***Wolbachia* induces costs to life-history and reproductive traits in the moth, *Ephestia kuehniella***

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**Abstract**

The intracellular endosymbiont *Wolbachia pipientis*is is well-known as one of the most common bacterial symbionts of arthropods. Recently, research has focused on the potential to utilize *Wolbachia* as a biocontrol agent of agricultural and medical pest insect species. *Wolbachia* blocks host infection from other pathogens and viruses in some species, however, it can also influence host life-history and reproductive traits. Therefore, in order to understand the biological impact and potential economic utility of *Wolbachia*, it is necessary to investigate the effects of *Wolbachia* infection on host traits. We compared life-history and reproductive traits between *Wolbachi*a-infected and cured population in Mediterranean Flour Moth, *Ephestia kuehniella*. *E. kuehniella* is well known as a pest of stored products, and when infected with *Wolbachia*, it exhibits cytoplasmic incompatibility between uninfected females and infected males. We found that *E. kuehniella* suffers costs as a result of *Wolbachia* infection, through decreased larval survival and adult longevity, and prolonged developmental period. Moreover, reproductive performance was greater in the uninfected population, when excluding the effect of cytoplasmic incompatibility. Our results indicate that *E. kuehniella* suffers deleterious effects on both life-history and reproductive traits as a result of being infected with *Wolbachia*. We suggest such costs should be considered when evaluating the efficacy of utilizing *Wolbachia* in pest control.

**Highlights**

・*Wolbachia* has potential as a biocontrol agent.

・Investigation of the effects on the host of *Wolbachia* is needed.

・*Ephestia kuehniella* suffers life-history costs when infected with *Wolbachia*.

・Use of *Wolbachia* in pest control may be not effective in *E. kuehniella*.

**Keywords**: biocontrol, cytoplasmic incompatibility, deleterious effect, life-history, *Wolbachia*

**1. Introduction**

The intracellular endosymbiont *Wolbachia pipientis*, of the *Rickettsia sp*. of bacteria, is well-known as one of the most common bacterial symbionts of arthropods, estimated to infect 40-50% of terrestrial species (Weinart et al., 2015; Zug and Hammerstein, 2012). *Wolbachia* are of particular interest to behavioral ecologists studying arthropods, due to its ability to manipulate the reproduction of its hosts. The endosymbiont is transmitted to all progeny, sons and daughters, from the infected mother via the cytoplasm of the egg; therefore, male sperms are essentially a dead-end for the bacterium (reviewed in O’Neill et al., 1997). As a result, *Wolbachia* have evolved a number of innovative mechanisms by which it increases its transmission (reviewed in Werren et al., 2008). In some amphipods, the bacteria feminizes genetic males. Phenotypically, and reproductively, the males resemble and act like females, thereby effectively increasing the proportion of females in a given population, which in turn increases the spread of the *Wolbachia*. Similarly, in some species of arrhenotokys wasp, *Wolbachia* induce parthenogenesis, again thereby increasing the proportion of females, and in turn maximizing its transmission. In some insects such as the butterfly *Hypolimnas bolina*, *Wolbachia* act as a ‘male-killer,’ killing males in a given brood early in development; again, this results in an increase of the proportion of females.

The most common mechanism by which *Wolbachia* increase its transmission is via cytoplasmic incompatibility (CI). When uninfected females mate with infected males, up to 95% of the resulting brood are rendered inviable, and die (O’Neill et al., 1997). Combinations of infected females versus infected or uninfected males are compatible, as a result of an unknown ‘rescue factor’ produced in infected females’ eggs which permit crossing with infected male sperm (Bourtzis et al. 1998; Riparbelli et al., 2007; Zabalou et al. 2008, but see Clark et al., 2008). As a result of CI, uninfected females have lowered reproductive success compared to infected females, who can mate with both infected and uninfected males. Thus, again, the bacterium promotes its transmission through the host population (Hoffmann et al., 1990).

