

Fitness Costs of the Aphid Endosymbiont, *Hamiltonella defensa*

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Abstract:

Black sage aphids, *Obtusicauda frigidae*, are infected by the bacterial endosymbiont *Hamiltonella defensa*. The bacteria confers a resistance to parasitism by the solitary endoparasitic wasp *Aphidius ervi*. The bacteria also confers a number of other advantages to the aphids. Despite the number of advantages conferred by bacterial infection, *H. defensa* is only seen at intermediate frequencies in the aphid population. Our experiment was designed to test whether or not there is a fitness cost to the aphid by infection with *H. defensa* which would explain the intermediate frequency of infection. I placed infected and uninfected aphids on plants in bags. I dyed the initial population of aphids in order to track the mortality of only that initial population. Then I surveyed each population at weekly intervals in order to gather data on the effect of infection on the fitness initial and subsequent aphids. I found that uninfected aphids had lower mortality and that their offspring had higher survivorship than those of infected aphids.

Introduction:

Symbiosis is defined as the living together of two dissimilar organisms in mutualism, commensalism, amensalism, or parasitism (Oliver, 2006). Endosymbiotic bacteria live inside the cells of their host and are present in numerous organisms from humans, to termites, to marine worms and are particularly prevalent and well studied in the insect world (Verberkmoes *et. al* 2009; Hongoh *et. al* 2006; Dubilier *et. al* 2001). Each endosymbiont can have both benefits and or costs to the host organism. Their roles range from antagonistic to mutualistic. In the case of *Wolbachia*, the endosymbiont is antagonistic to the insect, feminizes males, induces parthenogenesis, and causes cytoplasmic incompatibility between the sperm of an infected male and egg of an uninfected female resulting in zygote death (Werren, 1997). All of these adaptations have positive implications for the bacteria but deleterious ones for its insect host. The reproductive costs to the host are fewer offspring and less genetic recombination. However,

. They can perform many positive activities for their host organisms including amino acid production, cellulose digestion, and even sulfur oxidation (Douglas and Prosser, 1992; Zimmer and Topp, 1998; Distell *et. al* 1988). In the case of *Buchnera aphidicola* (Douglas, 1998) the bacteria synthesizes essential amino acids for its aphid host. Contained within this range are numerous relationships which exist in between antagonism and mutualism.

The relationship between the bacteria *Hamiltonella defensa*, its associated bacteriophage, *Acrythosiphon pisum* Secondary Endosymbiont (APSE), and aphids is both complex and little studied. What we do know is that *H. defensa* is a gram negative gamma-proteobacterium which is transmitted vertically through the maternal lineage and is found in multiple tissues (Dale and Moran, 2006; Degnan, 2008). *H. defensa* is transmitted vertically from mother to clonal offspring and horizontal transmission is rare (Oliver, 2007; Casteñeda, 2009). Vertical transmission is significant to the ecology of the bacteria because in order to maintain its presence in the population the bacteria must increase rather than decrease the fitness of its host. Horizontally transmitted bacteria are not dependent on the survival of one of their hosts for their continuation in the population. With vertical transmission the host must survive long enough to produce offspring in order for bacterial DNA to be transmitted. For this reason, *H. defensa* has evolved to confer many positive traits to aphids. *H. defensa* also prevents the solitary endoparasitic wasp, *Aphidius ervi*, from parasitizing black bean aphids, *Aphis fabae*, and pea aphids *A. pisum* (Vorburger 2009; Oliver *et. al* 2003). This resistance to parasitism occurs through the production of toxins by the bacteria that kill the wasp larvae (Degnan, 2008; Oliver, 2009). *H. defensa* has also been demonstrated to increase the fecundity of infected aphids in numerous species including, *A. fabae*, *O. frigidiae*, and *A. pisum* (Casteñeda, 2009; Erickson,

Furthermore, *H. defensa* has been shown to confer an increased resistance to heat shock in *A. pisum* (Russell and Moran, 2006), and cause them to be larger at maturity (Casteñeda, 2009). With so many beneficial effects resulting from bacterial infection we would expect *H. defensa* to move toward fixation in the aphid population. However, *H. defensa* has only been detected at medium to low frequencies in the population at large (Oliver, 2007). The fact that *H. defensa* is seen at intermediate frequency in the overall population of both *O. frigidus* and *A. pisum* (Erickson, unpublished; Sandström *et. al* 2001; Tsuchida *et. al* 2002; Oliver *et. al* 2006) taken together with the data that infection frequency has been demonstrated to decrease in the absence of parasitoids by laboratory experiments (Oliver, 2007) points to two possible hypotheses: 1) there is an unknown cost to harboring the bacteria, and 2) Only certain aphid genotypes accept the bacterial infection and are maintained at low to medium frequencies. These hypotheses are not necessarily mutually exclusive.

