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INVESTIGATING THE EFFECT OF A FACULTATIVE SYMBIONT, *HAMILTONELLA DEFENSA*, ON PEA APHID, *ACYRTHOSIPHON PISUM*, FECUNDITY AND BEHAVIOR IN ELEVATED CO_2 ATMOSPHERES

by

TYLER FOLLMAN

(Under the Direction of Edward Mondor)

ABSTRACT

As atmospheric carbon dioxide (CO_2) levels continue to rise, it is important to study how economically important organisms, like the pea aphid, *Acyrthosiphon pisum*, will react to these conditions. The pea aphid feeds on the phloem of crop plants like alfalfa, peas, and fava beans, where it not only directly harms the plant but also can spread plant viruses. A wide variety of factors can influence pea aphid fecundity and behavior. Some of these factors are abiotic, like atmospheric conditions, and some are biotic, like microorganisms with which the pea aphid has a mutualistic relationship. In this thesis, pea aphids with and without the facultative symbiont *Hamiltonella defensa*, were reared in either ambient CO_2 or elevated CO_2 concentrations. Pea aphid fecundity and behavior was assessed to determine if the presence of *H. defensa* impacted aphid fitness under atmospheric conditions associated with global climate change. I found that aphids harboring *H. defensa* had approximately twice as many offspring as uninfected aphids, but offspring production was not significantly influenced by CO_2 level, and there was no significant interaction between *H. defensa* and CO_2 level. In addition to offspring production, I found that behavior was also influenced by the symbiont. I found that aphids with *H. defensa* had greater responses to alarm pheromone compared to those without the symbiont. There was also a significant interaction on aphid dispersal behavior between the presence of *H. defensa* and CO_2 level; aphids with a symbiont had higher dispersal rates in ambient CO_2, but the dispersal response between infected and uninfected individuals did not differ at high CO_2 levels. As CO_2 levels continue to increase, variation in

phenotypic expression in response to these environmental conditions may become evident, resulting in altered predator-prey dynamics and associated community functioning.

INDEX WORDS: Pea aphid, Endosymbiont, *Hamiltonella defensa*, Carbon dioxide, Fecundity, Behavior, Alarm pheromone, Climate change

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DEDICATION

I want to dedicate this thesis to my parents, Ken and Kris, for the endless love and support, and to my cats, Toby and Opal, for the somewhat frequent love and assumed support.

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A very special thank you is required for my advisor, Ed Mondor. Your knowledge, patience, and kindness were always appreciated, and you have inspired me to be the best scientist and person that I can be.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Pea aphids, *Acyrthosiphon pisum* (Homoptera: Aphididae) are small, phloem feeding insects that damage crops like alfalfa, clover and broad bean (Van Emden & Harrington 2017). In addition to being key vectors of plant viruses, large groups of pea aphids can cause tissue damage to plants as a result of direct feeding (Maiteki & Lamb, 1985). Pea aphids have a life cycle that includes sexual generations as well as Parthenogenetic periods (Moran, 1992). In the summer months, aphids are Parthenogenetic, meaning that a female aphid makes clones of herself (Pickering & Gutierrez, 1991). Being clonal is not only beneficial to aphids in natural settings, but it also makes them an excellent model organism for scientists studying symbiosis, phenotypic plasticity, and ecology (Brisson & Stern, 2006). Being small, sedentary, and group-living, aphids are undoubtedly subject to high rates of predation. One unique characteristic of aphids is their abdominal appendages called cornicles that secrete a liquid that contains an alarm pheromone, (E)-β-farnesene (Eβf) (Nault et al., 1973; Wynn & Boudreaux, 1972). Aphids attempt to smear this secretion on a predator that is hunting in their colony so nearby aphids, which have an extremely high degree of relatedness, can be alerted that an attack is imminent (Mondor, 2001).

