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Effect of *Wolbachia* infection and temperature variations on the fecundity of the Uzifly *Exorista sorbillans* (Diptera: Tachinidae)

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Abstract *Wolbachia* are cytoplasmically inherited endosymbionts known to cause several reproductive alterations in insects which allow their spread in host populations. In the Uzifly *Exorista sorbillans*, endoparasites of silkworms, the prevalence of *Wolbachia* is high in the field. In the present study, we investigated *Wolbachia*'s effects on the Uzifly fitness traits by measuring fecundity and hatching rate in crosses involving infected and cured individuals. We found evidence for positive fitness effects associated with *Wolbachia* infection in females which could help promote the spread of *Wolbachia* in *E. sorbillans* populations. We tested two types of treatments for removing *Wolbachia*, antibiotic therapy and high temperature treatment and found an influence on the reproduction: females treated by antibiotics have a lower fecundity than females cured by high temperature which could indicate a negative effect of the antibiotherapy on females' fitness. Furthermore, the monitoring of the Uzifly populations during 2 years revealed seasonal variations of the offspring production which may be linked to temperature.

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1 Introduction

Many arthropods live in symbiosis with one or more endosymbiotic bacteria. Two types of bacterial symbionts are usually recognized: obligatory symbionts (primary symbionts) that provide essential nutrients when the host has unbalanced diets, leading to cooperative insect-microbial relationships (Douglas 2006), and facultative symbionts (secondary symbionts), acquired more recently. In contrast to obligatory bacteria, these symbionts are not essential to host survival. Their phenotypic effects are diverse and must allow their spread and maintenance in host populations despite the physiological cost for their host. Some facultative endosymbiotic bacteria have a mutualistic strategy since they confer direct fitness benefit to their host such as protection against natural enemies (Ferrari and Godfray 2003; Oliver et al. 2003), thermal tolerance (Chen et al. 2000; Montllor et al. 2002) or host plant specialisation (Tsuchida et al. 2004). Others manipulate the host reproduction in order to modify the fitness balance between infected and uninfected females since symbionts are maternally transmitted (for review, see Stouthamer et al. 1999).

The maternally inherited bacteria *Wolbachia* is one of the most common symbiont of invertebrates since it infects more than 60% of insect species, but also many species of arachnids, terrestrial crustaceans and filarial nematodes (Werren et al. 1995; Jeyaprakash and Hoy 2000; Hilgenboecker et al. 2008). In most arthropod associations, *Wolbachia* act as parasites manipulating the reproduction of their hosts in diverse ways including

feminization of genetic males (Rigaud et al. 1991a, b), male killing (Hurst et al. 1999), induction of thelytokous parthenogenesis (Stouthamer et al. 1990), but the most common is cytoplasmic incompatibility (Hoffmann et al. 1990; Breeuwer and Werren 1993; O'Neill and Karr 1990). Cytoplasmic incompatibility is a sperm-egg incompatibility expressed in crosses between infected males and uninfected females that lead to post-zygotic reproductive isolation. *Wolbachia* can also successfully invade host populations without inducing reproductive modifications. For example, in *Drosophila melanogaster*, the maintenance of *Wolbachia* in wild populations is explained by positive effects upon fitness traits such as a longer survival of infected flies (Fry and Rand 2002), protection against viral and fungal pathogens (Hedges et al. 2008) or a higher fecundity under nutritional stress (Brownlie et al. 2009). In the mosquito *Aedes albopictus*, infected females live longer, produce more eggs and have higher hatching rates than uninfected females (Dobson et al. 2002). In two extreme cases of insect species, the parasitoid *Asobara tabida* (Dedeine et al. 2001) and the bedbug *Cimex lectularis* (Hosokawa et al. 2010), *Wolbachia* is, like in filarial nematodes (for review see Taylor et al. 2005), an obligate mutualist. However, *Wolbachia* is more often associated with negative effects than with benefits.

