

THE EFFECT OF ANTS ON THE POPULATION DYNAMICS
OF *HAMILTONELLA DEFENSA*,

A PROTECTIVE SYMBIONT OF APHIDS

By

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To my parents,
for encouraging me to love monsters from a young age

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CHAPTER I

INTRODUCTION

Interspecific facultative mutualisms, associations where two species provide non-essential beneficial services to each other, are prone to conflicts of interest. Mutualists can become parasites or abandon the association altogether when greater benefits are provided (Sachs and Simms 2006). Nonetheless, many mutualisms resist conflict by having little cost to their participants, or by evolving sanctions, rewards, or other persuasive mechanisms that encourage mutualism and discourage parasitism (Douglas 2008). However, among facultative mutualisms, ambient conditions can cause a switch from mutualism to parasitism, or vice versa. For example, the appearance of a third species can cause a facultative mutualism to break down by displacing a mutualist or providing greater benefits than one of the original mutualists. Mutualists that provide benefits to the same partner can sometimes coexist and sometimes compete for access to that partner. Whether or not mutualisms can coexist depends on the nature of the services provided, the costs and benefits provided by each mutualism, and the abiotic and biotic conditions of the environment (Palmer et al. 2003).

Defensive symbioses are mutualisms wherein at least one partner provides protective services against natural enemies or stressful abiotic conditions. Aphids are sap-sucking insects and common agricultural pests that can engage in mutualisms with two types of defensive symbionts: intracellular bacteria and ants. The potential interactions between aphid-bacteria and aphid-ant mutualisms have not been well studied.

In this chapter, first previous research that tested competition and coexistence between mutualisms with shared partners is discussed. Then the bacteria and ants used in the study system are described. Next, the potential interactions between these aphid-ant and aphid-bacteria mutualisms are predicted. Finally, research questions are presented.

Inter-mutualism competition and coexistence

Facultative mutualists can exploit or abandon their partners when a greater fitness benefit can be gained from not participating in the mutualism (Sachs and Simms 2006), unless sanctions otherwise prevent the mutualism from disassociating (Douglas 2008). A facultative mutualist that is presented with two mutualists at the same time is presented with new conditions where benefits and costs might be greater or less than participating with only one of the original mutualists. When benefits and costs are greater or the same, the two mutualists coexist. When the benefits and costs are decreased, the facultative mutualism breaks down. Alternatively, two mutualists can compete against each other for a partner and one can displace the other. On the other hand, there may be a lack of cost in participating in the mutualisms or the mutualists may be prevented from disassociating by some other mechanism.

Coexistence between mutualisms

There are many examples of multiple mutualist species providing the same service to one species. Many flowers allow multiple pollinator types and species with no significant competition between pollinators and no significant effect on rates of pollination between flowers. Direct competition is limited when pollination is dispersed

and nectar rewards are relatively unlimited between flowers (Waser et al. 1996). The ability for redundant mutualisms to be separated by distance or time, perhaps by occurring only in different geographic patches or seasons, can allow frequent coexistence of redundant mutualisms without competition (Palmer et al. 2003, Thompson 2005).

In some cases, defensive strategies (including mutualisms) that co-occur appear to protect against the same natural enemies but in fact specialize on different closely related enemies (Dyer et al. 2000, Stachowicz and Whitlatch 2005). When each facultative mutualism provides a separate rather than redundant service, the defended mutualist gains nothing by breaking ties with one of the two defenders.

In some cases, when mutualistic defensive services are identical and rewards are limited, we expect either 1) a fitness disadvantage in participating in multiple mutualisms, or 2) competition for resources between mutualists leading to the removal of one mutualist. If multiple ant species protect plants with extra-floral nectaries (EFN) or protect an aphid species, one ant will often provide greater protection than the other ant species and in some cases replace the less aggressive ants. However, the presence of multiple ant species is still stable and does not significantly reduce the fitness of plants and aphids (Stadler and Dixon 2005, Miller 2007). Multiple ants are still able to maintain the relationship because of spatial and temporal heterogeneity between plants.

Finally, we expect reciprocal specialization of some mutualistic partnerships, and an antagonistic response to other closely related mutualists that are not of the correct “lock-and-key” match. Ants raise specific lineages of genetically distinct cultivar fungi for food that are in turn protected from parasitic fungi by the bacteria *Pseudonocardia* spp. Because *Pseudonocardia* specializes its antifungal toxins to not damage the cultivar

but specifically attack pathogenic fungi, we expect a lock-and-key fidelity of fungus-ant-bacteria mutualisms. Foreign cultivar fungi of other genotypes can invade and be used by *Acromyrmex* ants. In an experiment conducted by Poulsen and Currie (2009), the expectation was that the mutualist *Pseudonocardia* would deter foreign invasion of non-native mutualists not adapted to its antifungal toxins. The opposite was found: there was no inter-mutualist antagonism between fungi and bacteria with ants that decreased fungi or bacteria fitness. The lack of antagonism suggests a flexibility of ants to acquire new fungi and the possibility of multiple mutualist species to exist in relative harmony.

In the above examples, an expected antagonism between potentially competing facultative mutualisms was shown to be erroneous (in the case of toxins or specialization of defense) or prevented by spatial and temporal heterogeneity (floral pollination and ant attendants). However, there is evidence suggesting that facultative mutualisms with the same partners can be antagonistic.

Competition between mutualisms

Inter-mutualist antagonism is predicted to result from direct competition between mutualists for the same partner or by the overtaxing of shared partner resources. Ants tending aphids are often facultative defensive mutualists and switch between protecting and eating aphids. When multiple aphid species are present, ants will favor tending and protecting aphids with superior honeydew quality. In this case, aphids are predicted to compete for ant protection by producing higher quality honeydew (Fisher and Shingleton 2001).

Symbiotic microbes have a cost to their hosts, and when there are too many microbes, the added cost can outweigh the benefits and overwhelm the host. Multiple protective and nutritional bacteria symbiont species can coinfect one aphid, but can compete with each other (Sakurai et al. 2005) and or increase in density and overwhelm aphid hosts (Oliver et al. 2006), causing hosts to die off. A similar inhibition of multiple microbial symbionts occurs in plants, where protective foliar endophytes strongly diminish colonization by arbuscular mycorrhizal fungi in the grass *Schedonorus phoenix* (Mack and Rudgers 2008). This displacement is likely caused by poisonous endophyte alkaloids (normally used as defense against herbivores). Otherwise, the plant is overwhelmed and reduces use of the mycorrhizae in response to a change in the nutritional needs of the plant. These two causes of displacement mirror the expected displacement of fungi by *Pseudonocardia* (Poulsen and Currie 2009) and the overtaxing of resources by different bacteria species (Oliver et al. 2006)

We see competition and inhibition between mutualists is possible both at a macroscopic and microscopic scale. Either direct competition between mutualists or overtaxing of partner resources by multiple mutualists forces one symbiont to displace another or causes superinfected hosts to die off.

A thin line between coexistence and competition?

In each case of competition, we can imagine scenarios where coexistence between mutualists might have evolved instead if there were safeguards against conflict of interest. Costs and benefits of multiple mutualists are equally as important as spatial and temporal heterogeneity and the mechanisms of inhibition between mutualisms.

If multiple species of aphids produced an equal quantity and quality of honeydew, ant tending or predation behavior might not be significantly different between these aphid species. If aphid species dispersed more widely from each other or at different times of the year, competition for ants might be prevented altogether between aphids. In the case of bacteria competition within aphids, if symbiotic bacteria were housed in different parts of the aphid, they might never come into direct contact. If aphid resources were nearly unlimited like flower rewards, superinfection of bacteria might not have a serious effect on aphid fitness. Finally, endophytes in grass could have evolved alkaloids less predisposed to damaging helpful fungi.

Given the variety of examples of both competition and coexistence between mutualists, we questioned whether bacteria and ants that protect aphids against the same natural enemies would compete or coexist in natural settings. Do facultative aphid-bacteria and ant-aphid mutualisms have safeguards that maintain mutualism despite conflicts of interest? Or does one mutualist partner displace the other?

Aphids

Aphids (Order Hemiptera, Suborder Sternorrhyncha, Superfamily Aphidoidea) are small insects that suck the sap from plants in temperate regions of the world (Dixon 1998). Many species of these agricultural plant pests are successful due to their asexual cyclical parthenogenetic lifestyle and an ability to switch yearly from woody to herbaceous plants. Depending on the species and time of year, aphid mothers can rapidly colonize a new plant by reproducing asexually. One aphid mother can produce over a billion genetically identical descendants in one season. The descendants in turn do not

have the added cost and time of mating every generation. Aphid control is economically important because aphids are damaging to agriculture not only through direct herbivory but also through vectored plant diseases.

