THE EFFECT OF IRON ON THE DEFENSIVE MUTUALISM OF *Spiroplasma* BACTERIA AND *Drosophila* FLIES

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Effect of Iron on the Defensive Mutualism of *Spiroplasm*a Bacteria and *Drosophila* Flies.

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Maternally-transmitted associations between endosymbiotic bacteria and insects are pervasive in nature, and vary from commensalism to mutualism to parasitism. The association between the fruit fly *Drosophila melanogaster* and the maternally-inherited bacterium *Spiroplasma* involves reproductive parasitism and an apparent defensive mutualism. When attacked by the parasitoid wasp, *Leptopilina boulardi*, *Spiroplasma*-infected flies have a higher survival rate than their *Spiroplasma*-free counterparts. The mechanisms by which *Spiroplasma* prevents successful development of wasps are not understood, except that wasps exhibit slower growth when developing within a *Spiroplasma*-infected fly. One possible mechanism may involve competition between *Spiroplasma* and the wasp for a limiting resource. In this study, we investigated the role of iron in the *Spiroplasma*-mediated defense against the parasitoid wasp *Leptopilina boulardi*. Iron levels in the fly host were manipulated by rearing flies on diets that differed in the amount of iron available. Growth of the wasp larvae was monitored by measuring body length at 0, 72, and 144 hours post-oviposition. In the absence of *Spiroplasma*, iron levels had little effect on wasp growth. In the presence of *Spiroplasma* however, iron levels had a significant effect on wasp growth. The growth rate of wasps was lowest in the high-iron treatment and highest in the low-iron treatment. Indeed, in the latter treatment, the growth rate

of the wasp resembled that of wasps reared in *Spiroplasma*-free hosts. These results imply that while competition for iron does not seem to be the mechanism of host defense, iron plays a role in the *Spiroplasma*-mediated defense against *L. boulardi*.

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CHAPTER I

INTRODUCTION

The bacterial genus *Spiroplasma* (class Mollicutes) contains several strains of maternally transmitted endosymbionts of flies of the genus *Drosophila* (Haselkorn, 2010). Although the evolutionary consequences associated with *Spiroplasma* infection remain poorly understood, *Spiroplasma* has been shown to offer increased resistance to nematodes (Jaenike et al., 2010), as well as fitness benefits to its host when exposed to attack by the parasitoid wasps, *Leptopilina boulardi* (Xie et al., in review) and *Leptopilina heterotoma* (Xie et al., 2010). Wasp larvae in *Spiroplasma*-infected hosts experienced impaired growth as compared to their counterparts in uninfected hosts (Xie et al., 2011). The mechanism by which *Spiroplasma* protects its host is not currently understood. Xie et al. offer three possible explanations: a) competition between *Spiroplasma* and the wasp larvae for some limited resource; b) *Spiroplasma* produces a substance toxic to the wasp larvae; or c) *Spiroplasma* enhances the fly's immune response to parasitoid attack. Here we attempt to shed light on the first hypothesis; whether competition for a resource, specifically iron, between *Spiroplasma* and the developing wasp larva contributes to reduced growth, and ultimately death, of the wasp.

Although the role iron plays in insect immunity is not yet fully understood, recent studies have suggested an important role for iron sequestration in protection against bacterial and parasitic invasions (Gregorio et al., 2001; Yoshiga et al., 1997). Endosymbionts, such as *Wolbachia*, have also been found to play roles in the metabolism and utilization of iron by their hosts (Kremer et al., 2009; Brownlie et al., 2009). Genomic comparisons of different strains of the endosymbiont,

Regiella insecticola, in Pea Aphids found that strains with more genes for iron transporters and iron transport systems better protected their hosts from attack by the parasitic wasp, *Aphidius ervi* (Hansen et al., 2011). This suggests that endosymbionts may influence host immunity through changes in host iron metabolism.

CHAPTER II

METHODS

Fly strains, antibiotic treatment, and artificial infection

In this experiment, we used an isofemale line of *Drosophila melanogaster* prepared as described in Xie et al. (2010). All experiments were conducted at least five generations after antibiotic treatment and artificial infection of the flies to create *Spiroplasma*-infected lines and *Spiroplasma*-free control lines. Artificial infection of the antibiotic-treated flies was performed as described in Xie et al. (2010), through adult-to-adult direct hemolymph transfer using pulled mircocapillary tubes and a manual microinjector. Infection status of the transfected flies was verified as described in Xie et al. (2010), using PCR and/or dark field microscopy. Throughout the study, flies were maintained at 25°C on a 12:12 hour light:dark cycle.