Our experiment tested one of these hypotheses. We looked for an overall fitness cost to the population harboring bacteria. Infected aphids are demonstrably more fecund (Casteñeda, 2009; Erickson, unpublished; Oliver, 2007). What we want to know is whether bacterial infection causes the aphids to alter their reproductive strategy. Our hypothesis is informed by other insects and aphids such as *Podisus distinctus*, the spined soldier bug, which adopts a strategy of accelerated offspring production of lower fitness offspring in response to sublethal doses of pesticides (Guedes *et. al* 2009). Aphids have been shown to adopt the same strategy when exposed to bacterial infection and wounding (Altincicek, 2008).

A fitness cost resulting from bacterial infection could function as a barrier that prohibits facultative endosymbionts in aphids from becoming obligate endosymbionts. The current hypothesis regarding endosymbionts is that if they are beneficial they should become ubiquitous

both the host and endosymbiont (Dale and Moran, 2006).

If our work uncovers a fitness cost to infection it would suggest that the picture is more complicated. A fitness cost to bacterial infection would suggest that disadvantages to endosymbiont infection function as barriers to between obligate and facultative modes. Immunological disruption resulting from infection is a possible barrier to an endosymbiont becoming obligate. Whether or not an endosymbiont becomes obligate or stays facultative is a crucial factor in both bacterial and insect ecology.

Methods:

Field methods:

Our study was conducted in the Almont Triangle of the Gunnison National Forest. The Almont triangle is a dry sub alpine meadow 25 kilometers north of the city of Gunnison. We conducted our experiment in an open meadow which sits between 2500m and 2600m.

The experiment utilized the shiny black aphid, *O. frigidae* and their associated plant host, *Artemisia tridentata*, big California sage brush. *A. tridentata* is the dominant widespread shrub in Western North America (Beetle, 1960; McArthur *et. al* 1981) and functions as a plant host for a variety of hemipteran parasites. *O. frigidae* feed on the phloem of *A. tridentata*. Aphids in the Almont triangle are tended by the Northern thatch ant, *Formica obscuripes*. When the ants tend aphids they collect honeydew from aphids and protect them from predators (Way, 1963). *F. obscuripes* has been shown to have conflicting roles as both protector, and predator of other aphid species, *Pleotrichophorus utensis* and *Uroleucon escalantii* (Billick *et. al* 2007). It is possible that the *F. obscuripes* ants we allowed to tend *O. frigidae* ended up eating some of the

ments excluded ant tending so effects of predation should be equal between treatments.

O. frigidus is parasitized by multiple solitary oligophagous endoparasitic wasps including *Trioxys* sp., *Lysiphlebus* sp., and *A. ervi* (Erickson, Unpublished data). These wasps lay eggs inside aphids (Du *et. al* 1998). However, the bacterial endosymbiont and the bacteriophage APSE, confer a resistance to parasitism by *A. ervi* (Oliver *et. al* 2003; Ferrari *et. al* 2004).

Our experiment identified whether or not there is a cost to aphids from infection by the bacteria and its associated bacteriophage, APSE. We answered our question by quantifying the cost of the bacteria in terms of aphid mortality and the effect of the bacteria on effective fecundity.

Two treatments of 25 plant replicates (50 total replicates) with an initial population of 10 aphids were created in close proximity to the thatch mounds of the aphid's ant tenders, *Formica obscuripes*. The initial population of 10 aphids was dyed using powdered fluorescent pigment (Radiant Color, P.O. Box 4019, Richmond, CA 94804). The dyeing procedure was designed to track the mortality of the initial population of aphids separate from the mortality of the entire population. The aphids were marked by placing them in a Petri dish and painting them with a fine tipped paint brush. The aphids were then placed on a sage plant. The aphids were isolated by a mesh bag to prevent predation and to prevent aphid movement off of the plant. Mortality and effective fecundity, how many offspring survived, were measured at weekly intervals.