All aphids have an obligate symbiont, *Buchnera aphidicola*, that helps produce amino acids the aphid does not receive from its diet (Hansen & Moran, 2011). In addition to *Buchnera*, pea aphids may have secondary symbionts that are not essential for survival, but are mutualistic with the aphid, providing a benefit in novel conditions. These symbiotic bacteria are vertically transmitted both sexually and asexually and can offer the aphid defense against environmental factors (Moran and Dunbar, 2006), and influence aphid fecundity (Chen et al., 2000; Reyes et al., 2019; Russel and Moran, 2006). In North America, there are seven pea aphid secondary symbionts that can occur at different frequencies (Russel et al., 2013); *Hamiltonella defensa*, *Regiella insecticola, Serratia symbiotica,* X-type, *Rickettsiella viridis, Rickettsiella sp., and Spiroplasma sp.* (Doremus & Oliver, 2017; Rock et al., 2018). Some of these

secondary symbionts offer benefits against changes in abiotic factors, like increased temperature (Chen et al., 2000; Montllor et al., 2002). For example, pea aphids that were heat shocked and had the secondary symbiont *Serratia symbiotica* had more offspring and a faster development time compared to aphids that were heat shocked without having this symbiont (Oliver et al., 2010; Russell & Moran, 2006). Studying how symbionts and other abiotic factors interact may provide insight on how aphid populations will adapt to changing environmental conditions.

Aphids used for the experiments in this thesis possessed the secondary symbiont *Hamiltonella defensa. H. defensa* has been shown to reduce the success of aphid parasitoids, through egg encapsulation responses (Oliver et al., 2003). As a result, parasitoids must deposit more than one egg into a single host to overcome these defenses (Oliver et al., 2012). Another study showed that despite an overall decrease of *H. defensa* prevalence over time in large colonies without parasitism pressure, individual pea aphids that possessed the symbiont had a slightly higher fecundity than those without (Oliver et al., 2008). With regards to aphid behavior, aphids possessing *H. defensa* produced significantly less Eβf than aphids that were uninfected with a secondary symbiont (Oliver et al., 2012). It is currently unknown, however, if secondary symbionts influence alarm pheromone responses.

Aphids react to the alarm pheromone, (E)-β-farnesene (Eβf), through anti-predator responses in the form of dispersing or dropping off the plant (Keiser et al., 2015). Of these behaviors, dropping off the plant is the most detrimental to the aphid's reproduction (Nelson, 2007), and depends on a variety of factors such as predation risk, host plant quality, and the surrounding environment (Losey & Denno, 1998). Exposure to Eβf not only influences the aphid that senses the pheromone, but the offspring of that aphid as well (i.e., transgenerational responses). These effects include wing induction in their offspring (Podjasek et al., 2005), and altering the feeding positions of young aphids born after their mother was exposed to Eβf (Keiser & Mondor, 2013). Although experiments have been performed to determine if alarm pheromone responses differ between pea aphid biotypes (Ben-Ari et al., 2019), little is known about how endosymbiotic bacteria affects alarm pheromone-mediated aphid-predator interactions.

Community-level processes may be altered dramatically by altered atmospheric conditions associated with global climate change. When plants are exposed to elevated atmospheric CO_2 levels, nitrogen in the soil acts as the limiting factor of growth and other physiological processes, as nitrogen is in chlorophyll (Diaz et al., 1993). Chewing insects like caterpillars and beetle larvae decrease growth rate and increase mortality (Chen et al., 2005), while increasing food consumption (Wu et al., 2006) in elevated CO_2. Sucking insects, like aphids, have a more complex response to feeding in elevated CO_2, as studies have shown negative, positive, and neutral impacts across different aphid species (Bezemer & Jones, 1998; Hughes & Bazzaz, 2001). One reason for this mixed response to elevated CO_2 may be due to certain aphid species being specialists on certain plants (Bezemer et al., 1999). Legumes, however, which pea aphids primarily feed on, are not limited by the amount of nitrogen in the soil, thanks to symbiotic bacteria in their roots (Phillips, 1980). These bacteria can fix nitrogen for the plant by converting free nitrogen into nitrogenous compounds that are able to be utilized by the plant (Rogers et al., 2006). When comparing legumes to non-legume plants in elevated CO_2, legumes have higher carbon to nitrogen ratios because their rate of photosynthesis is not limited by the amount of nitrogen in the soil (Rogers et al., 2009). Some other aphid species raised on legumes in elevated CO_2 have either no significant differences in growth rate and fecundity, or an increased fitness than those that feed on non-legumes at ambient CO_2 levels (Hughes and Bazzaz, 2001; Robinson et al., 2012; Sun et al., 2015). For pea aphids feeding on specific cultivars of legumes, however, the opposite has been found, showing that elevated CO_2 conditions either did not have a significant effect on fecundity (Mondor et al., 2010; Ryalls et al., 2017), or a reduction in fecundity or feeding (Hughes and Bazzaz, 2001; Johnson et al., 2014).

