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Wolbachia effects in *Drosophila melanogaster*: in search of fitness benefits

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Abstract

Insect endosymbionts often influence host nutrition but these effects have not been comprehensively investigated in *Wolbachia* endosymbionts that are widespread in insects. Using strains of *Drosophila melanogaster* with the *wMel Wolbachia* infection, we showed that *Wolbachia* did not influence adult starvation resistance. *Wolbachia* also had no effect on larval development time or the size of emerging adults from a low nutrition medium. While *Wolbachia* may influence the expression of heat shock proteins, we found that there was no effect on adult heat resistance when tested in terms of survival or virility following heat stress. The absence of nutrition or stress effects suggests that other processes maintain *wMel* frequencies in natural populations of *Drosophila melanogaster*.

Keywords: *Wolbachia*; *Drosophila melanogaster*; Endosymbiont; Fitness; Stress; Heat; Nutrition

1. Introduction

Wolbachia bacteria are common in insects and increase their transmission by influencing the reproduction of their hosts, most commonly inducing cytoplasmic incompatibility (CI) which reduces the egg hatch rate of uninfected females if they mate with infected males (Hoffmann and Turelli, 1988). Levels of reproductive alteration combined with transmission rate accurately explain the population dynamics of *Wolbachia* in some associations, such as that between *wRi* and *Drosophila simulans* (Hoffmann et al., 1990). In other cases, specifically those in which reproductive manipulation is low or absent (Charlat et al., 2003; Hoffmann et al., 1994, 1996; Perrot-Minnot et al., 2002), the distribution of *Wolbachia* is not fully explained. These cases suggest that our understanding of the interaction between *Wolbachia* and its hosts is incomplete.

The maintenance of *Wolbachia* infection in systems where reproductive effects on hosts are minimal could be better explained if there were host fitness effects. Theory predicts that as a vertically transmitted bacterium, *Wolbachia* should be selected to increase its transmission by providing fitness benefits to its host (Lipsitch et al., 1995 but see Turelli, 1994). This theory is supported by several *Wolbachia* induced fitness benefits such as fecundity advantages in *Trichogramma bourarchae* (Vavre et al., 1999), a potential increase in longevity in *Drosophila melanogaster* (Fry and Rand, 2002), and increased fecundity and longevity in *Aedes albopictus* (Dobson et al., 2002). However, the majority of investigations for fitness benefits in insects have found no positive fitness effects or even negative effects of *Wolbachia* (Bordenstein and Werren, 2000; Giordano et al., 1995; Hoffmann and Turelli, 1988; Hoffmann

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et al., 1990; Schoenmaker et al., 1998; Johanowicz and Hoy, 1999). The majority of these experiments have tested for *Wolbachia*-induced host benefits under ideal conditions. Little is known about fitness effects under stressful conditions (see Olsen et al., 2001), despite the fact that other endosymbionts often aid hosts by increasing tolerance to stressful environments (Douglas, 1994; Montllor et al., 2002).

In *Drosophila melanogaster* populations in Australia, the incidence of *Wolbachia* suggests that the endosymbiont is providing environmentally dependent fitness benefits (Hoffmann et al., 1994, 1998). Southern populations have 15% infection levels, while in northern populations infection levels can be as high as 95% (Hoffmann et al., 1994). Such high infection levels in the north are surprising as CI levels drop off after males are three days old (Reynolds and Hoffmann, 2002) and there is imperfect transmission of this infection (Hoffmann et al., 1998). The variation in the frequency of infection suggests that *Wolbachia* is providing a fitness benefit in the north while providing no such benefit in the south. Field cage studies have shown that the fitness benefit is not due to either mortality or fecundity differences between infected and uninfected individuals (Olsen et al., 2001).

Here we investigate whether *Wolbachia* is providing fitness benefits that are linked to heat or nutritional stresses. Heat resistance seemed a likely source of the fitness benefit because data suggests that other symbiont species provide their hosts with resistance to extreme temperatures (Montllor et al., 2002) and *Wolbachia* has been shown to interact with the expression of heat shock proteins (hsp) in *Drosophila* sperm (Feder et al., 1999). On the other hand providing hosts with novel metabolic pathways (i.e., assisting with nutrition) has been suggested as a primary route to endosymbiosis for bacteria (Douglas, 1994). In a series of experiments, we found that *Wolbachia* was providing neither heat nor nutritional benefits to *D. melanogaster*.