Aphids have many predators that take advantage of their large numbers such as ladybird beetles, crab spiders, larvae of many insects, parasitoid wasps specialized to attack aphids, and entomopathogenic fungi. Aphids are relatively slow and have few physical defenses, aside from their innate host-immune system, kicking behaviors, sticky secretions, and risky escape mechanisms such as falling off their host plants (Gross 1993). Likely their strongest defenses against predators are defensive mutualisms with ants and bacteria.

Secondary symbionts of aphids

Bacteria can produce nutrients and toxins that many animals cannot produce on their own. It is predicted that bacteria can readily adapt to a mutualistic lifestyle in animal hosts because they can produce these beneficial products (Moran et al. 2008). Aphids are home to obligate and facultative mutualistic bacteria that are vertically transmitted from generation to generation (Russell et al. 2003). Obligate mutualistic bacteria, such as *Buchnera aphidicola*, provide essential amino acids such as tryptophan that are not present in aphid phloem diets (Buchner 1965). All aphid species have obligate bacteria like *B. aphidicola* and aphid growth is stunted and ultimately fatal without this primary symbiont. Aphids also have a number of secondary symbionts, or facultative symbionts non-essential to aphid survival, that provide defensive benefits. Different secondary symbiont species can protect aphids against parasitoid wasps (Oliver et al. 2003),

entomopathogenic fungi (Scarborough et al. 2005), high temperatures (Montllor et al. 2002). Like *B. aphidicola*, these bacteria are dependent on the host for transmission and mostly concentrated in specialized bacteriocyte cells clustered around aphid ovaries (Oliver et al. 2003). No evidence in the laboratory supports the capability of these symbionts to be horizontally transmitted from insect to insect (Russell et al. 2003). This thesis focused on one secondary symbiont of aphids.

Hamiltonella defensa

S. symbiotica, *R. insecticola*, and *H. defensa* bacteria are known to exhibit anti-parasitoid effects. *Hamiltonella defensa* is the best characterized (Oliver et al. 2006). Although the mechanism of defense is not clear, it is known *H. defensa* possesses a lysogenic bacteriophage APSE that encodes putative toxins that likely disrupt the development of parasitoid wasps and prevent aphid mortality (Oliver et al. 2009). APSE can be lost from *H. defensa* and is essential for the protective phenotype. Ecological surveys show *H. defensa* is not fixed in natural populations. Frequencies of infection and benefits that symbionts confer vary across aphid species (Russell et al. 2003), due to such factors as the presence or absence of natural enemies (Oliver et al. 2008), host genotype (Oliver et al. 2008; Vorburger et al. 2009), host nutrient status (Chandler et al. 2008), and temperature or other environmental conditions (Russell & Moran, 2006). Like with other protective symbionts, the costs and benefits of *H. defensa* mutualisms in aphids are sensitive to biotic and abiotic conditions. When aphids with and without *H. defensa* are in the presence of parasitoid wasps, the frequency of aphids with *H. defensa* increases; in the absence of wasps, *H. defensa* frequency decreases (Oliver et al. 2008).

Ant-aphid mutualisms

Associations between ants and nectar or honeydew-producing insects, including aphids, are ubiquitous in natural communities (Stadler & Dixon, 2008). Often these interactions are mutualistic: ants protect aphids from predators or pathogens in return for the sugary secretions they produce. Ant-attendance of aphids can be obligate or facultative. When ant-aphid mutualisms break down, ants eat aphids instead of eating aphid honeydew. For this reason, different aphid species are believed to compete for ant attendance through rapid production of superior honeydew with the trisaccharide melezitose (Yao et al. 2000, Yao and Akimoto 2001). This increased production of honeydew is suspected to weaken aphid growth and reproduction (Fisher and Shingleton 2001). If the cost of ant-appeasement is too great, aphids cannot stop participating in the mutualism because ants will eat them (Billick et al. 2007).

Potential interactions between ants and *H. defensa*

When ants engage in mutualisms with aphids, there is the potential for ants to be important in determining the interactions of secondary symbionts and aphids. Because ants can influence aphid ecology by altering mortality rate caused by predators and parasitism (Stadler & Dixon 2005, Vinson et al. 1991, Nielsen et al. 2009), dispersal rates (Kindlmann et al. 2007), population density (Breton and Addicott 1992), and fecundity and aphid size (Yao and Akimoto 2001), ants can affect the relative costs and benefits of secondary symbionts. By reducing parasitoid attack, ants might offset the benefit of protection conferred by *H. defensa* and change the prevalence of *H. defensa* in aphids.

H. defensa was also speculated to have the potential to change ant behavior towards aphids. *H. defensa* can change aphid physiology, in terms of growth and fecundity (Oliver et al. 2008, Vorburger and Gouskov 2011), and these traits in turn have been separately linked to aphid size and aphid honeydew production (Yao et al 2000). Ant predation of aphids is likely decided by aphid honeydew output (Sakata 1994, Yao and Akimoto 2001), so *H. defensa* could make aphids more or less edible to ant mutualists by changing honeydew quality. An effect of *H. defensa* on ant predation would then cause ant predation to alter infection by *H. defensa* in aphids in mixed populations.

Research Goals and Questions

- 1) Which ant-tended aphid species have secondary symbionts? A survey in which aphids were screened for bacteria and wasps is detailed in Chapter II.
- 2) Do *H. defensa* and ants provide duplicate services in natural populations? This question is answered in Chapter III by measuring the effect of ants and *H. defensa* on rates of parasitoid attack on aphids.
- 3) Do ants alter the frequency of *H. defensa* and APSE in natural populations of aphids? This question is also answered in Chapter III by manipulating the presence and absence of ant tending aphids and measuring the frequency of *H. defensa* in aphid populations.
- 4) Do ants prefer or avoid eating aphids infected with *H. defensa*? This question is explored in Chapter IV by surveying for the presence of *H. defensa* in ant stomachs.

CHAPTER II

SURVEY OF SECONDARY SYMBIONTS OF APHIDS AT THE ALMONT TRIANGLE, CO

Introduction

The aphid hosts of secondary symbionts are not well identified. Ecological experiments testing the interaction of ants with *H. defensa* or other symbionts are more difficult unless the aphids tested are known be infected with a high frequency of the bacteria. Furthermore, superinfection of secondary symbionts might alter interactions with ants. For this reason in the summer of 2008, ant-tended aphids were screened at the Almont Triangle for defensive secondary symbionts in order to select one aphid species for an ant manipulation experiment in 2009 (Chapter III).

Methods

In order to identify candidate aphid species with secondary symbionts, aphids were collected from plants, DNA was extracted, and aphid DNA was screened using polymerase chain reaction with primers diagnostic for the primary obligate symbiont *Buchnera aphidicola*, secondary facultative symbionts *H. defensa*, *Regiella insecticola*, and *Serratia symbiotica*, commensal or parasitic bacteria *Arsenophonus* spp., and the bacteriophage APSE (see Table 1). There is evidence these secondary symbionts provide protection to aphids against parasitoid wasps, fungi, and high temperatures (Oliver et al. 2009, Scarborough et al. 2005, Russell & Moran 2006). Aphids were also screened for

the presence of parasitoid wasp larvae using molecular bar-coding primers, and when possible parasitoid wasps were identified to species (Robert Kula, USDA, 2010).

Aphids were collected from the plants *Artemisia tridentata*, *Chrysothamnus viscidiflorus*, *Symphoricarpus albus*, *Populus tremuloides*, *Epilobium angustifolium*, *Lupinus sp.*, and *Linus sp.* Whole genomic DNA was extracted from single aphids using a technique modified from Bender et al. (1983). Genomic DNA was screened for bacteria and wasp DNA using PCR conditions modified from Abbot & Withgott (2004) and screened for APSE bacteriophage using protocols from Degnan and Moran (2008) (see appendix A). All diagnostic screens used *B. aphidicola* screens as positive controls because the bacteria are present in all aphid species. Products were visualized on 2.5% agarose gels stained with EtBr, purified for sequencing using a Qiagen QIAquick PCR purification kit, and sequenced at the University of Arizona's Laboratory for Molecular Systematics and Evolution on an Applied Biosystems 377 automated DNA sequencer.

Results

Seven aphid species were identified on seven different plants, and *H. defensa* and APSE infections were found in three aphid species on *A. tridentata* plants (see Table 2). *H. defensa* infection rates were highest in *Obtusicauda frigidae* aphids. Parasitoid wasp species *A. ervii*, *Lysiphlebus testaceipes*, and *Binodoxys sp.* were identified in *O. frigidae* aphids. Subsequent manipulative experiments in 2009 focused only on *O. frigidae* aphids.