Aphid behavior has also been reported to change under elevated CO_2 conditions. Adult aphids of other species showed significantly less dispersal in response to alarm pheromone at elevated CO_2 than at ambient CO_2 (Mondor et al., 2004; Sun et al., 2010), and pea aphids produced less alarm pheromone overall when reared in elevated CO_2 (Boullis et al., 2017). English grain aphids reared in elevated CO_2 had altered feeding behaviors as well, as they take more time to probe and ingest from their host plants

than aphids in ambient atmospheric CO_2 (Zhang et al., 2009). Differences in response to elevated CO_2 has even been seen between color morphs of the same species, as pink morph pea aphids have shown a higher rate of wing induction compared to green morphs (Mondor et al., 2005). It has been suggested that plant-aphid interactions in elevated CO_2 should move away from descriptive studies and move toward mechanistic studies (Sun & Ge, 2011).

The goal of this thesis is to better understand the possible mechanisms underlying aphid fitness under CO_2-enriched atmospheres. Here, I explore the fitness (offspring production) and behavior (alarm pheromone responses) of pea aphids harboring the facultative symbiont *H. defensa* in elevated, atmospheric CO_2 concentrations associated with global climate change, to better understand predator-prey interactions in the future.

CHAPTER 2

DOES THE SYMBIONT, *HAMILTONELLA DEFENSA*, ALTER PEA APHID, *ACYRTHOSIPHON PISUM*, FECUNDITY IN ELEVATED CO_2 ATMOSPHERES?

Introduction

Aphids are worldwide agricultural pests, causing direct feeding damage and vectoring plant diseases (Van Emden & Harrington 2017). As many aphid species are economically important, several have become model organisms for both agricultural and ecological research. Understanding more about the life histories of these insects can lead to novel insights for reducing their agricultural impacts. From an ecological standpoint, since most aphid species are Parthenogenetic for at least part of their life cycle, it provides the opportunity to study how populations of genetically identical individuals respond to different environmental conditions (Simon et al., 2002), such as the altered atmospheric conditions associated with global climate change (IPCC, 2014).

The pea aphid, *Acyrthosiphon pisum*, feeds on plant phloem from a variety of legumes (Akey & Beck, 1971). The pea aphid is a host to the obligate symbiont *Buchnera aphidicola*, which helps produce the amino acids that the aphid would otherwise not receive due to their diet (Hansen & Moran, 2011). In addition to *B. aphidicola*, pea aphids may also host secondary bacterial symbionts that, while not necessary for survival, may offer a benefit in novel situations such as high temperature, protection from parasitism, and defense from fungal infection (Oliver et al., 2008). During the Parthenogenetic stage of an aphid's life, these symbionts are vertically transmitted from mother to daughter, but they may also be transmitted sexually to an aphid's offspring (Moran & Dunbar, 2006). In North America, there are currently seven known secondary symbionts that pea aphids can host, but the frequencies of these secondary symbionts in aphid populations can change based on the prevailing biotic and abiotic conditions (Rock et al., 2018; Russell et al., 2013). While pea aphids and their symbionts are affected by climactic factors such as temperature (Montllor et al., 2002; Scarbourough et al., 2005; Sepúlveda et al., 2021), almost nothing is known about how symbionts are affected by altered atmospheric composition.