Interest in *Wolbachia* has increased rapidly since the 1990s, as increasingly widespread and cheap molecular technologies have allowed researchers to easily identify infected individuals. However, the focus of the field has switched over time, from merely estimating prevalence of *Wolbachia* in wild and laboratory populations, to its importance as an evolutionary force (Duron and Hurst, 2013), to its utilization as a biocontrol agent of agricultural and medical pest insect species (reviewed in LePage and Bordenstein, 2013). For example, it has been shown that *Wolbachia* blocks Zika virus isolates in the mosquito *Aedes aegypti* in Brazil, the location of the recent Zika epidemic (Dutra et al., 2016). *Wolbachia*-induced resistance against dengue virus has been also found in *A. aegypti* (Walker et al. 2011; Bian et al., 2010). *Wolbachia* infection is known to activate the host immune system against other virus and pathogens (Xi et al., 2008; Kambris et al., 2009). Further, Zhang et al., (2016) have shown promise in utilizing a combination of the Sterile Insect and Incompatible Insect Techniques in *Aedes albopictus* mosquitoes infected with three strains of *Wolbachia*, under semi-field conditions, in order to suppress host population productivity.

Yet, other than in model species such as *Drosophila melanogaster* (Champion de Crespigny et al., 2006), or species of high medical or economic interest such as *Aedes* sp. (e.g. Dutra et al., 2016; Zhang et al., 2016) and the Mediterranean fruit fly crop pest (e.g. Sarakatsanou et al., 2011), the basic behavioral and physiological biology of the *Wolbachia*-host interaction is still unknown for the majority of the hosts which *Wolbachia* is known to infect. It has been shown that, despite the costs of CI to uninfected females within a population, there can be benefits of harboring *Wolbachia*. For example, in some species *Wolbachia* is an obligate endosymbiont of the host. In the bedbug *Cimex lectularius*, the bacterium is essential for the growth and reproduction of the host, as a result of the provision of crucial B vitamins (Nikoh et al., 2014). Similarly, in the parasitic wasp *Asobara tabida*, *Wolbachia* is required for oogenesis (Dedeine et al., 2001). A number of studies have also shown that *Wolbachia* can confer an advantage to the host by affording protection against pathogens such as other bacterial symbionts, fungi, and viruses (reviewed in Zug and Hammerstein, 2015). In contrast, in some species, above and beyond the costs associated with CI, hosts can suffer additional costs to, for example, male reproductive competitiveness (e.g. Champion de Crespigny and Wedell, 2006), female fecundity (e.g. Perrot-Minnot et al., 2002), and adult longevity (e.g Carrington et al., 2010). The effects of *Wolbachia* on the host have also been examined in some more diverse taxa (e.g. in the spider mite; Vala et al. 2004, a *Drosophila* parasitoid; Fleury et al., 2000, *Tribolium confusum*; Wade and Chang 1995, and a bean beetle; Okayama et al., 2016). However, to understand the biological impact and potential economic utility of *Wolbachia* in pest control, it is necessary to investigate the effects of infection on host life-history and reproductive traits in a greater variety of taxa.

The Mediterranean Flour Moth, *Ephestia kuehniella,* is a cosmopolitan pest of stored products such as flour; the larvae infest the products and cause considerable economic damage. The adults are polyandrous and females produce over 100 eggs over the course of their lifespan. The sterile insect technique via radiation has been considered as a method of controlling *E. kuehniella* (Ayvaz et al., 2007), however the use of *Wolbachia* has not been. Potentially crossing males with strong CI-inducing *Wolbachia* could decrease uninfected female fitness, resulting in suppression of the population. It has been reported that *E. kuehniella* populations in Japan are infected by *Wolbachia* (Ikeda et al., 2003; Kageyama et al., 2010; Sasaki and Ishikawa 1999) and that they suffer CI as a result of *Wolbachia* infection. For example populations from Yokohama and Tsuchiura population exhibited CI of 83.1% and 39.2% respectively (Sasaki and Ishikawa 1999). Additionally, *Wolbachia* infected males exhibit a reduction in the numbers of sperm they transfer to females (Lewis et al., 2011a). However, nothing is known with regards the effects of *Wolbachia* on other life-history and/or reproductive traits in this species. Here, we examine the effect of *Wolbachia* on reproductive and life-history traits in *E. kuehniella*. By comparing *Wolbachia*-infected and uninfected populations, we assess the impact of *Wolbachia* on development time, adult body size, adult longevity, male mating performance, and female reproductive success.