Each replicate was surveyed each week for two weeks and the number of dyed aphids and new aphids was recorded. At every survey date third instar aphids or larger were re-dyed. The assumption being that large un-dyed aphids were original dyed aphids that had molted and small un-dyed aphids were newly born aphids.

Our experiment was part of a larger experiment designed to also assess the affect of artificial infection on aphid populations. For this reason all aphids were fed sugar solution A, (Prosser, 1999) which also contained a homogenate of either aphids infected with *H. defensa* or uninfected aphids.

Screening Procedure:

Five aphids were collected from ten plants having aphid aggregations no smaller than 100 aphids. Each group of aphids was screened for *H. defensa*. Also, each group of aphids were screened for the presence of *B. aphidicola* to test the success of the extraction procedure. Whole genomic DNA was extracted from aphids utilizing the techniques pioneered by Bender *et. al* 1983. The genome was subjected to PCR in 10 µl reactions including 4-8 ng of total DNA, 1X PCR buffer (200 mM tris-HCL [pH 8.4], 500 mM KCL; New England Biotechnologies), 0.25 mM of each dNTP, 3.75 pM of each primer, 3.0 mM MgCl₂, and 0.5 units of Taq DNA polymerase (New England Biotechnologies) (Abbot & Withgott 2004). The primers for the *H. defensa* genome included GYRB87F and GYRB729R yielded amplicons of 750 base pairs. For *Buchnera* the primers used included Buch757F and Buch1507R and will yield amplicons of 850 base pairs. The DNA was cycled, to begin with, for 2 minutes at 94 °C, and the subsequent 35 cycles consisted of 30 seconds at 94 °C. Annealing was accomplished with 30 seconds at 52 °C followed by a 30 second extension temperature of 72 °C. The final extension was 6 minutes at 72 °C. The gene product was visualized on 2.5% agarose gels stained with EtBr.

Statistical analysis:

Statistical analysis was accomplished using JMP and consisted of a Wilcoxon between the dependent variables, effective fecundity and mortality, and the independent variable,

performed more than one test on the same data we used a Bonferroni correction of $\alpha = 0.025$ to establish statistical significance. A Fishers exact test was also performed to discern whether or not infected aphid populations were more or less likely to go to extinction

Results:

Throughout the experiment the average size of all aphid populations declined (Figure 1). Furthermore, as would be expected with population decline, the mean mortality of the original population of ten aphids rose to almost ten in both infected and uninfected aphid treatments (Figure 2).

Even with the Bonferroni correction there was a significant difference between the mortality between infected and uninfected aphids at week 2 (see Table 1). There was also a difference between the two groups' effective fecundity at week 2, although not a statistically significant difference. There was no significant difference between mortality and effective fecundity at week 3 (see table 1).

Table 1. Summary of statistical analysis between infected and uninfected aphids including a Bon Ferroni Correction of $\alpha = 0.025$.

Test	Sample Size	Z Value	P value
Wilcoxon Mortality Week 2	34	2.38435	0.0171
Wilcoxon Mortality Week 3	34	0.83413	0.4042

between effective fecundity and infection status. Bonferroni

correction of $\alpha = 0.025$

Test	Sample Size	Z Value	P Value
Wilcoxon Effective Fecundity Week 2	34	2.19008	0.0285
Wilcoxon Effective Fecundity Week 3	34	1.12842	0.2591

The results of the Wilcoxon of effective fecundity did not show a significant difference depending on infection status with a Bonferroni correction of $\alpha = 0.025$ in either week. However, during week two the p value came close to our threshold for significance indicating that there could be a trend for higher effective fecundity in uninfected aphids. Effective fecundity here refers to the number of offspring that survive rather than simply the number of offspring that are born.