The pea aphid symbiont *Hamiltonella defensa* is a secondary symbiont that offers pea aphids resistance to attack from parasitoid wasps (Oliver et al., 2003; Oliver et al., 2012). In addition to protecting its host from parasitism, *H. defensa* increases the fecundity of aphids compared to pea aphids without the symbiont (Oliver et al., 2008). Despite these benefits, population cage experiments have shown that without the presence of parasitoids, *H. defensa* does not persist in populations (Dykstra et al., 2014).

Studies have shown that elevated CO_2 does not have a direct effect on the growth of insect species if the host plant is not under elevated CO_2 as well (Coviella & Trumble, 1998). Chewing insects have shown decreased growth rates and increased mortalities in elevated CO_2 (Chen et al., 2005), however, aphid reproduction in response to elevated CO_2 levels has not been as straightforward. As phloem feeders, aphids are sensitive to the quality of their host plant (Pritchard et al., 2007), but there is a lack of consensus on how aphid populations will change under elevated CO_2 (Newman, 2003). Some studies have found no significant difference in fecundity (Mondor et al., 2010; Ryalls et al., 2017), or a significant decrease in fecundity for aphids in elevated CO_2 (Hughes & Bazzaz, 2001; Johnson et al., 2014).

While aphid fecundity with a variety of endosymbionts has been assessed (Chen et al., 2000; Leonardo, 2004; Russell & Moran, 2006) and aphid population dynamics have been examined in response to elevated CO_2 levels (Li et al., 2021; Mondor et al., 2005) there is a lack of research on the interactive effects of elevated CO_2 and aphid symbionts. Better understanding how aphids with *H. defensa* grow and reproduce in elevated CO_2 will give insight into how the population dynamics of pea aphids with this specific symbiont may change in the near future; that is, will aphids retain the fecundity benefits from *H. defensa* under altered atmospheric conditions?

Since aphids in this experiment will not face competition for resources, I hypothesize that pea aphids with *H. defensa* will have increased offspring production compared to aphids without the symbiont, as seen in other lab studies (Oliver et al., 2008). I expect to see a positive effect on fecundity from *H. defensa* because there will not be stresses from competition acting on individuals, and host plants

will remain relatively healthy for the duration of the experiment since aphid numbers will not reach the point of causing significant damage to the plant. I further hypothesize that aphids in elevated CO_2 will have equal or lower offspring production compared to aphids in ambient CO_2 atmospheres. Previous experiments have tested aphid fecundity at lower CO_2 levels than this experiment (Hughes & Bazzaz, 2001), so I predict that CO_2 levels this high may have some direct effects on the aphids that hasn't been seen in other studies. Since I anticipate the presence of *H. defensa* to have a positive effect on fecundity and elevated CO_2 to have a neutral or negative effect on fecundity, I hypothesize that there will be a significant interaction between the presence of *H. defensa* and elevated CO_2. That is, the fecundity of aphids with *H. defensa* raised in elevated CO_2, will be lower than that for aphids raised in ambient CO_2 with the symbiont.

Methods

Pea aphids, *Acyrthosiphon pisum*, were reared on the Broad Windsor cultivar of Broad bean, *Vicia faba*, in growth chambers in ambient CO_2 at 24℃ with a 16:8 L:D photoperiod, to maintain parthenogenesis. Four colonies were maintained, all consisting of the same genotype; two colonies contained aphids with the secondary symbiont *Hamiltonella defensa* and two contained aphids without any secondary symbionts. Aphids with and without symbionts were kept in separate growth chambers and the colonies maintained on different days to ensure no cross-contamination. In addition, during the experiment, colonies were genotyped to verify infection status.