2. Materials and methods

2.1. Stocks

Isofemale lines of *D. melanogaster* were established from single inseminated field females caught in 2001. Twelve isofemale lines were created from each of two tropical Australian populations from Cape Tribulation (CT, latitude 16°02′S), and Innisfail (I, latitude 17°32′S). All Australian populations north of Brisbane (latitude 27°28′S) have greater than 70% infection frequencies and no detectable cytoplasmic incompatibility in the field (Hoffmann et al., 1998; but see Reynolds and Hoffmann, 2002). The 24 isofemale lines that we used were infected with the *wMel* strain of *Wolbachia* as determined by PCR assays (Hoffmann et al., 1998).

Uninfected sublines were created for each isofemale line by treating larvae with tetracycline. Larvae were raised for one generation on normal media supplemented with 0.03% tetracycline. Treated lines were given two generations to recover from the effect of tetracycline prior to use in experiments. The infection status of all lines was checked by PCR with *Wolbachia*-specific 16S rDNA primers. Infected and treated lines were maintained at a census size of at least 100 flies and were compared within four generations of tetracycline treatment, to minimize genetic divergence between lines due to drift.

2.2. Metabolic assays

2.2.1. Starvation

To determine tolerance to starvation, 30 flies were held in 40 ml glass vials with no food. The vials were kept humid by attaching each vial with flies to a second vial containing cotton moistened with 10 ml of water and separated from the water by gauze (Service et al., 1985). Ten CT lines and nine I lines were used in the assay. For each infected and uninfected subline of an isofemale line, we set up one vial with females and one vial with males. Flies were 3–4 days old at the time of testing, and had been sexed using carbon dioxide 24 h prior to the experiment.

2.2.2. Nutrition

The impact of *Wolbachia* on nutrition was investigated by rearing larvae on poor quality media. Flies were normally reared on laboratory medium consisting of 4.8% sucrose, 3.2% dead yeast, and 1.8% agar. Yeast is the primary source of essential amino acids for *D. melanogaster* larvae. Therefore, to stress the larvae, media with 1% or 100% of the yeast compared to the above media was prepared. Fifteen eggs were then spotted into each vial. For each treatment, three vials were set up for each subpopulation of three CT lines and three I lines (i.e., 36 vials per yeast treatment). To determine nutrition effects on flies from each treatment, time to emergence and the size of female wings was assessed.

To determine wing size, digital images were made of wings and these images were then landmarked using tpsDig written by F. James Rohlf. The centroid size of the wings was calculated by taking the square root of the sum of the squared distances of each landmark to the center of the wing.

2.3. Heat assays

2.3.1. Knockdown

The effect of *Wolbachia* on tolerance to heat stress was determined by measuring the knockdown time of

flies at 39 °C following Berrigan and Hoffmann (1998). Individual flies from 10 CT lines were placed in 1 ml glass vials, which were then submerged in a water bath at 39 °C. The effect of infection on acclimation ability was also examined by acclimating half the flies for 1 h at 33 °C 6 h before they were heat shocked. This treatment leads to increased heat resistance in *D. melanogaster* (Dahlgaard et al., 1998). Eighty vials were heat shocked at a time, consisting of one fly per line/subline per sex and acclimation treatment. The position of the treatments and lines in the water bath was randomized and nine replicates were tested in separate runs.

2.3.2. Reproduction

The effect of *Wolbachia* on reproduction following heat shock was investigated using virgin flies collected from 6 Innisfail lines. Flies were 3 days old when stressed in vials with laboratory medium. Vials were placed in a water bath at 28 °C. To reflect natural heating stress, the temperature of the water bath was ramped up by 1 °C every half hour for 5 h until the bath had reached 38 °C. Flies were left at 38 °C for 1 h before being removed from the water bath and returned to 25 °C. Ten flies per line/subline, treatment and sex were tested.