Table 1. Primer Sequences

Organism	Primer	Sequence	Length	Reference
<i>B. aphidicola</i> 16s ribosomal	Buch 757 F	GAGGAATACCYKTGGCGAAA	850 bp	
	Buch 1507 R	TACCTTGTTACGACTTCACCCCAG		
Aphid Bar-coding primer 16s ribosomal	LepF LepR	ATTCAACCAATCATAAAGATATTGG TAAACTTCTGGATGTCCAAAAAATCA	700 bp	Footit et al. 2008
Braconidae 16s ribosomal	WaspF1 WaspR	ACCTGTTTATCAAAAACATG CGAGGTCGCAATCTTTTTTA	465 bp	Jones et al. 2005
<i>H. defensa</i> DNA gyrase B	gyrB 87 F gyrB 729 R	ATTTCATACTCATCCCCAGGC TCTACCGCATCTCCCATCAAC	750 bp	Degnan and Moran 2008
<i>H. defensa</i> , <i>R. insecticola</i> , <i>Arsenophonus</i>	gyrB F3 gyrB R3	CAARCARGARCAATAYATYAAAG GKTCWACMGCATCWCCCAT	850 bp	Degnan and Moran 2008
<i>S. symbiotica</i>	gyrB-type-158F gyrBR-type-402R	GCCGACCACAATTTTAGCAT CGCTGAACAGCTACATGGAA	250 bp	Oliver et al. 2006
<i>Arsenophonus</i> spp. 16s gene	Ars1015F	ATCCAGCGAATCCTTTAG		Russell and Moran 2006
General bacteria 23s gene	Ars35R	CCTTCATCGCCTCTGACTGC		
APSE bacteriophage	APSE 30.6F APSE 31.9R	AGTGCAGAAGGGTAACAAAGAC GGCTCTGATATTTTAGCCATGC	1095 bp	Degnan and Moran 2008

Table 2. Aphid species and percentage with *H. defensa* and APSE

Aphid Species	Plant(s)	# Screened	% Infected with <i>H. defensa</i>	% <i>H. def</i> + with APSE
<i>Aphthargiella</i> <i>symphoricarpi</i>	<i>S. albus</i>	8	0%	
<i>Aphis lupini</i>	<i>Lupinus</i>	3	0%	
<i>Cedoaphis</i>	<i>S. albus</i>	5	0%	
	<i>Linus</i>			
<i>O. frigidae</i>	<i>A. tridentata</i>	74	40.5%	98%
<i>Pleotrichophorus</i> <i>pseudoglandulosus</i>	<i>A. tridentata</i>	34	18%	100%
<i>Artemisaphis</i> <i>artemisicola</i>	<i>A. tridentata</i>	17	18%	100%
<i>Uroleucon</i> <i>escalantii</i>	<i>C.</i> <i>viscidiflorus</i>	20	0%	

Discussion

These screens revealed *H. defensa* might be at an intermediate frequency in three aphid species on the same host plant. Small sample sizes of aphids were screened for bacteria, so it is not certain that some aphid species did not have the secondary symbiont. The presence of APSE bacteriophage at 100% frequency in the aphids hinted that the expected relationship between APSE and *H. defensa* was possible. APSE provides the toxin necessary for the defensive phenotype conferred by *H. defensa* on aphids (Oliver et al. 2006), although every strain of APSE does not necessarily provide strong defense against parasitoids (Oliver et al. 2009). This does not rule out the possibility that this APSE strain could be non-functional and parasitic to *H. defensa* with no added benefit.

Various theories have been proposed as to why *H. defensa* and other secondary symbionts should be present in some aphid species and genotypes and not others, including aphid immune system mismatching (Moran et al. 2008, Vorburger et al. 2010). *H. defensa* was found in three distantly related aphid species on *A. tridentata* (see Table 2). Ancient or recent horizontal transfer of bacteria through close contact between aphids or through the plant itself could explain this pattern. This prediction is qualified by the lack of horizontal transmission of *H. defensa* observed in laboratory studies (Russell et al. 2003), suggesting such transmission must be rare. Nonetheless, patterns of bacteria transfer between aphids might be more structured by shared plant hosts between aphid species than by the degree of aphid phylogenetic relatedness and disparate immune systems. This line of evidence warrants further attention.

Although no quantitative data was taken, *O. frigidus* appeared to be ant-tended, while less ant-tending was detected for the less common *P. pseudoglandulosus* (Erickson,

personal observation). *O. frigidae* was also the most numerous and easiest to detect aphid this summer. For this reason, *O. frigidae* was selected for future experimentation testing the effect of ant-tending on the frequency of *H. defensa* in aphids.

CHAPTER III

THE LACK OF EFFECT OF ANTS ON THE PREVALENCE OF *HAMILTONELLA DEFENSA* IN *OBTUSICAUDA FRIGIDAE* APHIDS

Introduction

F. obscuripes are predicted to deter parasitoid wasp attack like other ant species (Völkl 1992). *H. defensa* infected *A. pisum* aphids decrease in frequency when in competition experiments with uninfected aphids and the aphids are in the absence of parasitoid attack. Ants are thus predicted to decrease *H. defensa* frequency in nature by deterring parasitoid wasp attack. The effect of *H. defensa* and *F. obscuripes* ants on parasitoid wasp attack and the effect of *F. obscuripes* on *H. defensa* prevalence in *O. frigidae* aphids were tested by manipulating ants.

Methods

In the summer of 2009, the presence and absence of *F. obscuripes* ant-tending was manipulated on *O. frigidae* aphid aggregations and the effect of treatment on the frequency of *H. defensa* and APSE bacteriophage was observed in aphid aggregations.

Ant presence was manipulated and the effect of ant exclusion on aphid survivorship, parasitoid wasp attack, and *H. defensa* infection frequency was measured. In order to manipulate ant presence, ants were excluded from aphid aggregations of ten or more individuals with duct-tape and Tanglefoot® (Tanglefoot Company, Grand Rapids, MI), a sticky artificial resin, wrapped eight inches from the end of a plant ramet. 256

ramets were selected from 154 *A. tridentata* plants and the treatment was applied to half of the ramets. Tanglefoot® does not prevent flying predators from attacking aphids, and aphid mortality from Tanglefoot® is minimized by lack of aphid migration.

On four days between late June and early August, the number of aphids and number of aphid mummies on both ant-excluded and ant-tended ramets were measured. Aphid mummies are the hardened, hollowed-out remains of aphids used by parasitoid wasps to finish development. For DNA screens, aphids were collected from each ramet at the beginning and at the end of the experiment. Fecundity was measured in aphids by collecting third instar aphids from 80 plant ramets. Aphids were cut dorsally between thoracic and abdominal segments and embryos were disgorged by lightly pressing on separated abdomens. Sclerotized embryos were counted and DNA was extracted

Aphids were screened for *H. defensa*, APSE, and parasitoid wasps, and *B. aphidicola* was used as a positive control. Screens for parasitoids were used to estimate the rate of parasitoid oviposition in aphids. Parasitoid screens were supplemented with field counts of aphid mummies. Aphids were screened for APSE bacteriophage because APSE is essential to the protective phenotype and sometimes lost spontaneously by *H. defensa* (Oliver et al. 2009). 211 aphids were screened from 50 ramets for APSE phage and 538 aphids were screened for parasitoid DNA.

In statistical analyses, *F. obscuripes* ant exclusion was used as the predictor variable and aphid number per ramet, percent mummies per aphid per ramet, and percent *H. defensa* aphid infected per ramet were used as response variables. To test this, the data was transformed into a Euclidian distance matrix, 999 randomized permutations were generated, and a MANOVA was performed for each using the statistical package

DISTLM v. 4 (Anderson 2004). Dependence was tested between the response variables using a correlation matrix and relationships between ants and each response variable were tested using ANOVA or Wilcoxon tests using JMP v. 9.0.0 (SAS Institute Inc.). A Generalized Linear Model (GLM) function was implemented in the R v. 2.12.0 (R Foundation for Statistical Computing) to test the effect of ant-tending and *H. defensa* presence on parasitoid wasp DNA per individual aphid.

Results

In 2009, *H. defensa* prevalence in aphid populations was higher than 2008. However, *H. defensa* prevalence dropped significantly from the beginning to the end of the experiment, from 70% to 52% over four weeks. The ant-exclusion treatment did not have a significant effect on *H. defensa* prevalence (Fig. 1, ant effect on arc sin square root *H. defensa* frequency: Wilcoxon, $X^2 = 1.2010$, $df=1$, $P<0.3049$). The ant-exclusion treatment also had no association with APSE presence or absence in aphids (ant effect on APSE presence in 88 *H. defensa* positive aphids: $df=1$, $X^2 = 0.034$, $P<0.8352$). Most but not all aphids with *H. defensa* screened positive for APSE. The bacteriophage was found in 78% of 211 aphids positive for *H. defensa*.