Iron treatments

Flies were maintained on one of three food treatments: low, standard, or high iron. The standard food was plain cornmeal medium modified from the Drosophila Species Stock Center, San Diego, California, to make 1 L of food instead of 28 L. The exact recipe was as follows. In a covered pot, 630 mL distilled water was boiled for 10 minutes. In a separate container, 260 mL distilled water was mixed with 14.54 g Agar, 32.91 g enriched yellow cornmeal, 52.26 g sugar, and 18.4 g inactive dry yeast. This mixture was then added to the boiling water and cooked for 8 minutes, stirring constantly. Then 130 mL distilled water was added to the pot, and the mixture cooked for another 6 minutes. The pot was then removed from heat and allowed to cool to 60°C. Next, a solution of 4 g sodium propionate dissolved in 11 mL 100% ethanol was added. Lastly,

0.955 g of methylparaben was mixed into the food. The food was then poured into vials (~10 mL food/vial) and allowed to solidify overnight. The low iron food was made as described in Brownlie et al. (2009), except four Tetley tea bags were steeped in 630 mL of distilled water for 5 minutes. This water was then used to prepare the standard cornmeal medium. The high iron food was also made as described in Brownlie et al. (2009), by adding $FeCl_3$ to standard cornmeal food at a final concentration of 10mM.

Wasp growth rate

Prior to each experiment, adult male and female *Drosophila melanogaster* were placed in a vial and allowed to oviposit until approximately 100 eggs had been laid. The fly parents were then removed from the vial, and hatching larvae were allowed to mature for 2 days. Five young female *Leptopilina boulardi* were then placed in the vial and allowed to oviposit in the larvae for 2 days, after which they were removed from the vial (0 hours post attack). Five fly larvae were then selected at random and dissected in PBS buffer to isolate the wasp larvae. The wasp larvae were then fixed in 100% ethanol, and digitally photographed under a dissecting microscope with a stage micrometer (0.01 mm scale). We used Spot Basic (version 4.7; Diagnostic Instruments, Inc., Sterling Heights, MI) to measure wasp body length as the straight-line distance from the tip of the proximal end to the caudal end (excluding the caudal appendage). This was repeated at 72 hours and 144 hours post-wasp attack. We used 5 replicates for each food treatment and each infection status (overall: 2 endosymbiont treatments X 3 food treatments X 3 time points X ~5 reps ≈ 92 reps).

Statistical analysis

A General Linear Model (GLM, JMP) was used to examine the effect of infection state (fixed), hours post-wasp attack (fixed), food treatments (fixed) and all their two-way and three-way interactions. Due to the finding of significant interaction terms, the following analyses were performed. The effect of *Spiroplasma* was determined by a subsequent GLM analysis of infection state (fixed), hours post-wasp attack (fixed) and their two-way interaction in each food treatment. In addition, the effect of iron was examined by a GLM analysis of food treatments (fixed), hours post-wasp attack (fixed) and their two-way interaction in each *Spiroplasma* infection state. Subsequent post-hoc analyses with Tukey's HSD or student's t-test were used to compare the difference among the three food treatments and between *Spiroplasma* infection states in different time points.

CHAPTER III

RESULTS

In the overall model, *Spiroplasma* infection state, iron treatment, hours post-wasp attack, and their interactions all had highly significant (P<0.0002) effects on the growth rate of wasp larvae. Therefore, the effects *of Spiroplasma* infection state and the different iron treatments were examined further.