After being stressed, females were placed individually in a vial with an unstressed virgin male from the same line/subline. After 3 days for oviposition, the female was moved to a new vial to lay for another 3 days. Productivity was measured as the number of flies that emerged from each vial both per laying period and averaged over both laying periods. Stressed males were placed individually in a vial with three untreated virgin females that were 2–3 days old. A male was left with the females for 24 h. Each female was removed and placed in a vial for 3 days, and then transferred to another vial for 3 days. Virility was measured as the number of flies that emerged from each vial cumulative over the 3 females for both vials.

2.4. Analysis

All data sets were tested for normality. ANOVAs were used to compare the stress tolerance of infected and uninfected flies. To determine the size of effects that could have been detected in our tests, we examined the differences that led to a correct rejection of the null hypothesis with a probability of 80% (Thomas, 1997).

3. Results

3.1. Metabolic

3.1.1. Starvation

Infection status of the lines did not influence starvation resistance ($F = 0.345$, $df = 1, 68$, $P = 0.559$) (Fig. 1A). Furthermore, there was no interaction between infection and sex ($F = 0.045$, $df = 1, 68$, $P = 0.832$) or infection and population ($F = 1.076$, $df = 1, 68$, $P = 0.303$). As expected sex had a significant impact on ability to withstand starvation ($F = 50.045$, $df = 1, 68$, $P < 0.0001$); females survived 44% longer. A power test indicated that we could have detected a differ-

Fig. 1. Means for uninfected and infected strains for starvation, emergence and size. (A) Starvation survival time. (B) Time to emergence of flies on low yeast media. (C) Wing size of flies emerging from low yeast media. Error bars indicate standard errors.

Table 1
Mean values for wing size and emergence time on varying yeast levels

	Size		Time	
	1%	100%	1%	100%
Infected	507.15	586.33	17.52	8.48
Uninfected	502.15	590.75	17.47	8.80

Percentages refer to relative proportions of yeast in the media. Wing size is in centroid units while emergence time is in days.

ence in starvation resistance of 10.7% with 80% probability Table 1.

3.1.2. Nutrition

Wolbachia infection did not influence the fitness of larvae under poor nutrition conditions (Figs. 1B and C). There was no significant difference between infected and uninfected individuals for time of emergence ($F = 0.0157$, $df = 1, 28$, $P = 0.901$) or wing size ($F = 2.808$, $df = 1, 59$, $P = 0.099$). There was, however, a significant difference in both time of emergence ($F = 105.695$, $df = 1, 70$, $P < 0.001$) and wing size ($F = 1560.62$, $df = 1, 321$, $P < 0.001$) between nutritional treatments (Fig. 2), indicating that the low nutrition media did stress the flies. The poor nutrition conditions decreased size by 14% and increased development time by 98%. Power tests indicated that we could have detected a difference in development time under poor nutrition of 4.4% and a size effect of 1.2%, both with 80% probability.

3.2. Heat

3.2.1. Knockdown

Wolbachia infection had no significant effect on knockdown time ($F = 0.027$, $df = 1, 621$, $P = 0.868$) (Fig. 2A). As expected, acclimation had a significant influence ($F = 8.001$, $df = 1, 621$, $P = 0.0048$), increasing knockdown time by 9.8% overall. There was no interaction between *Wolbachia* and acclimation ability ($F = 0.807$, $df = 1, 621$, $P = 0.369$). A power test indicates that we could have detected a difference in knockdown time of 5.9%, with a probability of 80%.

3.2.2. Reproduction

Wolbachia showed no effect on reproductive ability following heat shock (Figs. 2B and C). The number of offspring sired by heat-shocked males was independent of infection ($F = 0.413$, $df = 1, 38$, $P = 0.525$) as was the percent of females mated ($F = 0.256$, $df = 1, 46$, $P = 0.615$). Just over half of all males, 55%, were able to sire offspring in all three females, and 80% of males mated at least one female. *Wolbachia* also had no influence on female fecundity, either per day or total fecundity ($F = 1.617$, df=1, 46, $P = 0.210$). We could have detected a difference of 34.5% in male virility and 37.3% in female fecundity with 80% probability.

