citrate buffer made with deionized water from a Millipore system (Millipore, MA, USA). It consisted of 25 mM sodium dihydrogen phosphate, anhydrous, 50 mM citric acid monohydrate, 1.4 mM octane sulfonic acid, and 1 mM EDTA. The pH was adjusted to 4.22 using sodium hydroxide and 6% (vol/vol) acetonitrile. The solution was degassed by vacuum filtration through a 0.22-µm-pore-size cellulose membrane. All neurotransmitter standards were prepared daily using 0.1 M perchloric acid. Data were quantified with ChemStation software (Agilent Technologies) by calculating peak height ratios of each compound relative to the internal standard DE and corrected for dilution. Compound identity was determined by retention time, and the final amount of neurotransmitter is reported as picograms per head. Averages of total OCT concentrations were compared using Student's *t* test (SPSS).

Gene expression. A quantitative reverse transcriptase PCR (qRT-PCR) assay was used to determine if *Wolbachia* influenced transcription of the tyrosine decarboxylase 2 (*dTdc2*) and tyramine β-hydroxylase (*Tβh*) genes that encode the OCT biosynthetic pathway relative to that of the housekeeping gene *Act88F* (Table 1). *Act88F* was determined to be stably expressed across all experimental conditions (data not shown). A

total of 25 male flies were assayed at 5 days of age. Flies were collected by CO₂ anesthesia, snap frozen in liquid nitrogen, and stored at -80°C until dissection. Five pools of 5 fly heads (25 heads total) were dissected in petri dishes on dry ice and placed into a 1.5-ml Eppendorf tube. Total RNA was purified using TRIzol according to the manufacturer's protocols (Invitrogen Corp., Carlsbad, CA), immediately after dissection. Total RNA was treated with 3 μl of DNase I (Roche) for 30 min at 37°C in a 30- μl reaction volume to eliminate genomic DNA. Approximately 0.5 μg of total RNA was reverse transcribed using random primers (Invitrogen, Mulgrave, Victoria, Australia) and reverse transcriptase (Invitrogen, Mulgrave, Victoria, Australia) according to the manufacturer's protocols. Gene expression was estimated with two technical replicates using a standard quantitative PCR (qPCR) assay (47). Each qPCR mixture contained 12.5 μl of 2 $\times$ SYBR premix (Qiagen), 1 μl of forward primer, 1 μl of reverse primer, 100 ng of DNA, and H₂O to a final volume of 25 μl . The expression of two genes was estimated relative to *Act88F* (Table 1) using the $\Delta\Delta C_T$ (where C_T is threshold cycle) method (48). Averages of expression were compared using Student's *t* test (SPSS).

RESULTS

Male aggression. Using a standardized chamber for measuring fly aggression, we assessed the impact that *Wolbachia* infection has on male *D. melanogaster* aggression, using three established parameters (1, 42): (i) the latency, or length of time, it took to initiate the first aggressive encounter, (ii) the total number of aggressive encounters, and (iii) the average length of an individual fighting bout (Fig. 1). A total of six fly lines were used in this study, all derived from the Canton-S background. Three fly lines were infected by one of three *Wolbachia* strains (*wMel*, *wMelCS*, or *wMelPop*). Three genetically paired fly lines that were cured of their *Wolbachia* infection following the application of tetracycline were independently generated (*wMel-T*, *wMelCS-T*, and *wMelPop-T*); all comparisons were made only between genetically paired lines. *wMelPop*-infected males demonstrated significantly reduced aggression compared to the behavior of the uninfected controls. Infected flies took, on average, three times longer to initiate their first aggressive encounter (Fig. 1A) [$F(19, 19) = 0.44$ and $P = 0.07$; *t* test, $P = 0.03$] and initiated approximately half the number of fight bouts within the period analyzed (Fig. 1B) [$F(19, 19) = 2.41$ and $P = 0.08$; *t* test, $P = 0.003$]. Once a fighting bout had commenced, however, *wMelPop*-infected males fought for the same length of time as controls (Fig. 1C) [$F(19, 19) = 0.42$ and $P = 0.08$; *t* test, $P = 0.75$]. Thus, we conclude that *wMelPop* reduced the frequency of aggressive encounters, but once fighting was initiated the duration of a bout was equivalent to that with uninfected male flies.