Experiments were conducted in an atmospheric simulator; the Atmosim 2100. The Atmosim 2100 consists of eight, 20-gallon aquariums, arranged in two rows; four upper-level and four lower-level. Each aquarium is self-contained, with a Plexiglas sheet covering the tops of the chambers. Sentinel Analyzers deliver regulated CO_2 levels to each aquarium, thereby ensuring that each aquarium received the appropriate concentration of CO_2. Four aquariums were set to maintain an average of 400 ppm of CO_2 to simulate present-day atmospheric levels, and the other four were set to an average of 900 ppm of CO_2, which was chosen as it falls within the upper end of the predicted range of atmospheric CO_2 concentrations for the year 2100 (IPPC, 2014). CO_2 levels were monitored via a sensor inside the tanks,

which was rotated to a new aquarium daily to ensure that the aquariums received the proper CO_2 level for their designated treatment.

Broad Windsor fava bean plants used in each experiment were grown from seed in the Atmosim 2100 at their respective (400 "ambient" or 900 "elevated" ppm) atmospheric CO_2 levels. Vipar Spectra model TC450 growth lights were used for the duration of the experiment at the same 16-hour light/8-hour dark cycle at which the aphids were reared. Six pots with two seeds per pot were grown in each chamber, and after the plants grew, the four individuals that were the most similar in height and number of open leaves were chosen for the experiment by cutting one plant in each pot out and discarding the remaining two pots. Aphids were placed onto the Broad bean plants when all plants had at least open pair of leaves, approximately 7 - 9 days after being planted.

There were four different treatments in this experiment: 1) plants grown in ambient CO_2 (400 ppm) with aphids not harboring *H. defensa*, 2) plants grown in ambient CO_2 with aphids harboring *H. defensa*, 3) plants grown in elevated CO_2 (900 ppm) with aphids not harboring *H. defensa*, and 4) plants grown in elevated CO_2 with aphids harboring *H. defensa*. Two aquaria were used for each treatment combination, each containing four plants, which resulted in a sample size of eight plants per treatment. The complete experiment was replicated 4 times.

To infest the plants with aphids, four, second instar aphids from the stock colony were placed on each plant using a small paintbrush. Second instar aphids were chosen to give the individual and their unborn offspring (as aphids have telescoping generations) as much exposure to the atmospheric treatment as possible, while also being less fragile to transfer onto the plant than first instar aphids. Since aphids can reach adulthood and start reproducing 9 to 11 days after their birth (Wale et al., 2000). After the aphids were placed on the plants, a mesh bag was placed around each plant, adhered with a rubber band, to keep the aphids restricted to the plant, while still allowing for the plant to be exposed to the appropriate atmospheric conditions. After allowing the aphids to feed and develop for one week, the number of aphids that reached adulthood, and offspring those adult aphids produced, were recorded. After that, the total number of offspring on each plant was divided by the number of adults on each plant so data could

be analyzed as number of offspring per adult.

After experiments were completed, plants shoots and roots were separated, cleaned, placed in paper bags, and dried in a Yamato gravity convection oven. Root and shoot biomass were measured and recorded, and leaves from three of the trials were tested for nutrient composition.

A three-way nested analysis of covariance was performed using JMP Pro 16. Independent variables were CO_2 (ambient vs. elevated), Symbiont (absent vs. present), CO_2 x Symbiont, Aquarium (nested within CO_2 and Symbiont), and Trial (1-4) as a covariate. The dependent variable, number of offspring per adult aphid, was transformed using natural log to fit the assumption of normality, prior to analysis. Statistics are presented on the transformed values, while figures are presented using the untransformed values.