On average, individual aphids with *H. defensa* contained more sclerotized unborn embryos (Fig. 2, ANOVA, $n = 28$, $F=16.4268$, $P<0.0004$). We used embryos as a surrogate for reproductive output, and this result suggests *H. defensa* increased aphid fecundity. We would expect high fecundity to increase aphid group size. However, aphid aggregations with high proportions of *H. defensa* infection tended to have smaller

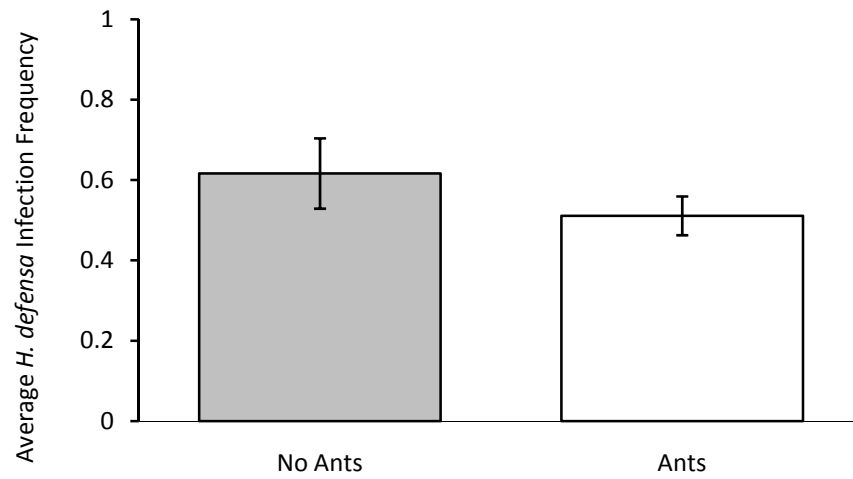


Fig. 1: Ant effect on mean $\pm$ SEM of *H. defensa* frequency per plant in week 4. Ant effect on arc sin square root *H. defensa* frequency (Wilcoxon test, $X^2 = 1.2010$, $df=1$, $P<0.3049$).

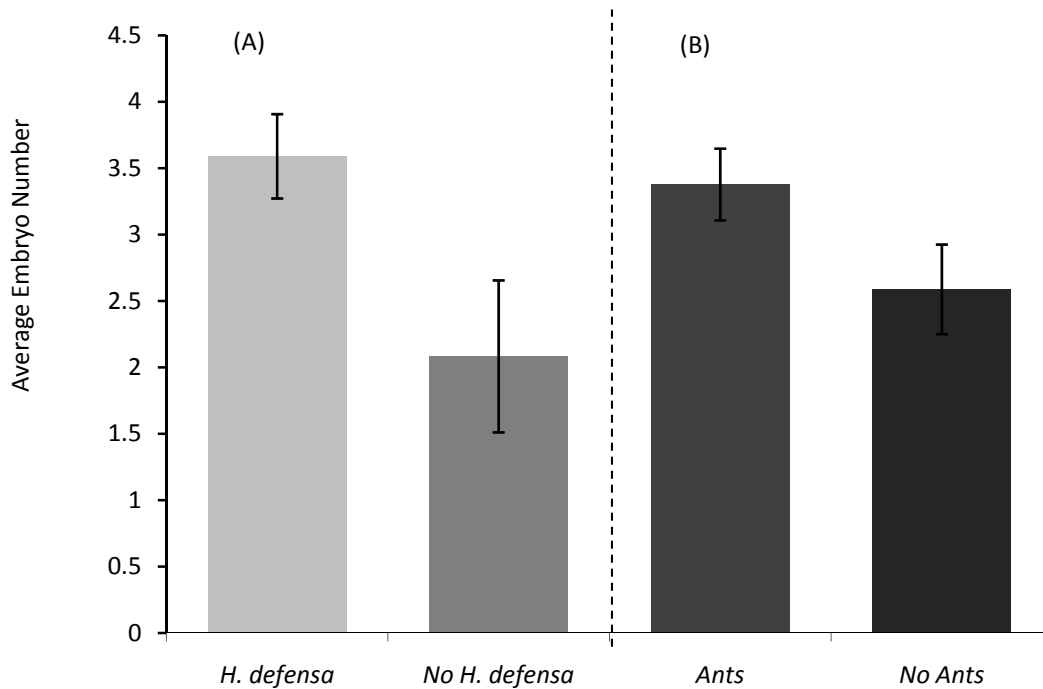


Fig. 2: Mean $\pm$ SEM of the number of embryos per aphid per treatment or infection group. (A) Effect of *H. defensa* infection on embryo numbers is significant. (B) Effect of treatment (ant exclusion) on embryo numbers shows no significant correlation between ants and aphid fecundity

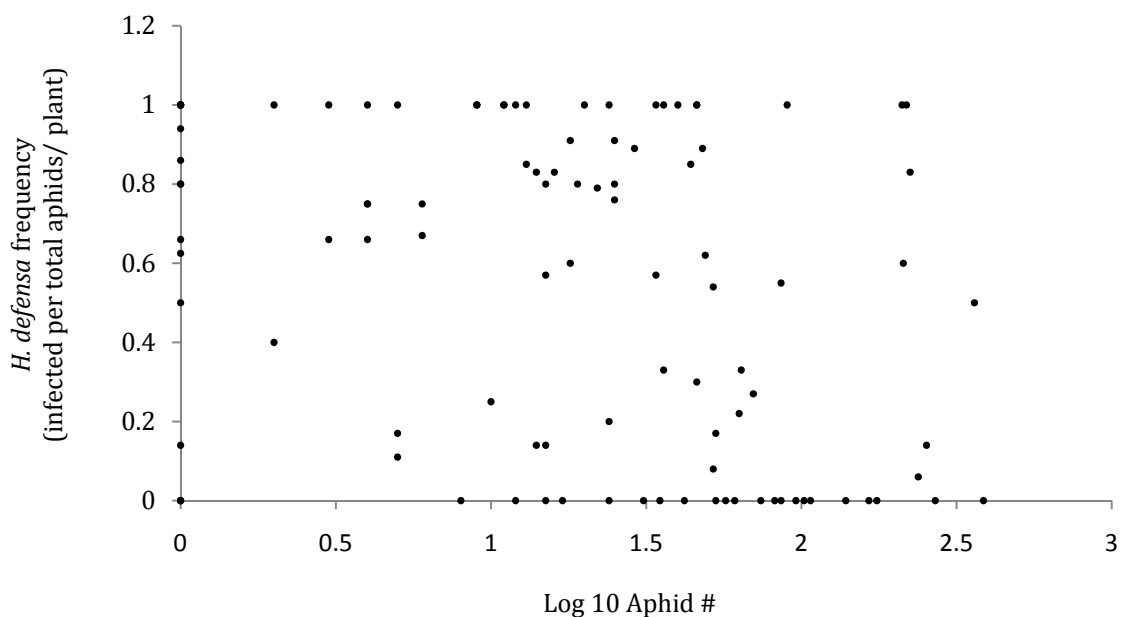


Figure 3: Correlation test of log aphid number per plant and frequency of *H. defensa* infected aphids on each plant. Aphid number per plant has a significant correlation with *H. defensa* frequency. $n=90$, Spearman's $Rho = -0.3614$, $P < 0.0005$.

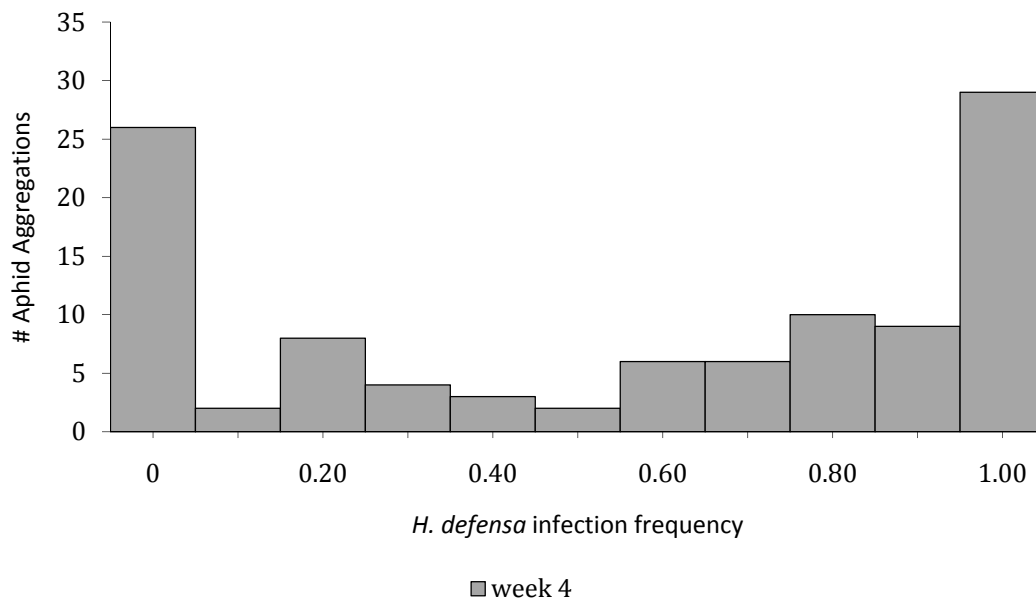


Fig. 4: Distribution of aphid group *H. defensa* infection frequencies.