Table 3. Summary of Fisher's exact test between infection status and population extinction

Test	Sample Size	R^2 value	P value
Fisher's exact test week 2	34	0.1076	0.0386
Fisher's exact test week 2	34	0.0162	0.6795

The results from the Fisher's exact test show that the infected populations were significantly more likely to become extinct than the uninfected ones. This is the trend you would expect with higher mortality.

we found that the effective fecundity of uninfected aphids, number of offspring that survived, was higher than that of infected aphids, although this was only a trend. The mortality of uninfected aphids was significantly lower than that of infected aphids. These results support our original hypothesis that aphids infected with *H. defensa* have higher mortality than uninfected aphids. Furthermore, we also found that the effective fecundity, or number of surviving offspring produced by our original aphids, was higher in the uninfected aphid population. However, this result was not statistically significant.

Higher mortality and lower effective fecundity of infected aphids contrasts with previous laboratory experiments (Casteñeda, *et. al* 2009; Oliver, *et. al* 2007) who found that infected aphid populations grew faster compared with uninfected populations. Our experiment was the first comparing survivorship between infection status in the field. However, the conditions of our experiment were artificial. Predators and parasitoids were excluded and there were no additional aphids let in by migration. The absence of parasitism is especially important because one of the major benefits of *H. defensa* is resistance conferred against parasitism. This resistance gives infected aphids an advantage in environments where parasitoids are present.

Despite the artificial conditions created by the experimental design, our results show a number of trends that point to the possibility that there could be a cost to infection by *H. defensa*. First, infected aphids had higher mortality in the original population of aphids than uninfected aphids in week 2 and infected aphid populations were more likely to go extinct than uninfected populations. These results indicate that infected aphids are not as resistant to stress and die more often than uninfected aphids. Second, uninfected aphids had higher effective fecundity than infected aphids during week 2. This result indicates that the offspring of uninfected aphids are

better than uninfected aphids. This result was not statistically significant with the Bonferroni correction of $\alpha = 0.025$. However, the p-value was around 0.028 meaning that it was close to significance and could indicate a trend of higher offspring fitness for uninfected aphids. All of our results together indicate that there is a cost to the aphids resulting from infection with *H. defensa* in the conditions imposed by our experiment.

All of our results were significant only for week two. Week three had minimal significance in all tests. As the experiment progressed many of the aphid populations became extinct. All of the replicates with zero aphids brought the means so close together that our tests could not discern differences between the two data sets.

There were several problems with our experimental design. First, we did not fully investigate or even successfully quantify the effect of the dye on the aphids. We observed behavioral changes in the aphids after they had been dyed. The aphids immediately removed their stylet from the plant and began to walk away from their original feeding site. Previous work suggests that some arthropods will respond to physical stressors like sub-lethal doses of pesticides by increasing their reproductive output and decreasing offspring quality (Guedes, *et. al* 2009). It is possible that the dye altered the normal reproductive dynamics of the aphids. Although we have no data to support this conclusion, it would be a valuable piece of information to investigate further. In the future a more effective way of tracking an initial population of aphids might be to carefully record the size of each individual in the initial population and then remove aphids from the subsequent F1 generation. This method would allow for a comparison of mortality without adding another factor, the dye, which could possibly confuse the results. Other problems with our experiment included the bag effect on the aphids and our inability to artificially infect the aphids with *H. defensa*.

the bag on the plant. We did not quantify the effect of the bag on the plant. However, it is possible that a ramet inside of a bag would receive less light and consequently be of a lower quality than a ramet outside of a bag. This alteration in quality could have different effects on the aphids depending on their infection status. We have no evidence to support this hypothesis but it would be an interesting phenomenon to investigate.

We were unable to artificially infect aphids with *H. defensa* despite trying a number of different procedures. In the future more success might be had by altering the procedure. First, smearing the Para-film the aphids were feeding through with crushed *A. tridentata* in order induce feeding. Second, grinding up some *A. tridentata* leaves into the homogenate in order to keep the aphids feeding on the homogenate might be more effective. Third, the procedure we used to isolate the bacteria from infected aphids was originally for isolating endosymbionts for analysis. When we tested our homogenate for the presence of *H. defensa* we did not find any bacteria, indicating that we had either removed the bacteria completely or reduced their numbers below the detectable threshold. On a subsequent infection attempt we made a homogenate where the aphids were ground up and not centrifuged. The resulting solution contained more aphid parts but also contained detectable levels of *H. defensa*. This new procedure could be a more effective method for transfecting aphids.