Results

Aphids with *H. defensa* had significantly more offspring per adult than aphids not harboring the symbiont ($F_{1, 108} = 18.83$, $p < 0.0001$) (Fig. 2.1). CO_2 treatment, however, did not significantly affect the number of offspring born per adult after seven days ($F_{1, 108} = 1.45$, $p = 0.23$) (Fig. 2.2). There was not a significant difference in fecundity between aphids in aquariums of the same CO_2 treatment ($F_{4, 108} = 1.49$, $p = 0.21$). There was no significant interaction between CO_2 level and symbiont presence ($F_{1, 108} = 0.48$, $p = 0.49$) (Fig. 2.3). There was, however, a significant difference in the number of offspring born per adult between trials ($F_{1, 108} = 16.68$, $p < 0.0001$). This was not unexpected as there may have been a small difference in plant ages between trials, which is why trial was entered as a covariate in my analysis. There were no other significant differences in the analyses (Table 2.1).

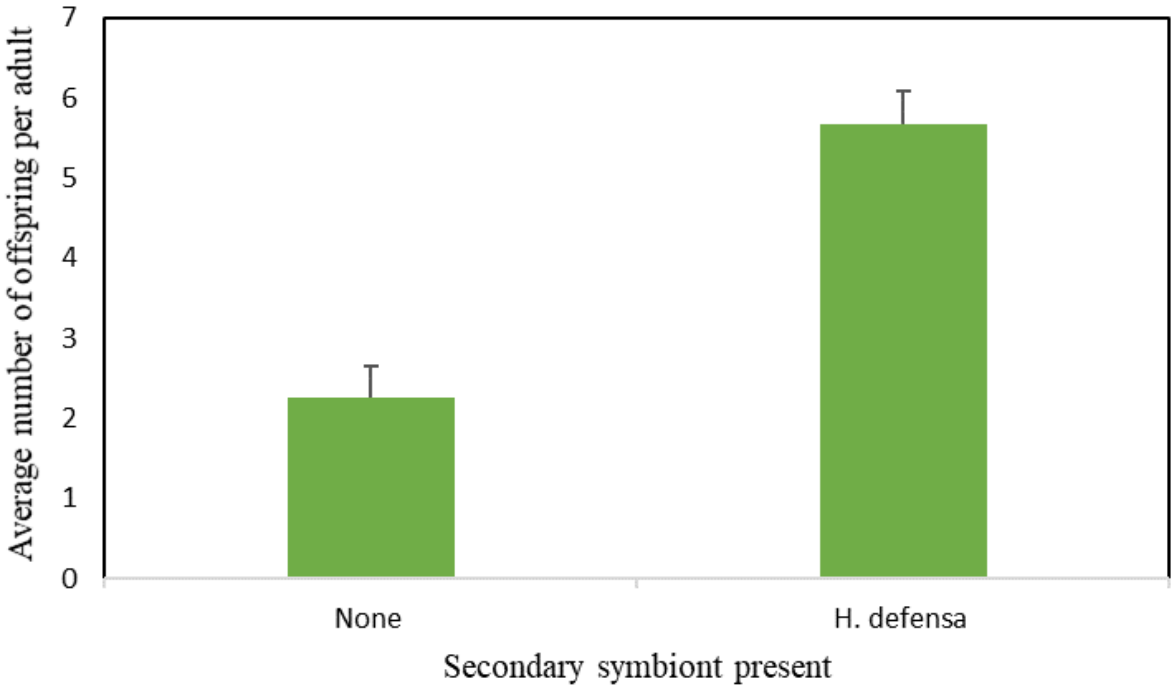

Figure 2.1. Average number of offspring for adult Pea aphids with vs. without the symbiont *Hamiltonella defensa* ($F_{1, 108} = 18.83$, $p < 0.0001$)