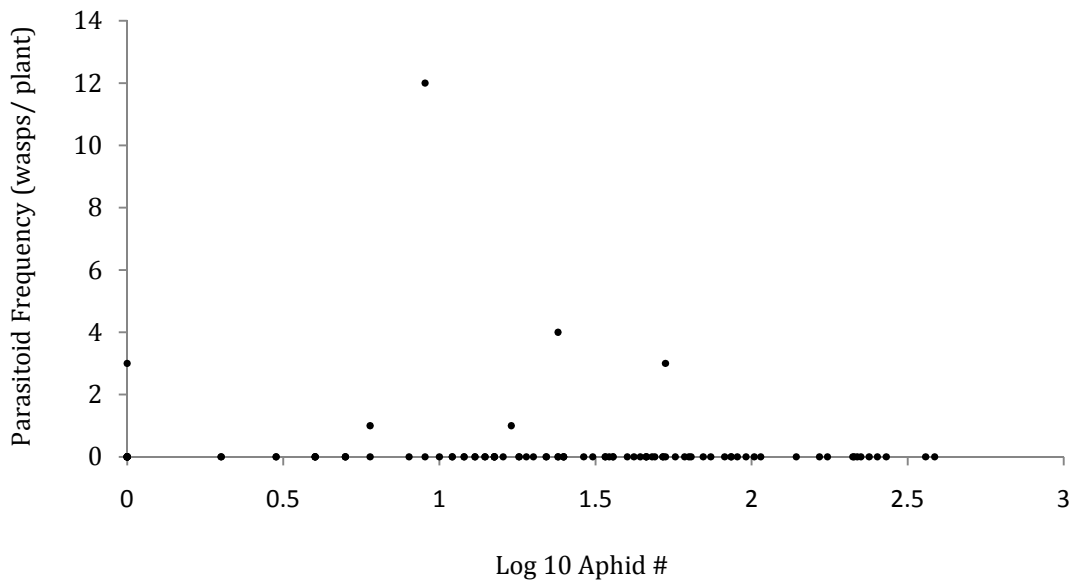


Figure 5: Correlation test between log aphid number per plant and parasitoid mummies per total number of aphids per plant. Aphid number from plants in week does not have a significant correlation with parasitoid mummy success. $n=90$, Spearman's $Rho = -0.0951$, $P < 0.3781$.

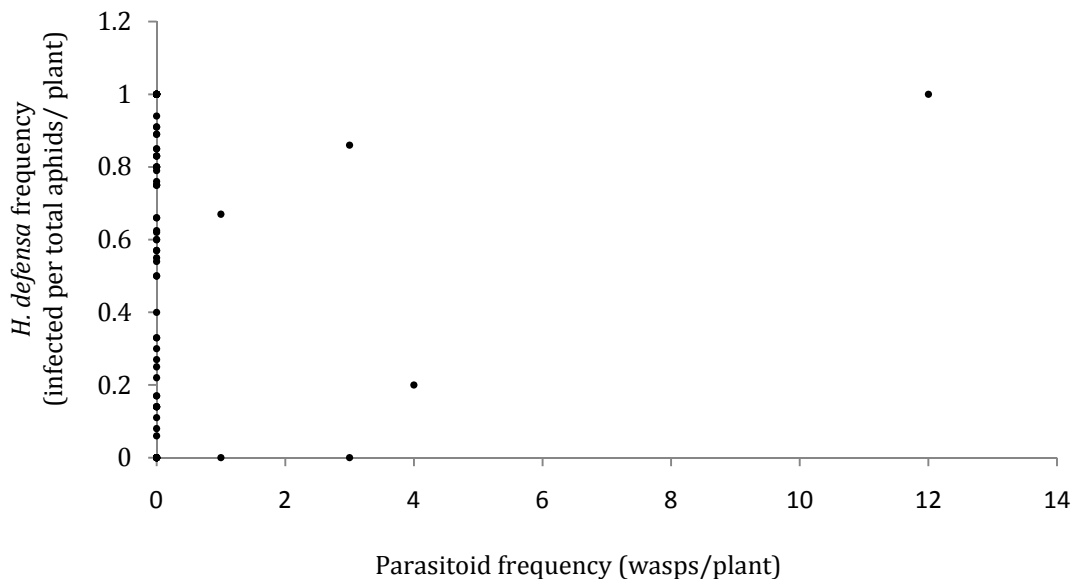


Figure 6: Correlation test of parasitoid mummies per total aphid per plant and frequency of *H. defensa* infected aphids on each plant. *H. defensa* frequency does not have a significant effect on mummy frequency. $n=90$, Spearman $Rho = -0.11$, $P < 0.3074$.

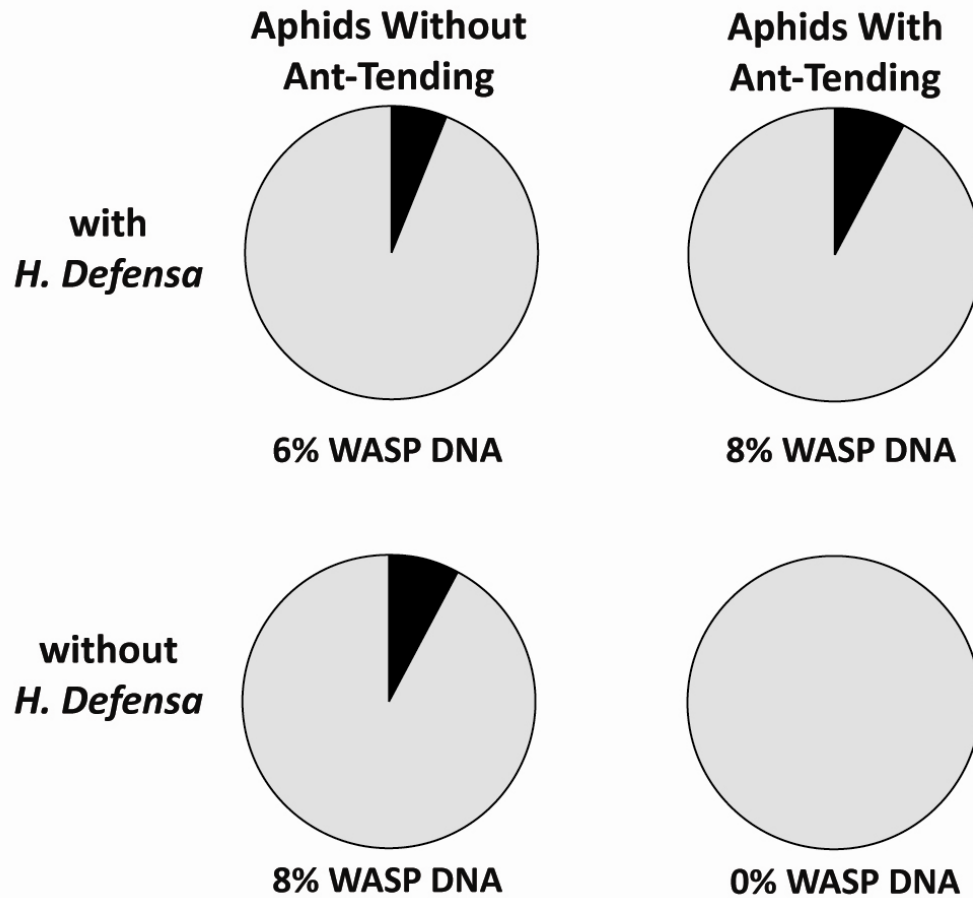


Fig. 7: Percentage of aphids with parasitoid wasp DNA detected per treatment and infection group. Among ant-excluded aphids, parasitoid DNA was not significantly less detected among infected or uninfected aphids (n=101 aphids, Fisher Exact test $P < 0.75$). Among 247 ant-excluded aphids, 127 were *H. defensa* positive, 120 were *H. defensa* negative. 10 out of 127 *H. defensa* positive were parasitoid positive, and 7 out of 120 *H. defensa* negative were parasitoid negative. Among ant-tended aphids, significantly less parasitoid DNA was detected in uninfected aphids (n=291 aphids, Fisher Exact test $P < 0.000254$). Among 291 ant-tended aphids, 155 were *H. defensa* positive, 136 were *H. defensa* negative. 13 out of 155 *H. defensa* positive were parasitoid positive, and 0 out of 136 *H. defensa* negative were parasitoid negative.

populations (MANOVA, $n=246$, pseudo- F 4.95531, permutation $P<0.02$; correlation matrix: Spearman's $Rho = -0.3614$, $P<0.0005$, see Fig. 3). *H. defensa* prevalences per aphid aggregation were skewed towards 100% and 0% frequencies with few groups of mixed aphid infection status (see Fig. 4).