Manipulation of reproduction and fitness effects associated with *Wolbachia* are influenced by the bacterial strain but also by the host genetic background. For instance, in a comparison of two populations of *D. melanogaster*, Olsen et al. (2001) demonstrated that genetic background can alter the effects of *Wolbachia* on fitness traits. Differences were found for fecundity and for viability in the eggs and larval stages. These effects were in turn influenced by environmental conditions. Indeed, under tropical conditions infected flies from both populations exhibited a fecundity deficit, but under cool temperature conditions one of the populations exhibited enhanced fecundity. More generally, environmental factors such as antibiotics, food quality, host density and temperature have a major influence on symbiotic populations. For example extreme temperatures can lead to symbiosis break-down (Stouthamer et al. 1990; Perrot-Minnot et al. 1996; Johanowicz and Hoy 1998; Van Opijnen and Breeuwer 1999). Clearly, temperature affects density (Mouton et al. 2006) and transmission efficacy of *Wolbachia* to a great extent (Stevens 1989; Rigaud et al. 1991a, b; Van Opijnen and Breeuwer 1999), suggesting that seasonal differences of *Wolbachia* infection can occur in the field.

In the present study, we explore the effects of *Wolbachia* on reproduction of the Uzifly *Exorista sorbillans*, a parasitoid of silkworms. In this species, most individuals are infected by *Wolbachia* in wild populations (B group of *Wolbachia*; Chatterjee et al. 2003; Guruprasad et al. 2011) thus indicating that, in this host species, *Wolbachia* either is able to induce reproductive manipulations or acts positively upon some fitness traits. To test these hypotheses, uninfected individuals are required. We obtained uninfected individuals by curing *Wolbachia* using treatments (antibiotherapy or heat) for two reasons: first, uninfected individuals are rare in the field; secondly it avoids bias due to the influence of the host genetic background. We conducted experiments to answer to the following questions: (i) Does *Wolbachia* affect the reproduction of *E. sorbillans* ?; (ii) Is there any influence of the type of treatment used for removing *Wolbachia* (antibiotic or heat treatment) on reproduction of the cured individuals ?; (iii) Is there seasonal variations in reproduction of the Uziflies?

2 Material and methods

2.1 Collection and rearing of *Exorista sorbillans*

The post parasitic maggots of Uzi flies were collected from a silkworm cocoon market in Ramanagaram (Karnataka state) soon after their emergence from infested host silkworm cocoons. The maggots were brought to the laboratory in a perforated plastic container with sand and allowed to metamorphose into pupae and then adults in wire mesh netted cages of 35×35×35 cubic cm (Manjunatha 1993). Upon emergence, male and female adults were separated based on genitalia and other morphological characters before mating crosses. Early fifth instar larvae of silkworm *Bombyx mori,* the hosts of the Uzi fly, were placed in the cages to stimulate oviposition (Uzi flies prefers to lay their eggs on the body of silkworm). The larvae were replaced every 12 h to maintain a density of 2–3 Uzi eggs on each larval body (Manjunatha 1993). The silkworm larvae, which have Uzi eggs on their body, were removed from cages and reared in the laboratory following the standard silkworm rearing technique at 26°C (Krishnaswamy 1978). Following eclosion, larvae penetrated silkworm hosts where they completed larval development. Upon reemergence larvae were collected, allowed to complete development and separated before mating.

2.2 DNA isolation and PCR assay for detecting *Wolbachia*

The DNA of the uziflies was extracted by proteinase K and SDS lysis method as in Sambrook et al. (1989). The genomic DNA was resuspended in 50μL of TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Detection of *Wolbachia* bacteria was done by polymerase chain reaction (PCR) assay based on amplification of the *Wolbachia wsp* (*Wolbachia* surface protein) gene using the primers 81F

(5′-TGGTCCAATAAGTGATGAAGAAAC-3′) (Braig et al. 1998) and 691R (5′-AAAAATTAAACGCTACTCCA-3′) (Zhou et al. 1998). PCR reactions were performed in 25μL volumes containing 2.5μL of 10X PCR buffer, 0.4 mM of each dNTP, 4 mM of $MgCl_2$, 1μL of both primers (5 pmol each), 0.5 IU of Taq DNA polymerase (New England Biolabs, England) and 20 ng of template DNA. Cycling conditions were an initial denaturation step at 94°C for 5 min followed by 35 cycles with a denaturation step at 92°C for 1 min, annealing at 55°C for 1.30 min, extension at 72°C for 1.15 min, and a final extension at 72°C for 10 min. PCR products were visualized by electrophoresis on 1.5% agarose gel stained with 0.5 μg/mL ethidium bromide under UV illumination to check for the presence/absence of *Wolbachia*. Some PCR products were sequenced to verify their identity.

2.3 Antibiotic treatment for removing *Wolbachia*

Antibiotic treatments were done on adult flies to eliminate *Wolbachia*. Uzi flies were maintained by administrating 0.02 mg/ml of Oxytetracycline with 8% glucose in distilled water soaked with cotton balls. The elimination of *Wolbachia* bacteria was confirmed by PCR diagnostic using the 81F/691R primer set as described previously. Control Uzi flies (i.e. flies infected by *Wolbachia*) were maintained by feeding 8% glucose in distilled water soaked with cotton balls.