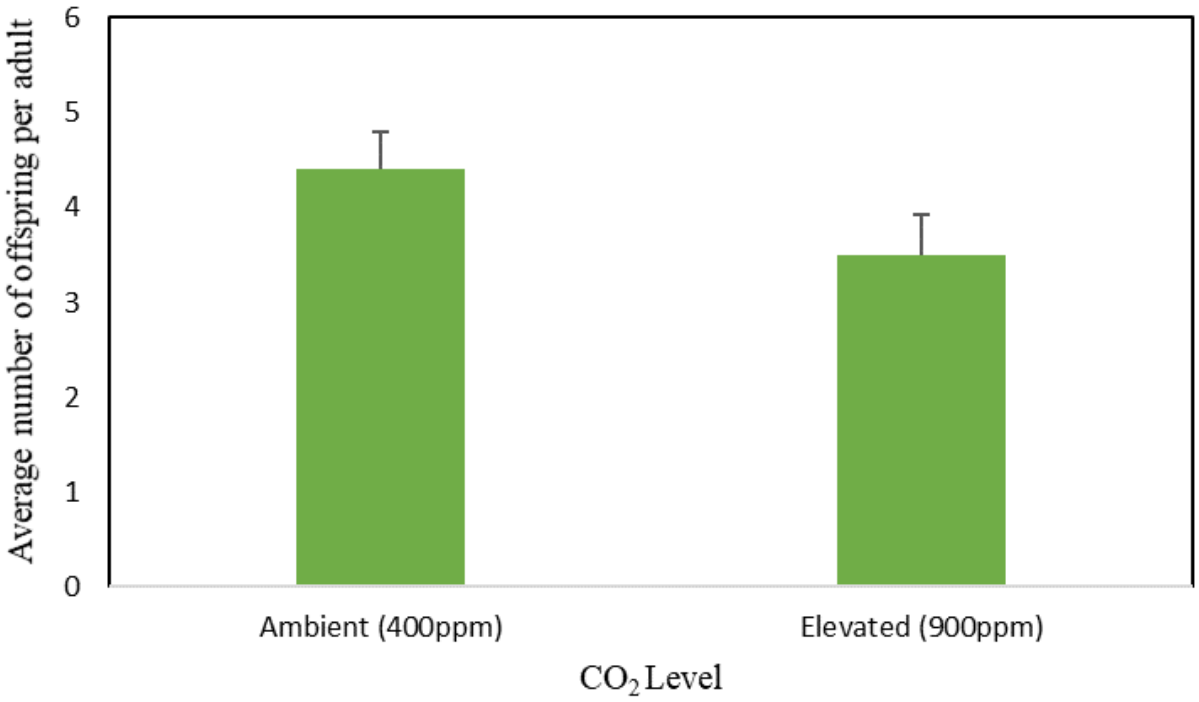

Figure 2.2. Average number of offspring for adult Pea aphids reared in ambient vs. elevated CO_2 ($F_{1, 108}$ = 1.45, p = 0.23)