Spiroplasma infection state

In the standard and high iron treatments (SF and HF, respectively), *Spiroplasma* infection state, hours post-wasp attack and their interaction had significant effects on wasp larvae growth (Table 1; $F_{(1, 25)}$ = 53.51, P<0.0001, $F_{(2, 25)}$ = 182.93, P<0.0001, $F_{(2, 25)}$ = 21.89, P<0.0001; $F_{(1, 24)}$ = 72.93, P<0.0001, $F_{(2, 24)}$ = 96.33, P<0.0001, $F_{(2, 24)}$ = 34.93, P<0.0001, respectively). These results are consistent with the previous finding of *Spiroplasma* killing wasps in standard food (Xie et al., in review) and indicate this protection also functions in the high iron treatment. However, in the low iron treatment (LF), the effect of *Spiroplasma* infection state on wasp larvae growth was not significant ($F_{(1, 25)}$ = 3.99, P = 0.0569, Table 1, Figure 1). At the last time point examined (144 hours), wasp larvae reached a similar length in both *Spiroplasma* infected and uninfected flies ($F_{(1, 8)}$ = 0.6522, P = 0.4427, mean length: 1.39 mm ±SE = 0.0898, mean length: 1.49 mm ±SE = 0.0898, respectively), indicating the protection conferred by *Spiroplasma* was lost in the low iron treatment.

Iron treatment

In the absence of *Spiroplasma*, the effects of iron treatment, hours post-wasp attack, and their interactions were significant (Table 1; $F_{(2, 36)} = 12.75$, $P<0.0001$, $F_{(2, 36)} = 558.06$, $P<0.0001$, $F_{(4, 36)} = 5.19$, $P = 0.0021$, respectively). The significant interaction indicates that wasp growth rate differed among the three food treatments. However, wasp larvae appeared to reach a similar length (1.50 mm ± SE= 0.025) at 144 hours post-wasp attack, but with a different trend ($F_{(4, 36)} = 5.19$, $P = 0.0021$, shown by the different letters in Figure 2). Overall, these results indicate that the iron content of food had effects on wasp growth around 72 hours post-wasp attack, but the effects disappeared later and thus, should cause no appreciable difference in wasp emergence in the absence of *Spiroplasma*.

In the presence of *Spiroplasma,* the effects of iron treatment, hours post-wasp attack and their interactions were significant (Table 1; $F_{(2, 38)} = 19.28$, $P<0.0001$, $F_{(2, 38)} = 90.42$, $P<0.0001$, $F_{(4, 38)} = 14.50$, $P<0.0001$, respectively). The significant interaction indicates that wasp growth rate differed among the three food treatments. Wasp larvae in the high, standard, and low iron treatments appeared to reach three very different lengths at 144 hours post-wasp attack (mean length: 0.61 mm ±SE= 0.081, 0.92 mm ±SE= 0.074, 1.39 mm ±SE= 0.081, respectively,) with dramatically different trends ($F_{(4, 38)} = 14.50$, P< 0.0001, shown by the different letters in Figure 2). Wasp growth in the high iron treatment appeared to be slow, indeed wasps hardly grew between 72 and 144 hours post-wasp attack, and only reached a final mean length of 0.61 mm (±SE= 0.081), whereas wasp larvae in the low iron treatment reached approximately the same length (mean length: 0.701 mm ±SE= 0.070) at 72 hours post-wasp attack. The growth of wasp larvae in the standard iron treatment appeared to be faster, reaching a greater length (0.89

mm ±SE= 0.070) at 72 hours post-wasp attack than the other treatments. However, growth stopped between 72 and 144 hours post-wasp attack (Figure 2). These results suggest that the time at which *Spiroplasma* kills wasps is earlier in the high iron treatment than in the standard iron treatment, although both maintain the *Spiroplasma* protection effect, whereas the protection effect was lost in the low iron treatment.

Table 1: GLM Analysis of Effect of Iron Treatment and *Spiroplasma* Infection State on Wasp Larvae Growth

Effects Tests:	Sprioplasma	Hour	SpiroplasmaXHour	Treatment	Hour	TreatmentXHour	Mean Larva Length ±SE
Treatment							
HF	$F_{(1, 24)}$ = 72.93, P <0.0001	$F_{(2, 24)}$ = 96.33, P <0.0001	$F_{(2, 24)}$ = 34.93, P <0.0001				In: 0.49 ±0.029
							Un:0.84 ±0.029
LF	$F_{(1, 25)}$ = 3.99, P = 0.0569	$F_{(2, 25)}$ = 184.46, P< 0.0001	$F_{(2, 25)}$ = 0.041, P = 0.96				In: 0.76 ±0.036
							Un: 0.89 ±0.037
SF	$F_{(1, 25)}$ = 53.51, P <0.0001	$F_{(2, 25)}$ = 182.93, P <0.0001	$F_{(2, 25)}$ = 21.89, P <0.0001				In: 0.72 ±0.040
							Un: 1.010 ±0.041
Infection State							
Spiroplasma Uninfected (Un)				$F_{(2, 36)}$ = 12.75, P <0.0001	$F_{(2, 36)}$ = 558.06, P <0.0001	$F_{(4, 36)}$ = 5.19, P = 0.0021	HF: 0.84 ±0.025
							LF: 0.89 ±0.025
							SF: 1.01 ±0.025
Spiroplasma Infected (In)				$F_{(2, 38)}$ = 19.28, P <0.0001	$F_{(2, 38)}$ = 90.42, P <0.0001	$F_{(4, 38)}$ = 14.50, P <0.0001	HF: 0.49 ±0.036
							LF: 0.76 ±0.035
							SF: 0.72 ±0.035