**2. Materials and Methods**

2.1. Stock culture

The stock population of *E. kuehniella* used in this study originated from adults collected in Yokohama, Japan, approximately 20 years ago, and is naturally infected with a single strain of group A *Wolbachia* inducing ~80% CI (Sasaki and Ishikawa, 1999; Lewis et al., 2011a). This population has since been maintained on a larval medium consisting of wheat bran, dried yeast and glycerol (20:1:2 w/w) at 25° C, 60% relative humidity, and with a photoperiod cycle of 16L:8D (see Sasaki and Ishikawa, 1999 for details). As the moths have a generation time of two months, we assumed that they had been maintained under these laboratory conditions for approximately 120 generations at the time of our experiment, which was conducted in 2011. The population has been maintained with several hundred adults per generation since it was established, thereby minimizing the possible effects of inbreeding.

2.2 Generation of the uninfected population

Due to partial CI expression in *E. kuehniella* (Sasaki and Ishikawa, 1999), the population utilized includes both *Wolbachia* infected and uninfected individuals. We therefore generated an uninfected population via curing with antibiotics; the comparison of infected and uninfected individuals is a standard method of examining the effects of *Wolbachia* on the host (e.g. Snook et al., 2000; Champion de Crespigny and Wedell, 2006). Briefly, an infected population was established by selecting only *Wolbachia*-infected males and females (see Lewis et al., 2011a), then a sub-set of the *Wolbachia*-infected population was cured by adding tetracycline hydrochloride to the larval medium at a final concentration of 0.04%, for two generations (Sasaki and Ishikawa, 1999). The “cured” uninfected population was maintained for two generations prior to the experiment, under the same conditions as the infected population, in order to allow it to recover from potential detrimental effects of antibiotic-supplementation to insect rearing media (Graf and Benz, 1970). Infection status of the uninfected and infected populations was subsequently confirmed by PCR for the universal *Wolbachia*-specific primers wsp81Fand wsp691R (Zhou et al., 1998).

2.3. Development time, adult longevity, and body size

All rearing experiments were conducted in a chamber maintained at the same rearing conditions described above. Females and males from the infected and uninfected populations were allowed to mate freely and lay eggs for twenty-four hours. Fifty eggs were then removed from each, and reared with an excess of the larval medium (100g) to investigate development time and survival from egg to adult. Sexual discrimination of larvae in the early developmental stages is impossible, therefore we pooled survivors from egg to pupa of both sexes. Development time was assessed daily. Upon emergence, each adult was moved immediately into a vial to prevent mating, and assessed for survival daily until death. This then gave a measure of adult longevity. After death, the length of the forewing was measured to indicate body size, in accordance with the protocol in Ingleby et al., (2010). Adults that lost the forewing prior to death, were excluded from subsequent analyses. We established three replicate blocks of infected and uninfected populations.