Aphid mummy prevalence was not correlated with either aphid group size ($n=90$, Spearman's $Rho = -0.0951$, $P<0.3781$, Fig. 5) or *H. defensa* prevalence per group ($n=90$, Spearman $Rho = -0.11$, $P<0.3074$, Fig. 6). Overall we detected low rates of parasitoid attack through diagnostic PCR screens and surveys for aphid mummies. We observed only 44 aphid mummies (less than 1% of total aphids) and 30 out of 538 aphids (~6%) were positive for parasitoid DNA. However, no parasitoid DNA was detected in aphids tended by ants and lacking *H. defensa* (Fig. 7). There was a significant interaction between the presence of ants and *H. defensa* on the presence of parasitoid wasp DNA in aphids. However, neither ants nor *H. defensa* had a significant effect on parasitoid wasp attack (GLM, $n=555$, ant effect $P<0.0515$, *H. defensa* $P<0.5684$, ant by *H. defensa* $P<0.0489$).

Discussion

No effect of *F. obscuripes* ant tending on *H. defensa* prevalence in *O. frigidus* aphids was measured. One explanation for this result could be that parasitoid wasp attack rate, potentially a major factor driving variation in *H. defensa* frequency between aphid populations, was not significantly influenced by *F. obscuripes*. Previous laboratory experiments showed that aphids with *H. defensa* declined in frequency in groups of aphids with and without *H. defensa* when parasitoid wasps were excluded (Oliver et al.

2008). *H. defensa* reduces longevity in aphids, and resulting reduced lifetime reproductive output could cause the observed decline in *H. defensa* frequency (Vorburger and Gouskov 2011). The lack of effect of *F. obscuripes* on wasp attack was surprising because *O. frigidae* appears to be strongly ant-attended and more than half of the aphid groups in the ant exclusion treatment succumbed within four weeks. However, only predation from ladybird beetles and salticid spiders was directly observed, and parasitoid wasp attack was low in frequency (6% aphids with parasitoid DNA, 1% mummies), suggesting ant protection was strong against some predators but might be weak against parasitoids. Alternatively, this wasp population could be similar to the mixed populations observed by Völkl (1992) where ant-attended *A. fabae* aphids were avoided by *Trioxys angelicae* wasps but attracted *Lysiphlebus cardui* wasps. Each attacking parasitoid species in this study could behave differently in the presence of ants and the mixture of behaviors could prevent the ant from significantly increasing or decreasing the overall parasitoid attack rate on aphids. If wasp behavior was mixed, the effect of *F. obscuripes* on wasps should not be indicative of the effect of all ant species. Other ant-attended aphids are known to be either strongly protected or susceptible to parasitism by wasps, but not both (Stechmann et al. 1996, Völkl and Stechmann 1998). If parasitoid-detering ants negatively covary with *H. defensa*, ants might explain large-scale patterns of *H. defensa* across aphid species.

Although an effect of *F. obscuripes* on wasp success was not found, the data suggested an interesting pattern connecting ant and *H. defensa* to wasp attack rate (Fig. 5). *F. obscuripes* was expected to lower parasitoid attack rates on all aphids and *H. defensa* was expected to have no effect on parasitoid attack rates. *H. defensa* would stop

parasitoid development but not prevent their oviposition. Surprisingly, it was determined that aphids protected by ants but without *H. defensa* were devoid of parasitoid wasp DNA. The most immediate explanation is parasitoid wasps did not attack aphids with ant protection and without *H. defensa*. However, wasps do not favor aphids with or without *H. defensa*, and *H. defensa* has been shown to increase wasp mortality (Oliver et al. 2003). To increase offspring fitness, wasps would be expected to prefer aphids without *H. defensa*, not avoid them. Furthermore, *H. defensa* alone does not explain wasp predation preference because wasp preference was not observed among aphids in the ant-exclusion treatment. Ants would have to be partially responsible for wasp avoidance of aphids without *H. defensa*, and there is no reasonable explanation ants would cause this wasp preference. For these reasons, there is little support that this pattern is explained by wasp preference towards aphids with *H. defensa*.

Another explanation is ants ate aphids containing developing parasitoids before these aphids could be collected for screening. Ants will sometimes protect aphids, but ants will often eat aphids if they do not produce quality honeydew (Sakata 1994, Völkl et al. 1999, Woodring et al. 2004). Wasp-infestation sometimes causes aphids to produce less honeydew (Völkl 1992) and the fire ant *Solenopsis invicta* eats wasp-infested aphids (Vinson et al. 1991). Although *F. obscuripes* has not been observed to eat wasp-infested aphid or aphids with less honeydew, *F. obscuripes* has been observed to eat aphids and reduce aphid population size (Billick et al. 2007). *F. obscuripes* is predicted to eat wasp-infested aphids, but *H. defensa* interrupts ant predation. The ant predation preference would be explained by *F. obscuripes* preferring to eat wasp-infested aphids that are moribund due to the lack of *H. defensa*. If *H. defensa* prevents the negative effect of

wasp-infestation on honeydew production by stunting and killing wasps, ants will still prefer to eat wasp-infested aphids, but only those aphids without *H. defensa*.

If ants prefer to eat all aphids without *H. defensa*, not just parasitized aphids, this prediction suggests ants should cause *H. defensa* frequency to increase, contrary to the finding that ants have no effect on *H. defensa* prevalence. However, this increase in *H. defensa* frequency is only suggested if aphids without *H. defensa* survive from wasp-attack when not protected by ants. Otherwise, aphids infested by wasps would succumb regardless of ant predation, and ants would not change the overall effect of *H. defensa* on aphid survival. The count of aphid mummies showed no sign aphids without *H. defensa* survived parasitoid wasp attack any more than aphids with *H. defensa*. To clarify these results and broaden understanding beyond *F. obscuripes* effect on *H. defensa*, further studies should explore the effect of secondary symbionts on the behavior of ants and the physiology of aphids in ant-aphid interactions.

Ant-aphid interactions with *H. defensa* might be isolated to specific aphid species and more rare than many factors driving *H. defensa* prevalence. A broader understanding of the factors driving the differences between *H. defensa* prevalences in different aphid populations could explain general population dynamics of facultative protective symbionts in many organisms, not just secondary symbionts in aphids (Haine 2008). *H. defensa* prevalence in aphid populations might be driven by many factors besides parasitoid wasps including ant presence, host genotypes (Vorburger et al. 2009), host nutrient status (Chandler et al. 2008), ambient temperatures (Russell & Moran, 2006), the presence of APSE bacteriophage (Degnan and Moran 2008), bacteria vertical transmission rates (Gundel et al. 2008), and predators (Oliver et al. 2008). However,

parasitoid wasps are likely the most important factor. Parasitoids have been shown to rapidly coevolve in response to *H. defensa* presence (Dion et al. 2011), and the boom and bust cycles of parasitoids described in nature (Godfray and Shimada 1999) are hypothesized to cause high and low *H. defensa* frequency populations that average into overall intermediate frequencies of *H. defensa* (Oliver et al. 2008). *H. defensa* frequency could be locally driven by either the protective benefits or metabolic costs of *H. defensa* that are in turn dependent on local parasitoid attack rates.

In nature, *H. defensa* frequency could fluctuate for many reasons, but the data suggest that *H. defensa* declined in prevalence because of an induced metabolic cost. *H. defensa* infected aphids were observed to have smaller group sizes despite the correlation of *H. defensa* with higher fecundity in individual *O. frigidus*. This observation could match the effects of *H. defensa* on *A. fabae* aphids in a laboratory setting. *H. defensa* caused higher instantaneous fecundity but lower lifetime reproductive output as a result of reduced longevity (Vorburger and Gouskov 2011). Previous experiments suggest low parasitoid wasp attack rate allows the metabolic cost of *H. defensa* to cause a decline in bacteria prevalence (Oliver et al. 2008). However, an effect of *H. defensa* on parasitoid wasp attack rates or aphid mummy frequency was not observed, suggesting *H. defensa* might not have any beneficial effect on *O. frigidus* aphid survival in nature. Little can be said for certain except *O. frigidus* were likely negatively affected by *H. defensa*. How could *H. defensa* maintain high frequencies in *O. frigidus* with a metabolic cost but no apparent benefit? Future work could answer why *H. defensa* still has a high frequency in the *O. frigidus* population, but should first confirm that the protection provided by *H.*

defensa occurs in other aphid species in natural settings and should test the importance of parasitoid wasps in regulating *H. defensa* prevalence.