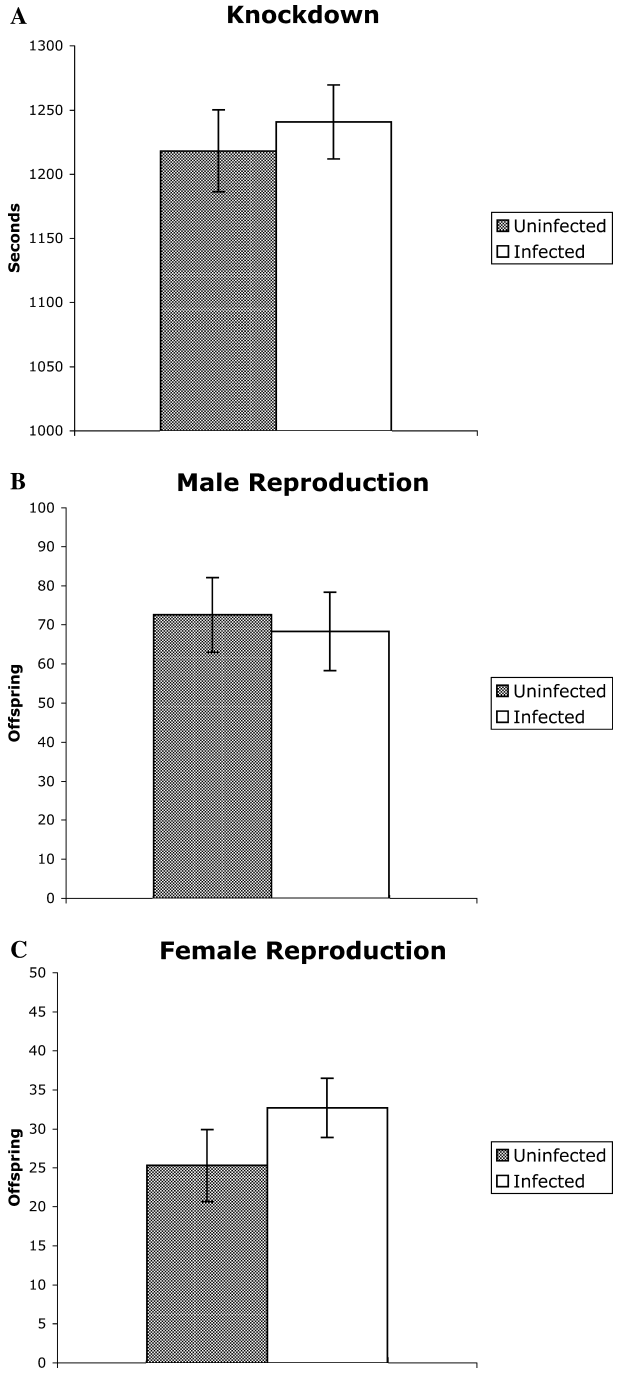

Fig. 2. Means for uninfected and infected flies in heat assays. (A) Knockdown time of flies at 39 °C. (B) Number of offspring produced by males following heat shock at 38 °C. (C) Number of offspring produced by females following heat shock at 38 °C. Error bars indicate standard errors.

4. Discussion

These negative results suggest *Wolbachia* is not providing a fitness benefit related to heat or nutritional tolerance for *D. melanogaster*. *Wolbachia* is therefore, not utilizing novel metabolic pathways to evolve towards

mutualism in *D. melanogaster*. The results also indicate that *Wolbachia*'s influence on hsp production does not cause an increase in heat shock tolerance in its hosts. Below we discuss the reasons for investigating each of these fitness benefits, and we consider the validity of our measures.

Wolbachia's ability to supplement nutrition was investigated because providing novel metabolic pathways has been proposed as a primary route to symbiosis (Douglas, 1994). The symbiotic relationships between bacteria and a wide range of hosts hinge on the provision of metabolic capabilities such as photosynthesis, nitrogen fixation, essential nutrient production, cellulose degradation, and consumption of hydrogen (Douglas, 1994). In insect hosts the majority of long-term endosymbioses described to date involve symbiont production of essential nutrients (Douglas, 1994). The prevalence of nutrition supplementation in other endosymbiotic systems suggested that *Wolbachia* might also influence this ability.