2.4 High temperature treatment for removing *Wolbachia*

Approximately 200 flies from the stock were reared at 33±1°C in an insect rearing chamber/BOD incubator for up to six generations. The elimination of *Wolbachia* was confirmed by diagnostic using the 81F/691R primer set as described previously.

2.5 Crossing experiments

Each treatment (heat and antibiotic treatments) were performed during six generations for removing *Wolbachia*. Individuals used for the crosses come from the 7th generation. Four combinations of experimental crosses using two day-old virgin flies with a ratio of 2:1 males and females were conducted: crosses between uninfected males and females; crosses between infected males and females; crosses between infected males and uninfected females and crosses between uninfected females and infected males. These flies remained together for 24 h. Uninfected individuals are individuals that have been cured by antibiotic therapy or high temperature treatment (33°C; the control flies have been reared at 26°C). These flies are referred in the present study as uninfected (UI) population and control flies as infected (I) population. For each type of cross, the fecundity and the hatching rate (proportion of adult emerging) were determined. Fecundity was measured by counting the number of eggs the uziflies laid on the body of silkworms. These eggs are visible to the naked eye. Hatchability was measured by counting the black scars on the silkworm body. These scars are due to the entrance of the larvae in their host body.

2.6 Effects of climatic variation on the Uzi fly

In order to analyze the effects of climatic variation on Uzi fly fitness traits a set of month wise experiments were conducted in Ramanagaram Uzi fly populations (Karnataka state in Southern India) from June 2005 to June 2007. The monthly mean temperatures are indicated Fig. 2. Individuals were collected in the field and directly used to perform crossing experiments. Two males and one female were put together for 24 h (12 replicates per modality). The rate of oviposition, fecundity and hatching rate were calculated as described previously.

2.7 Statistical analysis

For each cross 20 to 24 replicates were done three times except for the crosses involving individuals from the field (12 replicates). Means and standard errors were calculated and results were analyzed using the R statistical software (Team 2006). Data normality and homoscedasticity were verified by Shapiro and Bartlett tests respectively. Single factor and two-ways ANOVA were done for multiple comparisons and pairwise student's t-tests were used for two by two comparisons (probability level of significance: 0.05).

3 Results

3.1 Effects of antibiotic and high temperature treatments on *Wolbachia* infection in Uzi fly populations

It took up to six generations of treatments with high temperature (33°C) or antibiotics to cure the *Wolbachia* infection. The elimination of *Wolbachia* was confirmed by PCR realized on the *wsp* gene. The crossing experiments were performed on untreated individuals from the 7th generation.

3.2 Crossing experiments

3.2.1 Curing Wolbachia by antibiotic treatment

Measures of fecundity and hatching rate in crosses involving infected individuals and individuals treated by antibiotic treatment for removing *Wolbachia* are presented

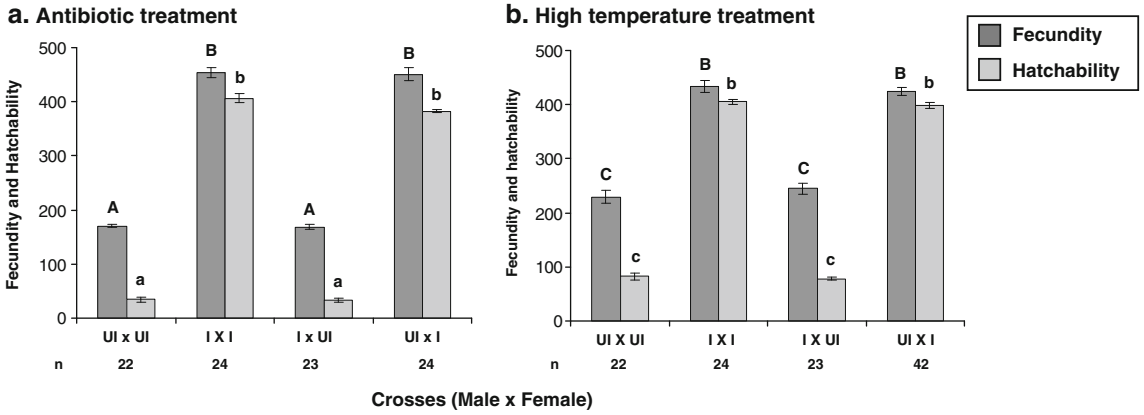

Fig. 1 Influence of the *Wolbachia* removal on fecundity and hatchability. Mean values of the fecundity (number of eggs laid) and the hatchability (number of adult emerged) measured in crosses between infected (I) and individuals cured by antibiotic treatment (UI). *Wolbachia* was removed by antibiotic (**a**.) or heat treatment (**b**.). n indicates the number of matings tested (3 replicates each time). Bars show standard errors. Means indicated with the same letter are not significantly different (pairwise student-t-tests; probability level of significance: 0.05)