The two benign *Wolbachia* strains, *wMel* and *wMelCS*, had no effect on *Drosophila* male aggression, with male flies displaying aggressive behaviors similar to those of their genetically paired control lines. Latency to the first aggressive encounter in flies infected with *wMel* (Fig. 1A) [$F(19, 19) = 0.91$ and $P = 0.88$; *t* test, $P = 0.91$] and *wMelCS* (Fig. 1A) [$F(19, 19) = 0.65$ and $P = 0.32$; *t* test, $P = 0.86$] was equivalent to the level in controls. Similarly, the total number of fights initiated and the length of each bout (Fig. 1B and C, respectively) were equivalent between flies infected with *wMel* [for total encounters, $F(19, 19) = 0.90$ and $P = 0.85$; *t* test, $P = 0.11$; for bout length, $F(19, 19) = 0.73$ and $P = 0.55$; *t* test, $P = 0.71$] or *wMelCS* [for total encounters, $F(19, 19) = 0.65$ and $P = 0.40$; *t* test, $P = 0.87$; for bout length, $F(19, 19) = 0.84$ and $P = 0.75$; *t* test, $P = 0.18$] and their uninfected controls. Thus, we

FIG 1 Effect of *Wolbachia* on *D. melanogaster* male aggression. Graphs show three measurements of aggression: latency, number of fights initiated (total encounters), and the average length of an individual fighting bout. Values were normalized to those observed for control lines. Error bars represent standard errors of the means. Significance was determined by Student's *t* test (SPSS). *, $P < 0.05$; **, $P < 0.005$.

conclude that neither the initiation nor the duration of male aggressive behavior was influenced by *wMel* or *wMelCS*.

Baseline locomotor activity. Changes to general locomotor behavior could influence aggressive behaviors in *Drosophila*. Previous studies had shown no effect on locomotor behavior in *Wolbachia*-infected flies (28), while our behavioral analyses identified no difference in the average lengths of fighting bouts. To further analyze the locomotor performance of our fly lines, we used CADABRA software to analyze recorded aggressive encounters and to determine the average velocity of adult male flies (45). No significant effects on velocity or speed were observed for male flies infected with *wMelPop* [$F(39, 39) = 1.67$ and $P = 0.11$; *t* test,

FIG 2 Effect of *Wolbachia* on locomotor behavior in *D. melanogaster*. Graphs show average velocity of *Drosophila* males in fighting arenas over a 20-min period normalized to that observed for control lines. Error bars represent standard errors of the means. Significance was determined by Student's *t* test (SPSS).

$P = 0.93$], wMel [$F(39, 39) = 0.86$ and $P = 0.63$; *t* test, $P = 0.11$], or wMelCS [$F(39, 39) = 0.90$ and $P = 0.34$; *t* test, $P = 0.12$] compared to levels in controls (Fig. 2). Thus, we conclude that the observed increased latency to initiate a fight in wMelPop-infected flies was not an accidental consequence of reduced locomotion, lethargy, or inactivity associated with infection by *Wolbachia*.

Total octopamine levels in the adult head. A *Wolbachia* strain has been shown previously to disrupt the biosynthesis of insect neurotransmitters (35). *Drosophila* male aggression is highly influenced by the monoamine neurotransmitter octopamine (43). Alterations to OCT have been shown to reduce aggressive latency in male *Drosophila* (49). Using HPLC, total OCT levels (pg/head) were determined in 5-day-old *Wolbachia*-infected *Drosophila* flies and compared to levels in their genetically paired *Wolbachia*-free controls (Fig. 3). Total OCT levels in wMelPop-infected flies were half those of the controls [$F(9, 9) = 0.38$ and $P = 0.16$; *t* test, $P = 0.04$]. As expected, where no change to aggressive behavior was observed, no difference in total OCT levels was observed in the heads of flies infected with wMel [$F(9, 9) = 0.56$ and $P = 0.40$; *t* test, $P = 0.90$] or wMelCS [$F(9, 9) = 0.47$ and $P = 0.28$; *t* test, $P = 0.91$] compared to levels in the controls.

FIG 3 Total head octopamine in *Wolbachia*-infected *D. melanogaster*. Total octopamine levels were determined by HPLC analysis (pg/head) and normalized to those observed for control lines. Error bars represent standard errors of the means. Significance was determined by Student's *t* test (SPSS). *, $P < 0.05$.