2.4. Mating performance

As above, two hundred newly laid eggs were collected from each population, and reared with an excess of larval medium (200g) to assess reproductive traits. At fifth-instar larval stage, the larvae were separated according to sex as the male testes are visible through the cuticle at this stage. We thereby generated virgin males and females. All virgin individuals of the two populations – infected and uninfected - were less than a day post-emergence. We established four types of pairs: uninfected female × uninfected male (UF x UM), infected female × infected male (IF x IM), uninfected female × infected male (UF x IM), and infected female × uninfected male (IF x UM). We established three replicate blocks of each type. We placed twenty females and twenty males for each type of pair into a plastic container. The group was continuously observed for one hour to investigate mating success. Mating pairs were removed from the container, and individually maintained into a petri dish until copulation ended. All observations were recorded with a digital video camera (Victor GZ-MG880) in order to measure copulation duration. Copulation duration is commonly used in insects as a proxy for male investment in a given copulation (Lizé et al., 2014). Mating pairs were checked every hour, and when the female finished copulation, she was removed from the petri dish and moved into a new petri dish, and allowed to lay eggs until death. The total number of eggs laid was counted. Of these females, we also collected a sub-sample of 10 eggs per female, to assess egg hatchability. Lifetime reproductive success of each female (LRS) was scored as the total number of eggs laid × hatchability. Females that mated but did not lay eggs were excluded from the analysis.

2.5. Statistical analyses

Logistic regression analyses were conducted on larval survival rate and copulation success. In the analysis of larval survival rate, survival (= 1) or death (= 0) were used as the dependent variables. Infection status (uninfected or infected) and replicates nested within the infection status, were used as the independent variables. In the analysis of copulation success, whether pairs mated (= 1) or did not (= 0) were used as the dependent variables. *Wolbachia* status of pairs (uninfected female × uninfected male, infected female × infected male, uninfected female × infected male and infected female × uninfected male) and replicate nested within pair status, were used as the independent variables. Development time, adult longevity, and body size were analyzed using an analysis of variance (ANOVA) with sex, infection status, and replicate nested within the infection status as the independent variables. Copulation duration, egg number, hatchability and female lifetime reproductive success (total number of egg × hatchability) were analyzed using ANOVA with pair status and replicate nested within pair status, as the independent variable. Non-significant replicate effects and interaction terms were removed from the model by stepwise deletion (Grafen and Hails, 2002). If there was a significant effect on pair status, Student’s *t*-test or χ2 test was used for pairwise comparisons, and the significance level was corrected for multiple comparisons by the sequential Bonferroni method (Rice, 1989). All analyses were carried out using JMP 7 (SAS Institute, 2007).

**3. Results**

Larval survival rate was significantly higher for the uninfected compared to the infected populations (infection status, df = 1, χ2 = 7.8559, *P* = 0.0051; Fig. 1a). Development time was significantly shorter in individuals from the uninfected than the infected populations, while there was no significant difference between sexes (infection status, *F*1, 163 = 7.7696, *P* = 0.0059; replicate, *F*4, 163 = 4.5553, *P* = 0.0016; sex, *F*1, 163 = 2.3223, *P* = 0.1295; Fig. 1b). Adult longevity was significantly longer in the uninfected than the infected populations and was longer in the male than the female (infection status, *F*1, 167 = 10.1941, *P* = 0.0017; sex, *F*1, 167 = 49.2443, *P* < 0.0001; Fig. 1c). Body size was unaffected by infection status and sex (infection status, *F*1, 159 = 1.7236, *P* = 0.1911; replicate, *F*4, 159 = 3.6756, *P* = 0.0068; sex, *F*1, 159 = 0.2553, *P* = 0.6141; Fig. 1d).

There was a significant difference between pairs of different infection status in copulation success (paring, df = 3, χ2 = 9.4760, *P* = 0.0236). Multiple comparisons showed that the rate was significantly higher in the pair UF x UM, than IF x UM (Fig. 2a). We found also a significant difference between pairs in copulation duration (type of pair, *F*3, 81 = 2.9831, *P* = 0.0361), which was significantly shorter in the pair UF x UM, than IF x IM (Fig. 2b).