Fig. 1a. Results are significantly different among crosses for both traits (One-way ANOVA: $F_{3,8}$=416.6, p<0.0001 for the fecundity, $F_{3,8}$=1538.6, p<0.0001 for the hatching rate). Two-way ANOVA results revealed that these differences are only explained by the infection status of the female, without any influence of the infection status of males, for both fecundity and hatching rate (Table 1). Indeed, the same results are obtained in crosses between infected females and infected or uninfected males, and in crosses between uninfected females and infected or uninfected males. On the other hand, results are significantly different among crosses that involve infected or uninfected females (Fig. 1a). Infected females lay around 450 eggs and the hatching rate is higher than 80% while the fecundity of uninfected females is close to 170 eggs with 20% of hatching rate. For all the crosses, sex-ratio ranges from 51 to 55% (percentage of males).

3.2.2 Curing Wolbachia by high temperature treatment

Results obtained in crosses involving individuals that have been cured by high temperature treatment (Fig. 1b) are similar to the results obtained with individuals that have been cured using antibiotic treatment (Fig. 1a). There is a significant difference among crosses for the two fitness traits (one-way ANOVA: $F_{3,8}$=119.8, p<0.0001 for the fecundity, $F_{3,8}$=1422.8, p<0.0001 for the hatching rate), which is not due to the infection status of males but only to the fact that females are infected or not by *Wolbachia*, as indicated by statistical comparisons (two-way ANOVA, Table 1). Fecundity of infected females is around 1.5 times higher than the one of uninfected females (around 430 eggs and less than 300 eggs respectively) and the hatching rate more than twice higher (around 94% for infected females and between 31.90 and 35.38 for uninfected females). Males represent between 50.5 and 55% of the offspring.

3.3 Comparison between the antibiotic therapy and the heat treatment

We have investigated whether the type of treatment performed to remove *Wolbachia*, antibiotherapy or heat treatment has an influence on the fecundity or the hatching rate. Similar results were obtained in crosses between infected males and females (Pairwise-*t*-test, p=1; Fig. 1a, b)

Table 1 Statistical analysis by two-way ANOVA for fecundity and hatching rate measured in crosses between infected and individuals cured by antibiotherapy or heat treatment. These comparisons were performed to test for the influence of the factors "infection status" of males and females. Bold typeface indicates significant effects

Treatment	Source	Fecundity		Hatchability	
		F	*p*	F	*p*
Antibiotic treatment	Male	$F_{1,8}$=0.015	p=0.904	$F_{1,8}$=4.971	p=0.056
	Female	$F_{1,8}$=1249.7	***p*<0.0001**	$F_{1,8}$=4605.4	***p*<0.0001**
	Male x Female	$F_{1,8}$=0.110	p=0.748	$F_{1,8}$=5.255	p=0.05
Heat treatment	Male	$F_{1,8}$=1.362	p=0.277	$F_{1,8}$=0.029	p=0.869
	Female	$F_{1,8}$=357.9	***p*<0.0001**	$F_{1,8}$=4267.2	***p*<0.0001**
	Male x Female	$F_{1,8}$=0.119	p=0.739	$F_{1,8}$=1.102	p=0.324

which can be considered as control experiments, the data obtained in these two sets of experiments can thus be compared. While comparable results have been obtained in crosses involving infected females (Pairwise-*t*-test, $p>0.98$), they significantly differ in crosses that involve uninfected females, whatever the infection status of the male (Pairwise-*t*-test, $p<0.0004$): females cured by antibiotic treatment lay around 170 eggs with a hatching rate of 20% while the fecundity of females cured by high temperature is higher, between 244 and 300 eggs, with a hatching rate comprised between 31.9% and 35.8%. These data suggest that the type of the treatment used for removing *Wolbachia* infection has an impact on these female fitness traits.