Aphids exert a large influence on crop production all over the world and cause billions of dollars in agricultural losses every year(citation? Can't find one). Predators and parasitoids can and have been used to control aphids all over the world (Sisgaard, 2002; Schmidt *et. al* 1905). Understanding the dynamics of a system that renders these methods of bio-control ineffective or less effective has great socioeconomic importance.

Hamiltonella informs our understanding of other crucial endosymbiotic bacteria like *Buchnera aphidicola*, *Regiella insecticola*, *Serratia symbiotica*, and *Arsenophonus sp.* It also informs our understanding of more phylogenetically widespread endosymbionts like *Wolbachia*. Our experiment suggests that there is a fitness cost to infection by *H. defensa*. This is an important piece of information because the mechanism for infection by endosymbionts is poorly understood. We do not know why most endosymbionts are distributed the way they are and know much less about the factors govern their evolution. We do not even fully understand how endosymbionts evolved in the first place. The cost to infection demonstrated by our results is an important piece of information that can be used to understand what drives the evolution of endosymbionts. For example, this cost sheds light on why some endosymbionts have evolved facultative relationships with their hosts while others have evolved obligate relationships. The cost to infection could function as a barrier to *H. defensa* becoming an obligate endosymbiont which would explain the relatively low frequency seen in aphids in the Almont triangle and in pea aphids (Erickson, unpublished data; Oliver *et. al* 2006).

While the experiment told us many things about *H. defensa*, a very important question to ask is: What does the experiment not tell us? Because we were unable to artificially infect aphids and did not control for aphid genotype by only using one clone we do not have any information on whether or not there is a genotype interaction between the bacteria and the aphid. I think there is a cost to being infected but I do not know if that cost is affected by aphid genotype. Also, some of the aphids were placed on new plants which also could have affected survivorship. Infected aphid genotype could interact with plant genotype and affect survivorship. However, there were a number of different aphid clones on a number of different plants which should have controlled for genotypic interactions between the aphids and the plants. Lastly, we observed a

the dye and collected observational data but did not find any statistically significant evidence that the dye affected aphid survival.

Our study found that aphids infected with *H. defensa* have higher mortality and that their young have lower fitness than uninfected aphids under the physiologically stressful conditions of our experiment. The existence of a cost to infection by *Hamiltonella defensa* demonstrated by our experiment is a crucial piece of information necessary for understanding the ecology *H. defensa*, aphids, and even other endosymbionts. We recommend repeating this experiment with the changes discussed and that further investigation be conducted.

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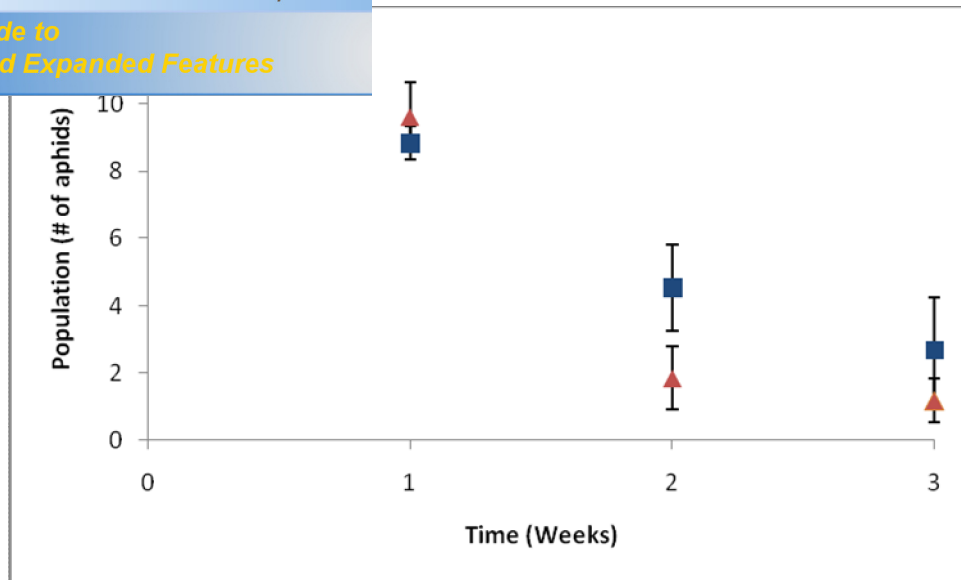