There was a significant difference between pairs in the total number of eggs produced (pairing, *F*3, 69 = 9.0914, *P* < 0.0001), egg hatchability (pairing, *F*3, 69 = 56.7202, *P* < 0.0001) and LRS (egg number × hatchability) (pairing, *F*3, 69 = 19.7496, *P* < 0.0001). The number of eggs produced was significantly smaller in the pairs IF x IM and IF x UM, than UF x UM and UF x IM (Fig. 2c). Egg hatchability was significantly lower in the pairs UF x IM than in the others (Fig. 2d). LRS was significantly larger in the pairs UF x UM than in others, and it was significantly larger in the pairs IF x IM and IF x UM than UF x IM (Fig. 2e).

**4. Discussion**

We found that survival rate was lower in infected larvae than uninfected. Development time was also longer in infected individuals; there was no difference between the sexes. Adult longevity was shorter in infected individuals, and in males compared to females. Body size was unaffected by status or sex. The longer development time and shortened adult longevity of the infected population are disadvantageous, because it results in fewer reproductive opportunities compared to individuals from the uninfected population (Stearns, 1992). The fact that infected adults died faster is likely to have a downstream impact on reproductive success, particularly for males. It has been shown in the closely related Indian meal moth *Plodia interpunctella* that adult longevity is an important predictor of LRS for males, but not for females (Lewis et al., 2011b). Potentially, the presence of the endosymbiont induces metabolic costs on the host, as has been reported in *D. melanogaster* infected with the *Drosophila* C virus (Arnold et al., 2013; although see Evans et al., 2009), which may then in turn impact on growth and survival.

We predicted that copulation success would be reduced for the incompatible UF x IM cross. However, our results indicate that copulation success was also lower for the cross IF x UM, despite being a compatible cross. Infected females produced fewer eggs compared to uninfected females, irrespective of mate infection status. Hatchability between UF x IM was lower than other pair types due to cytoplasmic incompatibility. As a result, LRS differed significantly across types of mating pairs; UF x IM, i.e., incompatible pairs had the lowest LRS, and uninfected pairs showed the greatest. Although IF x UM exhibited an intermediate LRS, they still suffered lower mating success compared with other mating pairs. Therefore, assortative pairings of infection status had greater fitness compared to disassortative pairs. In addition, the fitness of uninfected pairs was greater than those of infected pairs. It remains unclear which pair, UF x IM or IF x UM, has greater fitness, however, our results suggest that the fitness of uninfected females is relatively higher than infected females in *E. kuehniella*.

Prolonged copulation durations in IF x IM and UF x IM reinforce the results of a previous study, which showed that *Wolbachia* causes a reduction in sperm production in a number of insect species (Lewis et al., 2011a). This in turn can result in reduced sperm competitive ability (e.g. Champion de Crespigny and Wedell, 2006). As copulation duration is thought to correlate with the numbers of sperm transferred to the female in insects, our result suggests that males may compensate for their reduced ability to produce sperm, by mating for longer, as suggested by Watanabe et al., (1998). If infected males invest more in copulation duration, he could suffer costs such as increased predation risk (Rowe, 1994; Sih et al., 1990; Ward, 1986), or decreased longevity due to allocation trade-offs between survival and reproduction (Brown et al., 2009). In contrast, shorter copulation duration may benefit males because they can then access a new mate faster (Sakurai et al., 2012). Therefore, infected males are disadvantaged in reproduction compared to uninfected males.

By releasing CI-inducing *Wolbachia*-infected males into a pest population, it is expected to suppress numbers as the fitness of wild uninfected females is decreased due to CI (Brelsfoard and Dobson 2009; Zabalou et al., 2004). Considering our results in conjunction with those of Lewis et al. (2011a), *Wolbachia*-infected males suffer disadvantages in their longevity and investments in reproductive traits. Therefore we suggest that the utilization of CI-inducing *Wolbachia* may not be effective as a pest control agent in this species.