Because APSE is essential to *H. defensa* protection against wasps (Oliver et al. 2009), the lack of effective APSE bacteriophage may have prevented *H. defensa* protection against wasps. There were no screens for APSE presence in mummies, although APSE was in a majority of aphids with *H. defensa* (78%) and aphid groups with *H. defensa* that had mummies were highly infected with APSE (22/24 aphids). Even if most aphids with *H. defensa* had APSE, various APSE strains provide less protection than others (Oliver et al. 2009), and neither the APSE strain nor the purported defensive toxins in these aphids were identified. This particular strain of APSE may have provided too little protection to detect in this experiment. *O. frigidae* has also never been shown to gain protection from *H. defensa* and APSE, meaning the aphid species is possibly incompatible with the two symbionts. Investigation into *H. defensa*-aphid incompatibility could possibly explain the factors limiting the interspecies spread of secondary symbionts in general.

This experiment has demonstrated *F. obscuripes* ants are not a driving factor of *H. defensa* frequency. Nonetheless, *F. obscuripes* might not be representative of the potential influence other ant species have on parasitoid wasps and thus the large-scale effect of ants on *H. defensa* prevalence in aphid species. Furthermore, this study with *F. obscuripes* might inform other studies on the interaction of ants and secondary symbionts. The effect of *H. defensa* on the interaction of *F. obscuripes* with wasp-infested aphids was unexplained and suggests that other secondary symbionts might affect mutualist ant interactions with aphids in unexpected ways.

CHAPTER IV

THE EFFECT OF *HAMILTONELLA DEFENSA* ON ANT PREDATION OF APHIDS

Introduction

Secondary symbionts are predicted to affect aphid physiology by taxing aphid growth and or honeydew production. Ant predation of some aphid species is largely based on aphid honeydew quality and quantity, so it is predicted that *H. defensa* might influence the rate of ant predation of aphids infected with the bacteria. The effect of *H. defensa* on *F. obscuripes* predation of aphids was surveyed by screening for bacteria and aphid DNA in ants to see if ants preferred or avoided eating aphids with *H. defensa*.

Methods

In the summer of 2010, *F. obscuripes* ants and *O. frigidiae* aphids were collected from *A. tridentata* plants at the Almont Triangle. Plants were selected for aphid collection by walking a 50-meter transect and spotting plants with aphids around ant mounds. Ants and aphids were stored in 100% alcohol, DNA was extracted, and both aphids and ants were screened for *B. aphidicola* and *H. defensa* DNA (see Table 1). A Goodness of Fit Fisher exact test was conducted on *H. defensa* frequency in ants and aphids. The frequency of detection of *H. defensa* in ants was expected to be the same as the prevalence of *H. defensa* in aphids collected from the same plants as the ants.

As a control for field experiments *A. pisum* aphids infected with *H. defensa* were fed to *Tapinoma* ant species to guarantee that the bacteria could be detected and did not degrade in ant stomachs. During the spring and summer 2011, *A. pisum* aphids from clones 82BT and 5A0 (Oliver et al. 2009) infected with *H. defensa* were raised on fava bean plants in humid conditions in a greenhouse at ~28 degrees Celsius. These aphids were fed to *Tapinoma* spp. ants by placing 5 aphids on cut leaves in 50 ml tubes with 10 ants each. *Tapinoma* workers were collected from their natural habitat and left with aphids for at least two nights to ensure predation. Ants were then screened for bacteria.

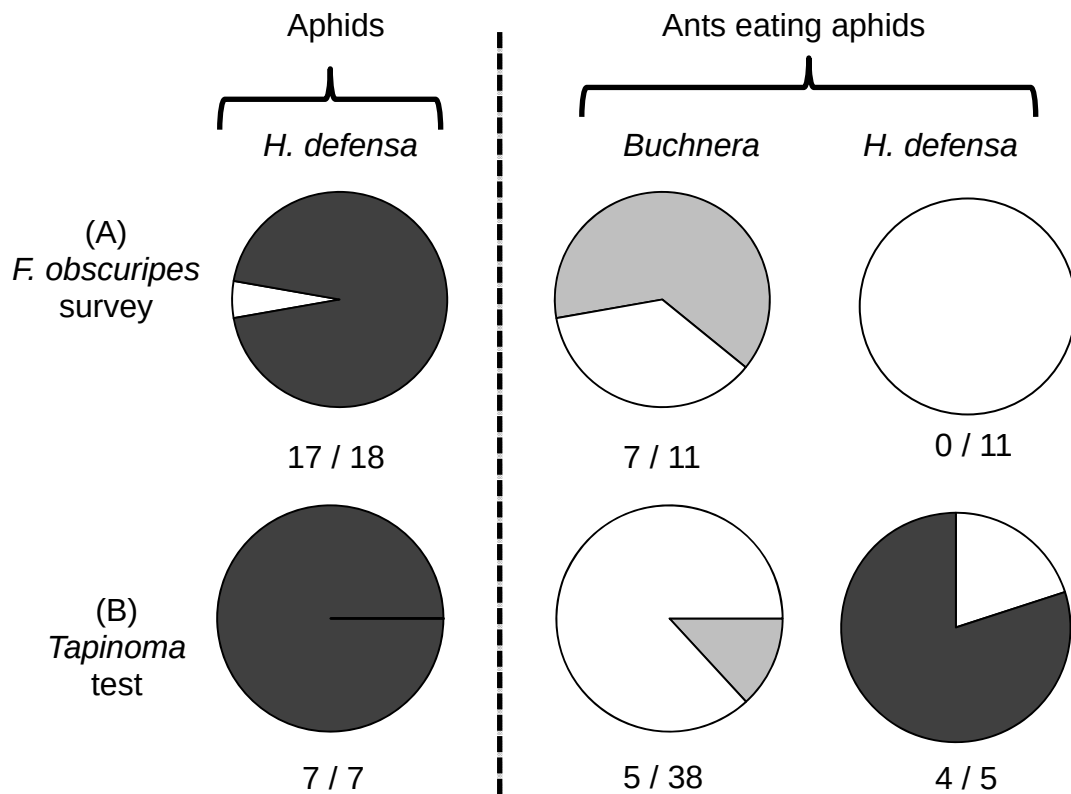


Fig. 8: Frequency of (A) *O. frigidus* aphids and *F. obscuripes* ant predators and (B) *A. pisum* aphids and *Tapinoma* ants that screened positive for *Buchnera* and *H. defensa*. (A) 17/18 *O. frigidus* aphids tended by 18 ants screened positive for *H. defensa*; 7/11 of these ants were positive for *Buchnera* DNA, while 0 were positive for *H. defensa*. (B) *A. pisum* aphids fed to *Tapinoma* were 100% infected with *H. defensa*; only 5 out of 38 ants fed aphids detected positive for *Buchnera*, and 4 of those 5 *Buchnera* positive aphids were positive for *H. defensa*.

Preliminary results

Sixty *F. obscuripes* ants total were screened from 20 plants. From 4 plants where 17 out of 18 *O. frigidus* aphids had *H. defensa* infections, 7 out of 11 ants contained *Buchnera* and aphid DNA, and 0 out of these 11 ants contained *H. defensa* DNA (see Fig. 8). Ants that ate aphids had significantly less *H. defensa* than expected (Goodness of Fit, two-tailed Fisher Exact Test, $P < 0.03$).

Forty aphids infected with *H. defensa* were fed to 38 *Tapinoma* ants, and 5 ants detected positive for *Buchnera*, 4 of which also detected positive for *H. defensa* bacteria (see Fig. 8). Ants that ate aphids and detected positive for *Buchnera* did not have significantly different *H. defensa* frequencies than the groups of aphids eaten.

Discussion

Preliminary results of the behavior of ants eating or avoiding aphids infected with *H. defensa* are far from conclusive. Seven ants with *Buchnera* are a small sample size and are not sufficient enough to say that *F. obscuripes* ants do not have *H. defensa* inside of them despite eating aphids. However, these ants were tending aphid groups with almost 100% *H. defensa* infections. Ants could have removed the uninfected aphids from these plants or consumed uninfected aphids from elsewhere. Either way, the result suggests these ants avoided eating aphids with which they were in constant contact.

The expected correlation of ant infection frequencies with aphid infection frequencies was based on an assumption that ants ate aphids from plants they visited frequently. It was assumed that individual *F. obscuripes* ants return to the same plants repeatedly due to experiments suggesting individual *F. obscuripes* ants continue to forage

in the same spots for weeks on end (Herbers 1977). Because of this assumption, it was also predicted that ants consume aphids that are from plants the ants normally visit for honeydew, and frequencies of detection of *H. defensa* in ants should correlate with the infection frequencies of aphids that the ants protect and eat. Ants may sometimes veer from their original path and eat aphids from plants other than the plant they were collected from. If this is true, then the frequency of *H. defensa* among all aphids tended should still correlate to *H. defensa* infection frequency in ants on the whole.