Figure 1. Mean population of infected and uninfected aphids compared with time. (y Mean population of uninfected aphids. z Mean population of infected aphids)

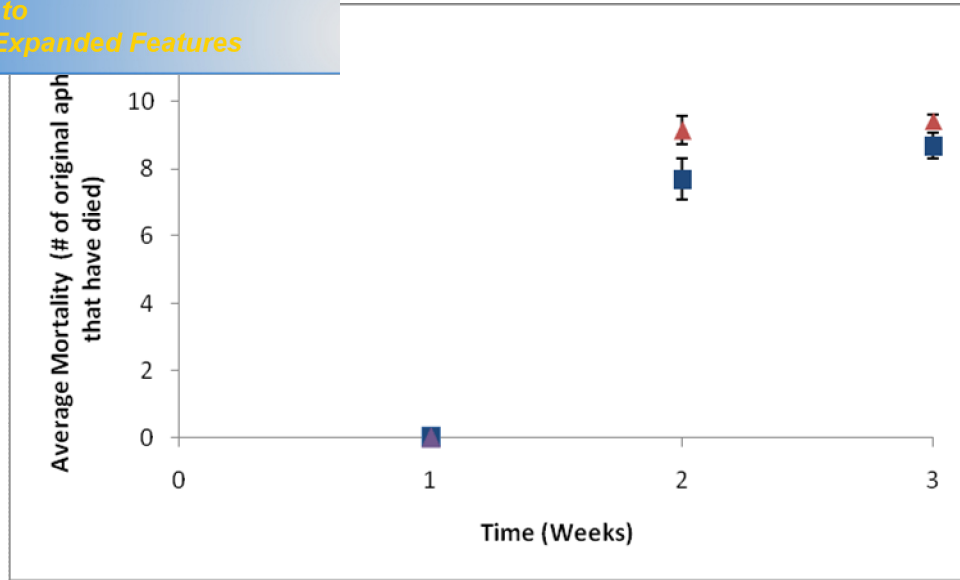


Figure 2. Mean mortality of original population of ten aphids compared with. time. (y' Uninfected aphids, z Infected aphids).

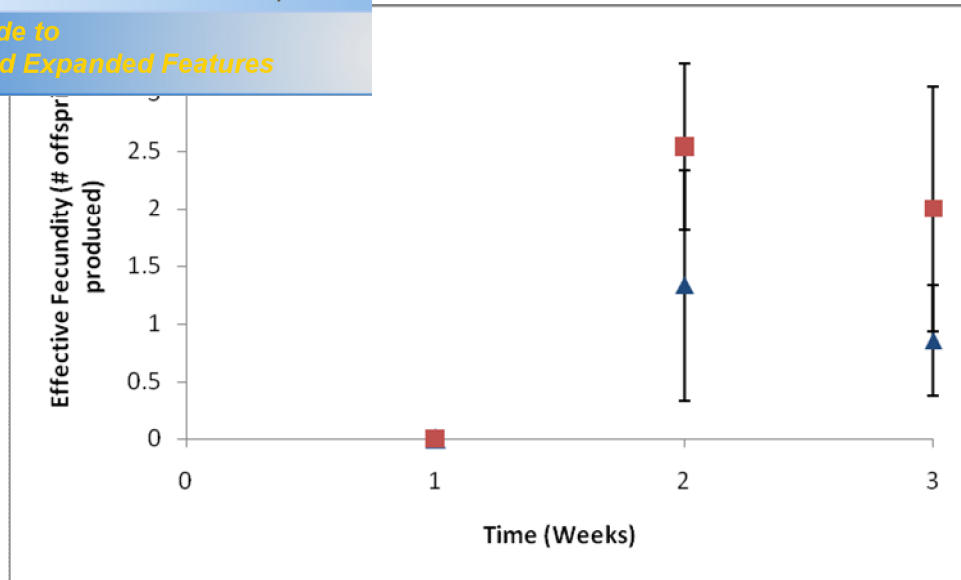


Figure 3. Effective fecundity, the mean number of offspring produced by the original aphids compared with time. (y Uninfected aphid population, z Infected aphid population).