FIG 4 Response of octopamine biosynthesis pathway to *Wolbachia* infection. RT-qPCR analysis of mRNA expression of the octopamine biosynthetic pathway in *Drosophila* lines was performed. Graphs show average gene expression of *dTdc2* (A) or *Tβh* (B) relative to the *Act88F* ratio normalized to that observed in the control fly lines. Error bars represent standard errors of the means. Significance was determined by Student's *t* test (SPSS). *, $P < 0.05$.

Transcriptional profiling of the octopamine biosynthetic pathway. To determine how wMelPop reduced neural OCT and thus increased aggressive latency in adult flies, we examined the expression of two genes that encode the rate-limiting enzymes within the OCT biosynthesis pathway using a quantitative reverse transcriptase PCR assay (qRT-PCR) (Fig. 4). Expression of *dTdc2*, which is primarily expressed in the brain (50) and converts tyrosine to tyramine (TA), was decreased by half in wMelPop-infected flies compared to levels in the controls (Fig. 4A) [$F(4, 4) = 0.60$ and $P = 0.21$; *t* test, $P = 0.006$]. Additionally, the expression of *Tβh*, which converts TA to OCT, was also decreased by half (Fig. 4B) [$F(4, 4) = 0.36$ and $P = 0.35$; *t* test, $P = 0.002$]. As expected, no difference in expression levels was observed for the OCT biosynthetic pathway in flies infected with wMel (Fig. 4A and B) [for *dTdc2*, $F(4, 4) = 0.95$ and $P = 0.94$; *t* test, $P = 0.32$; for *Tβh*, $F(4, 4) = 1.23$ and $P = 0.84$; *t* test, $P = 0.33$] or wMelCS [for *dTdc2*, $F(4, 4) = 0.71$ and $P = 0.75$; *t* test, $P = 0.17$; for *Tβh*, $F(4, 4) = 0.87$ and $P = 0.90$; *t* test, $P = 0.16$] compared to levels in the controls. Taken together, our data demonstrate that wMelPop infection reduced expression of the OCT biosynthetic pathway, leading to a reduction in total OCT production, which was correlated with significantly increased aggressive latency behavior.

DISCUSSION

Previous studies have described how parasitic microbes that transiently infect their hosts manipulate host behavior in order to complete their life cycle (7, 8, 51–54); more recently, a growing body of evidence has shown that stably maintained symbionts can also influence animal behavior (54–58). This study provides evidence that a microbial symbiont, a strain of *Wolbachia* that infects *Drosophila melanogaster*, correlates with altered male aggressive behavior of its host. Infection of male flies with *wMelPop* led to increased latency specific to the initiation of aggressive encounters without significantly affecting other observed behaviors, including the length of time their fights lasted or general locomotor behavior. These observations suggest that *wMelPop* reduces a fly's motivation to enter an aggressive encounter without affecting its ability to fight once the encounter commences. The mechanism by which the initiation of aggressive encounters was reduced by *wMelPop* was correlated with reduced gene expression of a specific neurotransmitter biosynthetic pathway and subsequent production of a specific neurotransmitter.

Wolbachia has been shown to increase general locomotor activity in a parasitoid wasp (59) and in artificially transinfected *Aedes aegypti* mosquitoes (60). In *D. melanogaster*, however, *Wolbachia*-mediated changes to *Drosophila* olfactory-motivated locomotor activity are highly varied and are determined by the surrounding environment and host species although baseline locomotor behaviors are apparently not affected by infection (27, 28). As *wMelPop* is known to establish high infection densities and halves the adult life span of its host when flies are reared at 29°C (24), the observed reduction in aggressive behavior may be due to a general malaise associated with infection. As all experiments were conducted at 24°C, 5-day-old flies infected by *wMelPop* showed no reduction of adult life span, having survival rates equivalent to those of controls at the time of experimentation (survival of 5-day-old *wMelPop*- and *wMelPop*-T-infected flies reared at 24°C was 100%; $n = 120/\text{line}$ [data not shown]), nor was locomotor function affected (Fig. 2), consistent with other studies that had shown no effect of *wMelPop* on general locomotion function (28, 61). Thus, we conclude that the changes in male aggression associated with the *wMelPop* infection were not due to changes to general locomotor activity or possible higher bacterial loads but due, rather, to other points of interaction between *wMelPop* and its male host.