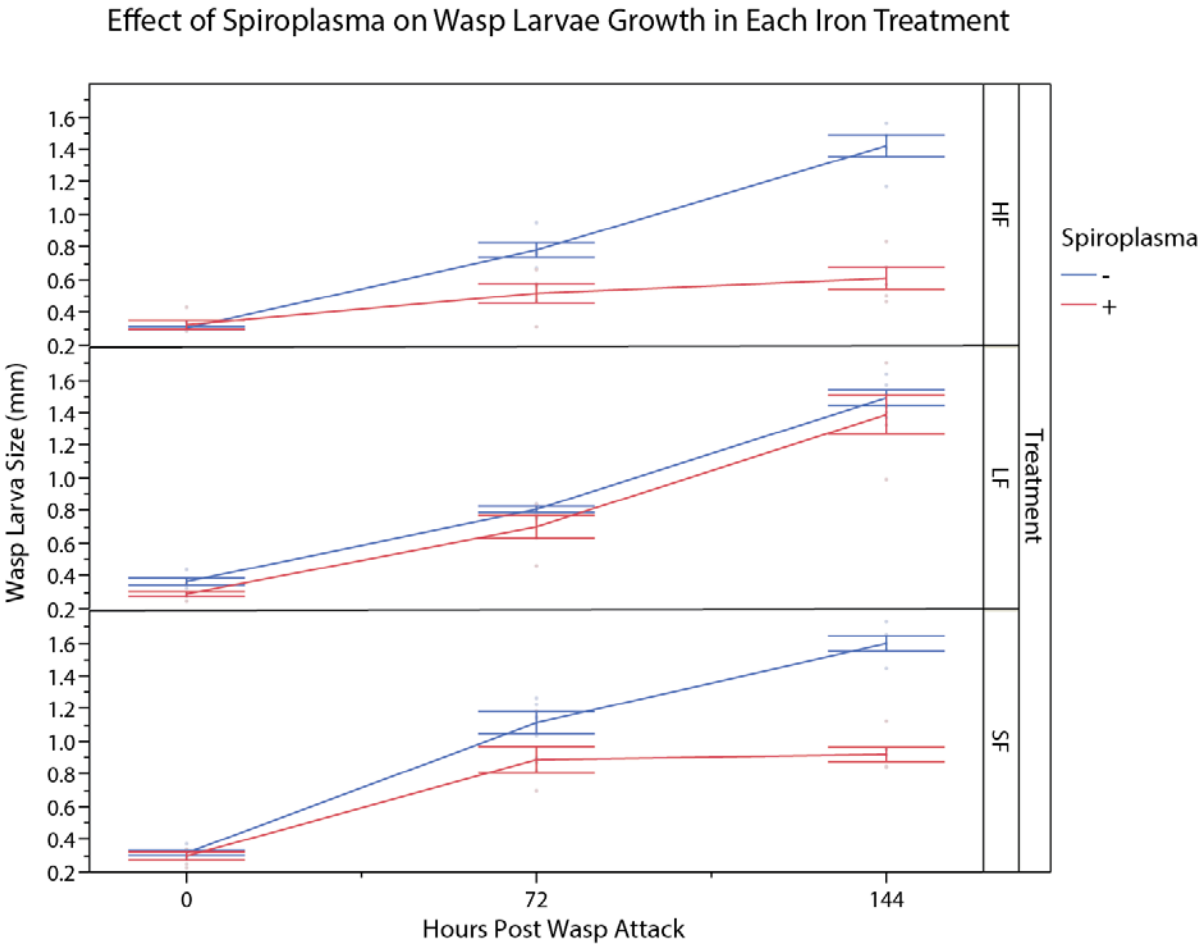

Figure 1: The effect of *Spiroplasma* infection state on wasp larvae growth in each iron treatment. Error bars show one standard error from the mean. Note loss of *Spiroplasma* protection in the low iron treatment.