3.4 Effects of climatic variation on *E. sorbillans* fitness traits

Monthly temperatures recorded between June 2005 and June 2007 in Ramanagaram were highly variable (one-way ANOVA: $F_{11,12}=123.0$, $p<0.0001$): the average was 27.3°C with a range from 21.5°C in December to 34°C in May (Fig. 2).

The results also revealed a seasonal variation in the fecundity and the hatching rate of the Uzi flies (ANOVA: $F_{11,12}=122.98$, $P<0.0001$ for fecundity; $F_{11,12}=200.73$, $P<0.0001$ for hatching rate) (Fig. 2), with a constant sex-ratio (from 50.6 to 52.1% of males in the offspring). The highest and the lowest fecundities and hatching rates were observed respectively in summer, where moderate temperatures are observed, with a maximum in September (fecundity: 452.45±0.75, hatching rate: 425.8±2.1), and in spring (253.5±3.7 of eggs laid in February and 211.15±3.7 of adults emerging in May). Interestingly, the lowest fecundity and egg viability were observed in the months (May and June) following the highest temperatures recorded (April and May).

4 Discussion

4.1 Effect of *Wolbachia* infection on reproductive fitness traits of the Uzi fly *E. sorbillans*

Crosses between infected and uninfected (cured) males and females did not indicate any evidence for cytoplasmic incompatibility in *E. sorbillans*. On the other hand, results clearly revealed the existence of a strong direct benefit of *Wolbachia* infection for females as previously described (Puttaraju and Prakash 2005). The infected females' fecundity (number of eggs laid) and the viability of their eggs (the hatching rate) are significantly higher than that of cured females. Therefore, the widespread occurrence of *Wolbachia* in natural populations of *E. sorbillans* seems to be due to the positive effects of *Wolbachia* infection and not to reproductive manipulation by the bacteria. Such cases of "mutualistic" associations with *Wolbachia* are rather rare in arthropods. They have been found in the mosquito *Aedes albopictus* (Dobson et al. 2002) and in the fly *D. melanogaster* (Fry et al. 2004; Brownlie et al. 2009). In insects there is, at the time, two descriptions of extreme mutualistic interactions between *Wolbachia* and its host since, in these cases, the bacteria is necessary for normal reproduction: in the parasitoid wasp *Asobara tabida* (Dedeine et al. 2001) and in the bedbug *Cimex lectularius* (Hosokawa et al. 2010). However, these examples are atypical and usually *Wolbachia* manipulates its host's reproduction. Further studies should determine how *Wolbachia* induces reproductive benefit in *E. sorbillans*. A recent study revealed that, in *D. melanogaster, Wolbachia* can confer compensatory effect during conditions of nutritional stress (Brownlie et al. 2009). Experiments on infected and uninfected females reared in various nutritional conditions should be done to determine whether *Wolbachia* could play a role in metabolic provisioning of

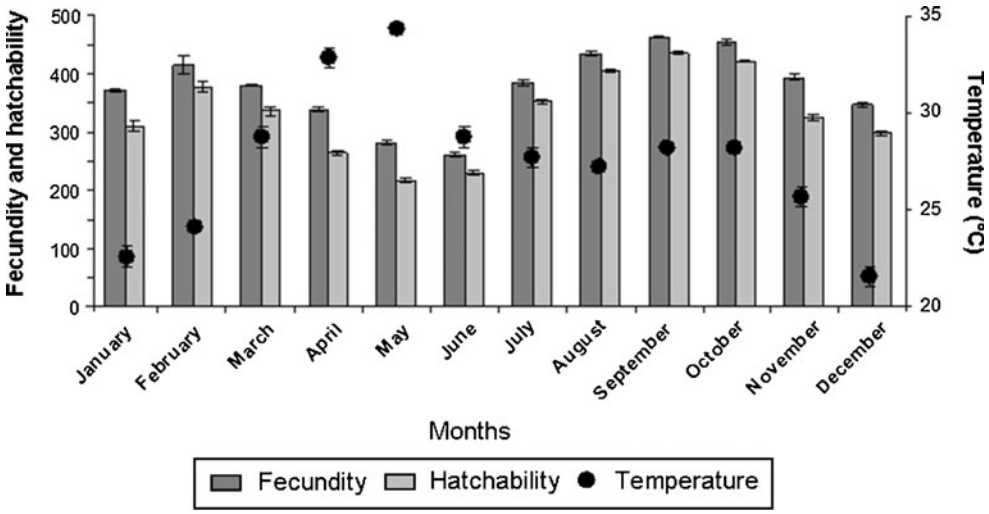

Fig. 2 Fecundity and hatchability of the Uzi fly *E. sorbillans* across year and monthly temperature. Mean monthly values of the fecundity (number of eggs laid) and the hatchability (number of adult emerged) of the Uzi fly and monthly averages of temperatures in Ramanagaram (Karnataka). Bars show standard errors. 12 replicates were performed for fecundity and hatchability and 2 measures per month were realized for calculating the mean temperatures

the uzifly. The influence of *Wolbachia* could also be investigated on non-reproductive fitness traits such as survival or protection against natural enemies.