Our experiments tested two aspects of nutrition. The starvation assay tested whether *Wolbachia* influences the level of sugar or fat stores in adult flies. These energy sources should be important in adult flies as they search for food. The nutrition assay tested *Wolbachia*'s ability to provide essential amino acids to larvae. *Drosophila* use plant food sources which lack most amino acids and therefore the larvae rely on yeasts for most of their protein. Reducing the yeast in the experiment would have limited the supply of many essential amino acids. Both assays tested nutrient level by measuring fitness effects, and could have detected changes of 1–10%. The 10% change that could be detected for starvation tolerance equates to an increase in *Drosophila* survival of only 5 h.

Wolbachia's ability to provide heat shock resistance was investigated because data suggests a connection between *Wolbachia* and tolerance to extreme heat. Endosymbionts commonly produce high levels of heat shock proteins, which can increase host fitness under extreme temperatures (Lee et al., 2001; Montllor et al., 2002). Feder et al. (1999) found an interaction between *Wolbachia* and heat shock proteins, by demonstrating that the expression of heat shock proteins in the sperm of *D. simulans* is altered by *Wolbachia* infection. Additionally, the pattern in Australia of increasing levels of *Wolbachia* infection in populations exposed to higher temperatures (Hoffmann et al., 1994) suggests that *Wolbachia* influences heat shock tolerance. Heat stress tolerance can be investigated with a wide variety of methods (as reviewed in Hoffmann et al., 2003). The two methods used in this experiment provide information about different aspects of tolerance. First, knockdown time was assessed including following acclimation known to induce expression of heat shock proteins. This test would have uncovered differences if *Wolbachia* either exported hsps to its host only in times of stress or influenced the expression of host hsp expression. The brief nature of the heat shock makes it unlikely that the results were confounded by desiccation tolerance. We could have detected differences in hsp production that increased knockdown time by a single minute. Additionally, heat stress was used to assess whether *Wolbachia* influenced the heat tolerance of reproductive systems. Reproductive ability following heat stress may be ecologically relevant (Patton and Krebs, 2001), particularly as *Wolbachia* fitness has been tied to host fecundity (Hoffmann and Turelli, 1988; Hoffmann et al., 1990; Vavre et al., 1999). The large variation in reproductive ability lead to greatly reduced power in this experiment. Our results clearly indicate that *Wolbachia* does not cause any substantial increase in *Drosophila* reproductive ability following heat shock, however, because of the low power we are unable to entirely rule out the existence of slight improvements in reproduction due to *Wolbachia*.

Our results suggest that the pattern of *Wolbachia* distribution in Australian *D. melanogaster* is unlikely to be explained by heat or nutritional fitness benefits. One alternative explanation of the pattern is that northern populations of *Drosophila* may experience higher levels of CI. *Wolbachia* has recently been found to induce CI in *D. melanogaster* when males are young (Reynolds and Hoffmann, 2002). If males in northern populations mate at a younger age than males in the south then CI would be stronger in the north. A second explanation is that *Wolbachia* provides a fitness benefit other than those tested, such as protection from natural enemies. It should also be noted that the fitness effects provided by *Wolbachia* may involve a complex interaction of several variables. Such a complex interaction would be difficult to detect in controlled laboratory experiments. Olsen et al. (2001) found that the association between *Wolbachia* and *D. melanogaster* varied extensively between the laboratory and field cage experiments. Future studies should include both laboratory and field work.

Fitness benefits are unlikely to explain all cases of *Wolbachia* infection in the absence of reproductive effects. In some cases such as that of the non-expressing *wAu* strain of *Wolbachia* in *D. simulans* (Hoffmann et al., 1996) and the Wolbachia infection in *D. yakuba* (Charlat et al., 2004), the persistence of *Wolbachia* infection is likely governed by stochastic processes. In other cases such as those in which *Wolbachia* strains rescue the CI phenotype but do not induce it (Mercot and Poinsot, 1998), infection may be maintained by the reproductive manipulation of other *Wolbachia* strains. However, in associations such as that between *Wolbachia* and *D. melanogaster*, fitness benefits seem the most plausible explanation of infection levels. Further studies of *Wolbachia* fitness effects in stressful environments are therefore likely to enhance our understanding of *Wolbachia* population dynamics.

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