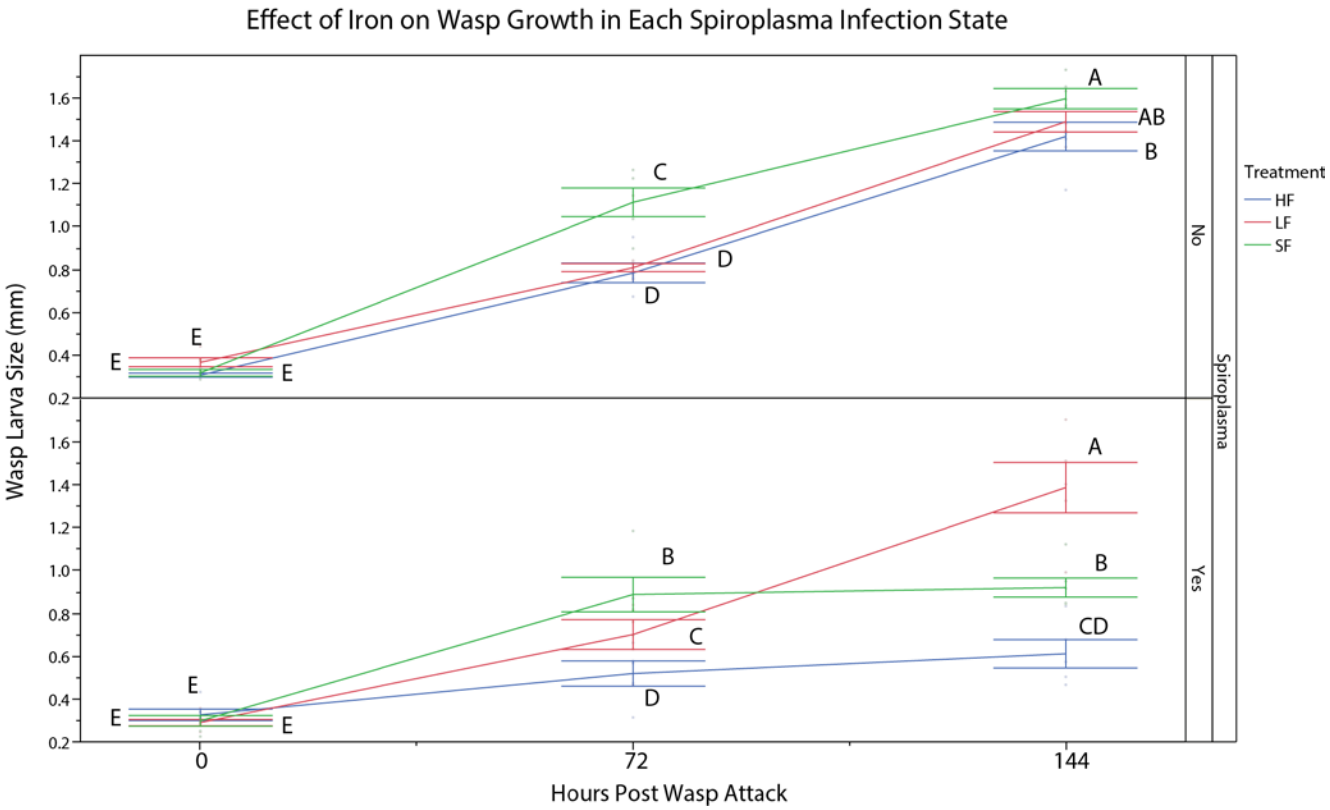

Figure 2: The effect of iron treatment and *Spiroplasma* infection state on wasp larvae growth, grouped by infection state. Error bars show one standard error from the mean. Different letters indicate where values are significantly different.

CHAPTER IV

CONCLUSION

The purpose of this study was to investigate the role of iron in the defensive mutualism between *Drosophila melanogaster* and *Spiroplasma* bacteria when the flies are attacked by the parasitoid wasp, *Leptopilina boulardi*. More specifically, we wanted to determine if competition for iron between the bacteria and the developing wasp larvae represented a mechanism by which *Spiroplasma* confers fitness benefits to wasp attacked flies.