Octopamine (OCT) and octopaminergic neurons are the primary regulators of male aggression in *Drosophila* (6, 36, 62, 63), with reduced OCT levels correlating with reduced aggression (62). The production of OCT in the brain is controlled by two enzymes: tyrosine decarboxylase (*dTdc*), which converts L-tyrosine to tyramine (50), and tyramine- β hydroxylase (*T β h*) which converts tyramine to OCT (64). *Drosophila* infected with *wMelPop* displayed reduced expression of both the *dTdc2* and *T β h* genes, which correlated with reduced head OCT levels and reduced male aggression. Neither *wMel* nor *wMelCS* had a significant effect on the expression levels of *dTdc2* and *T β h* or on neural OCT levels, correlating with the observed behaviors.

Several studies have shown host gene expression to be modulated by the presence of *Wolbachia* (65–74). The microRNA pathway, an important transcriptional regulatory system in animals, including invertebrates, has been shown to be modified in some insect hosts by *wMelPop*-CLA (75–77), a strain that is more than

99% genetically identical to *wMelPop* (78). Thus, potentially, *wMelPop* may be modifying the microRNA pathways, resulting in suppressed transcription of the OCT biosynthetic pathway, reducing both OCT production and male aggression. Alternatively *wMelPop*, which expresses a functional type IV secretion system (38), could directly influence transcription via the secretion of effector molecules. Such processes have been observed in *Culex* mosquitoes, where *wPip* influenced mosquito gene expression via the secretion of *wtrM*, a putative transcriptional regulator (71).

A second, and not mutually exclusive, possibility is that changes to OCT production are due to the high infection density *wMelPop* establishes compared to low-density infections of strains such as *wMel* and *wMelCS* (24, 47, 78). The higher infection density may result in increased consumption of host metabolites, including amino acids upon which *Wolbachia* growth and maintenance are dependent (38, 79). Normally, tyrosine is a non-essential amino acid synthesized from the essential amino acid phenylalanine (80). Phenylalanine is both a metabolite consumed by *Wolbachia* (38, 79) and a precursor of several neurotransmitters, including OCT and dopamine (81). Previous studies have shown that phenylalanine deficiency results in decreased dopamine synthesis (82). Thus, there remains the possibility that localized competition between symbiont and host for amino acids, such as phenylalanine, results in an overall reduction in neural concentrations of neurotransmitters. Alternatively, the higher bacterial loads may result in localized damage to the brain. Previous studies have shown that when *wMelPop*-infected *Drosophila* flies were reared at 29°C, there was considerable damage to all somatic tissues, including the brain (24), and more recently localized overreplication and subcellular changes have been observed in flies reared at 25°C (83). While we conducted our experiments in the wild-type fly line Canton-S reared at 24°C and observed no reduction in adult life span associated with the *wMelPop* infection (survival of 5-day-old *wMelPop*- and *wMelPop*-T-infected flies reared at 24°C was 100%; $n = 120/\text{line}$ [data not shown]), we cannot eliminate the possibility that this behavioral phenotype is the result of *Wolbachia* changes to neurophysiology or localized damage to the brain, specifically octopaminergic neurons. Such changes could be detected through the use of colocalization studies to determine if *wMelPop* preferentially infects octopaminergic neurons and/or whether infection correlates with a reduction of those neurons.

Wolbachia bacteria are maternally transmitted from mother to offspring; however, for *Wolbachia* bacteria to increase their frequency within a population requires either an increase to reproductive output or success. By far the most common mechanism *Wolbachia* employs is cytoplasmic incompatibility, which is induced between *Wolbachia*-infected males and uninfected females only. Thus, this mechanism relies heavily on *Wolbachia* males being as reproductively successful as uninfected males. Studies have shown that *T β h2* knockout flies with reduced OCT and aggression had reduced mating success when they competed with wild-type flies (62). Given that *wMelPop* also reduced OCT levels and male aggression, if similar effects are observed in the wild, then *wMelPop*-infected males are likely to be less reproductively successful than uninfected flies or those infected by benign *Wolbachia* strains. This could explain why attempts to establish *wMelPop*-CLA, a strain derived from *wMelPop* (84), in wild populations of *Aedes aegypti* mosquitoes failed, yet *wMel* was successfully established (85).

Symbiotic microorganisms are often overlooked as a component of animal behavior and of the mechanisms that control these behaviors. We have shown that a common bacterial symbiont of *Drosophila* can modify male aggressive behavior by modifying biosynthetic pathways and reducing neural OCT levels. Given that previous work has shown that a strain of *Wolbachia* can influence dopamine in novel insect hosts (35), there remains the possibility that other behaviors, such as sleep, learning and memory, or attention are also modified and affected by *Wolbachia*.

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