In our experiments, we used a standard method for curing *Wolbachia* from the host, but it is difficult to remove the possibility of other microorganisms affecting the host. However, our study is one of the few to report multiple deleterious effects to the host of being infected with *Wolbachia*. In *Drosophila* *melanogaster*, the ‘popcorn’ strain of *Wolbachia* has extremely virulent effects, causing early death (Min and Benzer, 1997). However, in the majority of cases *Wolbachia* tends to have relatively benign effects on most life-history traits (e.g. Hoffmann et al., 1996; reviewed in Zug and Hammerstein, 2015), outside of the more dramatic *Wolbachia*-induced modifications to host reproduction, such as male-killing and feminization. Increasingly researchers are investigating the potential for harnessing endosymbionts such as *Wolbachia* in the biocontrol of pests that impact human health and food security (reviewed in LePage and Bordenstein, 2013). In order to maximize the efficacy of such techniques, we need first to understand the basic biology of the pest, and the effects of the interactions with the endosymbiont in question. Although it is important to investigate the effects of endosymbionts on the host biology, such information is still lacking, outside of a limited number of model organisms. Such a profound ecological and evolutionary driver as *Wolbachia* may prove a key tool in our battle against problem arthropods and other organisms.

In conclusion, we show that the endosymbiont *Wolbachia* induces a number of deleterious effects on life-history and reproductive traits of the product pest *E. kuehniella*. We suggest that more of such information regarding the basic biology of the interaction between endosymbionts and their arthropod hosts is needed, in order to effectively control pest species.

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**Figure legends**

Fig. 1

Mean values of life history traits in *Wolbachia*-infected and uninfected populations: (a) larval survival rate, (b) developmental period, (c) adult longevity and (d) body size. Open and grey bars show *Wolbachia*-uninfected and infected, respectively. Bars and numbers in parentheses represent standard errors and total sample sizes of three replicates, respectively.

Fig. 2

Mean values of reproductive traits in *Wolbachia*-infected and uninfected populations: (a) copulation success, (b) copulation duration, (c) the number of eggs, (d) hatchability and (e) lifetime reproductive success (the number of egg × hatchability). U and I in the x-axis respresent infection status, *Wolbachia*-uninfected and infected individuals, respectively. Bars and numbers in parentheses represent standard errors and sample sizes, respectively. Replicates were pooled as analyses indicated no significant effects of replicate.

Figure 1

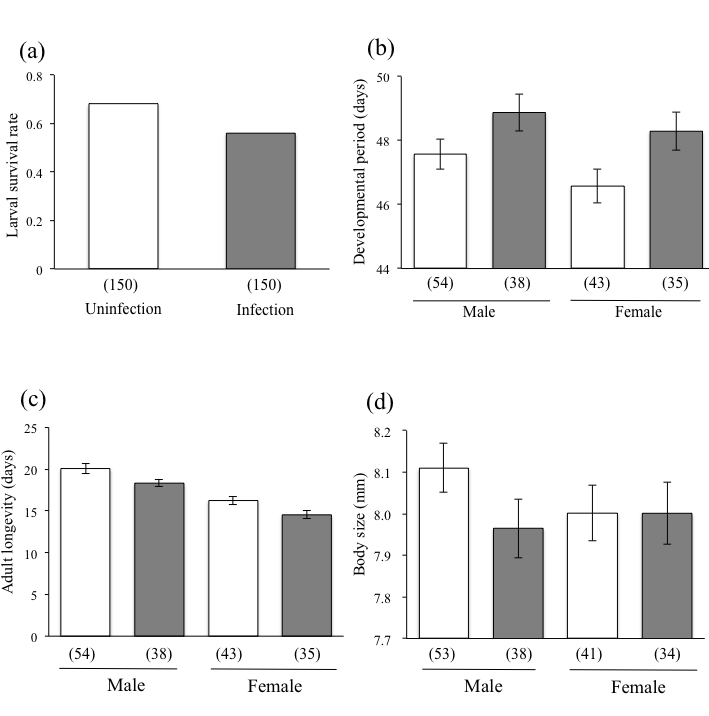


Figure 2