In *Spiroplasma* uninfected flies, iron treatments seemed to have little effect on wasp larvae growth. However, another study in which the exact number of wasp and fly adults emerging are examined may be needed to confirm that the effects seen at 72 hours post-wasp attack do indeed disappear at 144 hours post-wasp attack, as suggested by the current study.

In contrast to the uninfected flies, in *Spiroplasma* infected flies, iron treatments had significant and varied effects on wasp larvae growth. Wasp larvae in the high iron treatment did the worst of all of the treatments, while those in the low iron treatment grew to sizes comparable to their counterparts in uninfected flies. These results are in direct opposition to what would be expected if competition for iron between wasp larvae and the bacteria was contributing to the *Spiroplasma*-mediated protection of wasp attacked flies, in which case, wasp larvae in the high iron treatment should grow to be the same size or larger than their counterparts in the standard or low iron treatments. Therefore, we reject the hypothesis of competition for iron being the mechanism of *Spiroplasma* conferred protection of flies attacked by the parasitoid wasp, *L. boulardi*.

The marked contrast in wasp development between the iron treatments suggests that iron nonetheless plays an important role in the defensive mutualism between *Spiroplasma* and wasp attacked flies. It is possible that iron is required by *Spiroplasma* to replicate and reach a threshold titer in the host in order to confer protection. Accordingly, protection does not occur when iron levels in the diet are too low to support sufficient replication of the bacteria. In this case, another study in which *Spiroplasma* titers are compared across treatments may be able to shed more light on the role iron plays in this system. The results of the current study and the proposed future studies shed light on the mechanisms by which mutualisms and other symbioses may arise and be maintained in populations.

REFERENCES

Brownlie, J. C., Cass, B. N., Riegler, M., Witsenburg, J. J., Iturbe-Ormaetxe, I., McGraw, E. a, & O'Neill, S. L. (2009). Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia pipientis*, during periods of nutritional stress. *PLoS Pathogens*, *5*(4), e1000368. doi:10.1371/journal.ppat.1000368

De Gregorio, E., Spellman, P. T., Rubin, G. M., & Lemaitre, B. (2001). Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(22), 12590-5. doi:10.1073/pnas.221458698

Drosophila Species Stock Center. (2013). *Standard Cornmeal Recipe.* Retrieved from https://stockcenter.ucsd.edu/info/food_cornmeal.php

Hansen, A. K., Vorburger, C., & Moran, N. A. (2012). Genomic basis of endosymbiont-conferred protection against an insect parasitoid. *Genome Research*, *22*(1), 106-14. doi:10.1101/gr.125351.111

Haselkorn, T. S. (2010). The *Spiroplasma* heritable bacterial endosymbiont of *Drosophila*. *Fly 4*:80-87.

Jaenike, J., Unckless, R., Cockburn, S. N., Boelio, L. M., & Perlman, S. J. (2010). Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. *Science*, *329*(5988), 212-5. doi:10.1126/science.1188235

Kremer, N., Voronin, D., Charif, D., Mavingui, P., Mollereau, B., & Vavre, F. (2009). *Wolbachia* interferes with ferritin expression and iron metabolism in insects. *PLoS Pathogens*, *5*(10), e1000630. doi:10.1371/journal.ppat.1000630

Xie, J., S. Butler, G. Sanchez, and M. Mateos. (In review) Male killing *Spiroplasma* protects *Drosophila melanogaster* against two parasitoid wasps.

Xie, J., Tiner, B., Vilchez, I., & Mateos, M. (2011). Effect of the *Drosophila* endosymbiont *Spiroplasma* on parasitoid wasp development and on the reproductive fitness of wasp-attacked fly survivors. *Evolutionary Ecology*, *53*(5), 1065-1079. doi:10.1007/s10682-010-9453-7

Xie, J., Vilchez, I., & Mateos, M. (2010). *Spiroplasma* bacteria enhance survival of *Drosophila hydei* attacked by the parasitic wasp *Leptopilina heterotoma*. *PloS One*, *5*(8), e12149. doi:10.1371/journal.pone.0012149

Yoshiga, T., Hernandez, V. P., Fallon, a M., & Law, J. H. (1997). Mosquito transferrin, an acute-phase protein that is up-regulated upon infection. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(23), 12337-42.