Another means of acquiring aphid DNA is through trophallaxis, or exchanging of food meals between ants using the secondary gut. In this case, all aphids tended and eaten by an ant nest should contribute to a communal gut. If aphids with *H. defensa* are present among the aphids tended by an ant nest, then the frequency of *H. defensa* among all aphids should correlate to *H. defensa* infection frequency in ants on the whole.

When ants eat aphids with *Buchnera* and *H. defensa*, the ants are expected to yield positive screen results for both bacteria. Both bacteria are contained in the same bacteriocyte cells, and the DNA have little reason to degrade at different rates. As a control, *Tapinoma* ant predation of infected aphids was tested, and so far both *Buchnera* and *H. defensa* were detected 4 out of 5 times. This result suggests but is not conclusive that *F. obscuripes* ants that eat *H. defensa* infected aphids should screen positive for *H. defensa*. However, if *H. defensa* cells were fewer in number than *Buchnera* cells, a long period of time between ant predation of aphids and collection of ants might allow *H. defensa* DNA to degrade before *Buchnera*. Future tests would have to measure the effect of time on bacteria detection.

No conclusions can be drawn regarding cause and effect with *H. defensa* and ant predation because even with greater sample size supporting the results, *F. obscuripes* ants have never been directly observed consuming *O. frigidae* aphids. An experiment manipulating *H. defensa* presence in *O. frigidae* aphids and then quantifying *F. obscuripes* predation of aphids would be necessary to support the prediction that ants avoid eating *H. defensa*.

If ants avoided eating aphids with *H. defensa*, this finding could explain previous results showing that parasitized aphids without *H. defensa* were never detected (see Fig. 7) because ants preferred to eat these aphids. However, the result would call into question the lack of effect of ants have on *H. defensa* frequency in aphids in natural populations (see Fig. 1). If ants do not eat *H. defensa* in aphid populations, then *H. defensa* infected aphids should increase in number in tended populations with mixed infection status.

There are plans to collect and screen more *Tapinoma* and *F. obscuripes* and *O. frigidae* aphids in the summer of 2011, and also repeat ant-feeding experiments with *F. obscuripes* and *A. pisum* aphids instead of *Tapinoma* ants.

CHAPTER V

CONCLUSIONS

The original purpose of the thesis was to test for a potential antagonism between ant-aphid and aphid-bacteria mutualisms. However, the assumption that *H. defensa* and APSE would protect *O. frigidae* aphids from parasitoid wasps was based on previous laboratory data with *H. defensa* in *A. pisum* aphids and was not supported by field data (Chapter III). The lack of service provided by *H. defensa* undermines the basis of the thesis and explains the lack of interaction of ants and *H. defensa*. Instead of solid conclusions, more questions have been created.

If the mutualism between *H. defensa* and *O. frigidae* is in fact a parasitism and exacts a cost on aphids rather than a benefit, how does this particular strain of *H. defensa* maintain intermediate to high levels in *O. frigidae* aphids? *H. defensa* is assumed to be transferred vertically from mother to daughter in *O. frigidae* aphids, with rare horizontal transmission (Russell et al. 2003) but this was only tested in *A. pisum* aphids. If this assumption is supported, a vertically transmitted symbiont is theorized to maintain its prevalence in its host through beneficial services or by manipulating host reproduction (Gundel et al. 2008). Otherwise, frequent horizontal transmission is the main mode of survival for parasites. In general, parasites increase rates of transmission with increasing chance of mortality in their hosts. If *H. defensa* is not beneficial and is a parasite in *O. frigidae*, then according to current theory the maintenance of *H. defensa* at intermediate frequencies is dependent on horizontal transmission or reproductive manipulation.

Alternatively, this strain of *H. defensa* could have acquired another beneficial effect on *O. frigidus* that this experiment would never detect, perhaps because the beneficial effect is only relevant to aphids in another setting. If any of the above examples were true, it would imply this strain of *H. defensa* has acquired a novel means of survival or transmission that is different from multiple related strains of the bacteria (Oliver et al. 2009, Degnan and Moran 2008).

The thesis has also hinted at an effect of *H. defensa* on ant predation of aphids. Lack of wasp attack on aphids without *H. defensa* and tended by ants is predicted to be in fact caused by ant predation of parasitized aphids without *H. defensa* (Chapter III). Later, a small sample of *F. obscuripes* ants was determined to likely have never eaten aphids with *H. defensa*, despite eating aphids and being in close contact with aphids with *H. defensa* (Chapter IV). These results are not conclusive enough to confirm that aphids with *H. defensa* have a positive advantage when in the presence of ants, nor does previous data suggest this trend. However, intermediate frequencies of *H. defensa* found in nature could be explained if aphids have a positive advantage because this unknown strain of *H. defensa* deters ant predation, even if no protection against parasitoid wasps is provided. This finding would in turn suggest that certain protective benefits of *H. defensa* would be lost in aphids if *H. defensa* are in contact with ants.

Instead of *H. defensa* disappearing from its aphid hosts, the bacteria would adapt to match its surroundings. This increased adaptability of bacteria defenses and traits is possible given previous examples such as evidence of transfer of *H. defensa* toxins by APSE bacteriophage (Degnan and Moran 2008), the easy acquisition of defense against parasitoids in *R. insecticola* bacteria that normally protects against fungus (Vorburger et

al. 2010), and the shift of *S. symbiotica* into becoming a nutritional symbiont fulfilling some functions of *Buchnera* (Lamelas et al. 2008). On one hand, increased adaptability of bacteria defenses might be a form of safeguard against mutualism breakdown, allowing vertically transmitted bacteria species to maintain close symbiosis with an insect species despite changing environments (Douglas 2008). On the other hand, evolutionary switches from dependence on host reproduction to frequent horizontal transmission might be less constrained than once thought, and allow symbiotic bacteria to become parasitic to prevent extinction.

Multiple possibilities explaining the maintenance of intermediate frequencies in this strain of *H. defensa* have been outlined.

- 1) This strain has lost the protective phenotype and will go extinct (Chapter III).
- 2) This strain is parasitic and has frequent horizontal transmission.
- 3) This strain provides an unknown benefit, or deters ant predation (Chapter IV).

Future Directions

There are still no field studies that show *H. defensa* provides defense against parasitoid wasps in *A. pisum*, much less in our non-model aphid *O. frigidae*. To guarantee field studies can in fact match laboratory studies, research should first be conducted on model aphids in the field and in multiple geographic locations and times of the year while taking into account parasitoid wasp attack. Because dynamics of aphid populations and their predators will vary between field populations, and aphid lines will migrate rapidly, actually keeping track of the benefits and costs of *H. defensa* from aphid line to aphid

line might be difficult. More efficient and practical methods of measuring aphid infection or genotype would broaden avenues into this field of study.

If *H. defensa* or any secondary symbiont is found to affect ant predation, bacteria effect on aphid size and aphid honeydew production is the most likely mechanism causing *H. defensa* to affect ant-aphid interactions. If any kind of secondary symbiont can change aphid physiology, there is a strong potential for the bacteria to affect ant behavior towards these aphids. Analysis of *H. defensa* effect on aphid physiology would be the obvious next step in analysis of aphid-bacteria-ant interactions.

Inter-mutualist antagonism between ants and defensive bacteria is still an open-ended question. However, the question of antagonism might not be relevant yet if ant mutualists and the defensive phenotypes conferred by bacteria in aphids have not yet been shown to coexist in natural surroundings. Ants might prevent secondary symbionts from co-occurring if ants protect against the same natural enemies. Surveys of extant aphid species with secondary symbionts and their known associations with ant mutualists might shed light on the rate of ant and secondary symbiont co-occurrence. Nonetheless, as this thesis demonstrates, an accurate survey would require in-depth understanding of field populations and evidence that each secondary symbiont population provides protection against natural enemies. The switch from a mutualist to a parasitic or non-functional lifestyle may be widespread among secondary symbionts. Investigating the causative factors behind such switches would inform the evolution of mutualism in general.

APPENDIX A

PROTOCOLS FOR DIAGNOSTIC PCR SCREENS

All PCR reactions were carried out in 10 µl reactions containing 4-8 ng 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).

Cycling conditions were an initial 2 minutes at 94 °C, and then 35 cycles of 30 seconds at 94 °C, 30 seconds annealing at 52 °C, 30 second extension temperature at 72 °C, with a final extension of 6 minutes at 72 °C.

For APSE bacteriophage PCR screens, cycling conditions were an initial 2 minutes at 94 °C, and then 10 cycles of 30 seconds at 94 °C, consecutively decreasing temperature “touchdown” annealing at 56-46 °C for 50 seconds, and 1 minute and 30 second extension temperature at 72 °C. This was followed by 24 cycles of 30 seconds at 94 °C, 50 seconds annealing at 46 °C, 1 minute and 30 second extension temperature at 72 °C, with a final extension of 5 minutes at 72 °C (via Degnan and Moran 2008).

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