4.2 Influence of the treatment used for removing *Wolbachia*

Both antibiotic (as previously observed by Kyei-Poku et al. 2003) and high temperature (33°C) treatments allowed the elimination of *Wolbachia* from *E. sorbillans*. Results obtained with these two methods are congruent since a decrease in the number of offspring is observed for cured females. However, both the fecundity and the hatching rate greatly differ according to the treatment used for removing *Wolbachia*: females treated by antibiotics lay 1.5-fold fewer eggs than females cured of *Wolbachia* by heat. The same difference in the hatching rate is observed. It is unlikely that heat treatment increases reproduction. On the contrary, the energy-demanding processes developed in response to heat stress are likely to induce costs, including detrimental effects on reproduction (Krebs and Loeschcke 1994; Silbermann and Tatar 2000). The differences observed could be more probably explained by a negative effect of the antibiotic therapy on the host physiology. Effects of antibiotic treatments have been studied previously in other insect species. In *Drosophila simulans*, *D. melanogaster* or *Asobara tabida* the use of tetracycline to remove *Wolbachia* did not affect the host fitness (Poinsot and Merçot 1997; Dedeine et al. 2001; Fry et al. 2004). However, we cannot discount the possibility that antibiotics affect the *E. sorbillans* fecundity. Moreover, it is possible that tetracycline treatment kills other bacteria acting on the Uzifly fecundity.

4.3 Effects of seasonal variation on reproductive fitness traits of the Uzifly *E. sorbillans*

The monthly monitoring of temperatures over a period of 2 years revealed variations across seasons, with a range from 21 to 34°C. The fecundity and the hatching rate of the Uzi fly also varied over the year: they are high when moderate temperatures are observed, from July to October, and they are low in May and June when the highest temperatures were recorded in April and May. Therefore, there may be a link between the temperature and the reproductive capacities of *E. sorbillans*: since the generation time of the uzifly is 25 to 28 days it seems that high temperatures during the development (in April and May) lead to a decrease of the female fecundity and egg viability (observed in May and June). These results may be explained by a direct effect of temperature on the *E. sorbillans* fitness since temperature has profound effects on all biological and physiological processes of ectotherms (Cossins and Bowler 1987; David et al. 1983). Typically, performance increases with temperature up to some maximal or optimal level and then declines (Huey and Berrigan 2001). Many studies demonstrated the impact of temperature on insect fecundity, for instance in *Drosophila* species and their parasitic wasps (Ris et al. 2004). The influence of temperature on *E. sorbillans* reproduction can also be indirect by acting on *Wolbachia* density. Indeed, temperature affects the maternal transmission efficiency and the replication rate of micro-organisms (Douglas 1994). In the field, all individuals of the uzifly harbour *Wolbachia*, whatever the season, but the bacterial density may change which can affect the associated phenotype, as demonstrated in the wasp *Leptopilina heterotoma* (Mouton et al. 2004; Mouton et al. 2006). The direct or indirect influence of temperature on the uzifly reproduction could be investigated in the lab by measuring the fecundity and the *Wolbachia* density of females reared at different temperatures. Finally, the phenotypes observed probably result of complex interactions between host and bacteria, both subject to direct effects of temperature.

5 Conclusion

In summary, *Wolbachia* infection positively affects the fecundity and the hatching rate of *E. sorbillans* females. Moreover, we observed a seasonal variability of the offspring production linked to temperature variations. Further investigations should focus on determining direct and indirect impacts of environmental factors on the Uzi fly fecundity. More studies are also required to understand how the presence of *Wolbachia* leads to a reproductive fitness benefit. This knowledge could be exploited for effective control strategies of the Uzifly, a serious menace of silkworm *Bombyx mori*. Combining molecular science with ecological studies could result in the development of novel, cost-effective, and efficient pest control strategies.

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