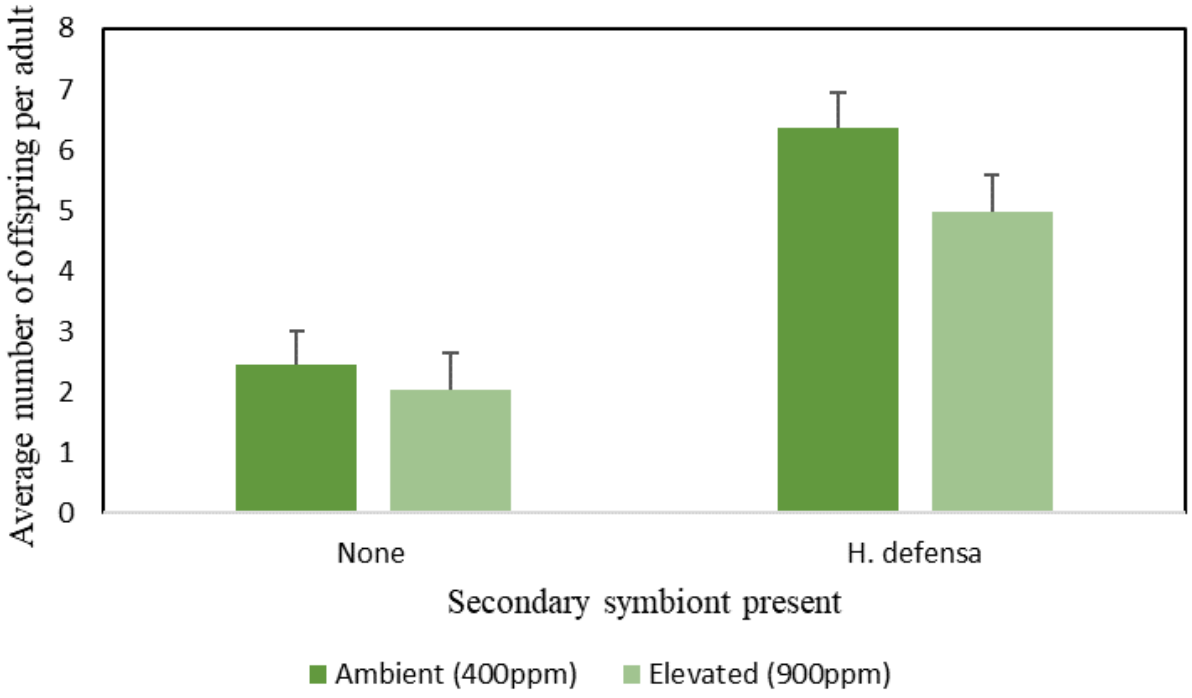

Figure 2.3. Average number of offspring for adult Pea aphids with vs. without the symbiont *Hamiltonella defensa* in ambient vs. elevated CO_2 ($F_{1, 108} = 0.48$, $p = 0.49$)

Effect Tests					
Source	**Nparm**	**DF**	**Sum of Squares**	**F ratio**	**Prob > F**
CO_2	1	1	0.946519	1.4484	0.2314
Symbiont	1	1	12.30758	18.8342	<0.0001
CO_2*Symbiont	1	1	0.313546	0.4798	0.49
Aquarium#[CO_2, Symbiont]	4	4	3.905647	1.4942	0.209
Trial	1	1	10.899333	16.6791	<0.0001

Table 2.1 Results of all effect tests from the analysis of covariance with offspring per adult aphid as the dependent variable

Discussion

Confirming my first hypothesis, aphids possessing the symbiont *H. defensa* had more offspring compared to aphids not harboring the symbiont. Vertically transmitted endosymbionts like *H. defensa* need their host to reach sexual maturity in order to increase its own fitness; that is, by enhancing reproduction of the host, it enhances the spread of the symbiont (Brownlie & Johnson, 2009). This symbiont-mediated increase in offspring production may explain why under lab rearing conditions, where the environment is ideal, infection rates of *H. defensa* are much higher than in field settings (Oliver et al., 2005). Analyzing the long term (i.e., over multiple generations) fecundity of aphids with *H. defensa* may give us a better idea of how this symbiont spreads in populations, as opposed to just looking at the first generation.

My second hypothesis was rejected, however, as aphids in elevated CO_2 did not have fewer offspring compared to aphids in ambient CO_2. Rather, there was no significant difference in aphid fecundity between CO_2 treatments. This could be due to differences in methodology as other studies have used different systems to achieve an elevated atmospheric CO_2 level as well as different host plants and cultivars for the aphids. Elevated CO_2 can maximize legume growth when other factors are not limited (Rogers et al., 2009), which may explain why aphids feeding on non-legume plants in elevated CO_2 show decreased fecundity compared to pea aphids in elevated CO_2 (Hughes and Bazzaz, 2001). Despite seeing a significant difference in some plant parameters between CO_2 treatments (see Appendix A), I suspect these plant growth differences did not result in a significant difference in aphid fecundity. It is likely that even though plants grown in elevated CO_2 had a higher dry shoot mass and shoot to root ratio, there were not enough aphids infesting the plants to make competition between aphids a factor. In fact, as part of my experimental protocol, I purposely restricted the number of adults per plant to avoid resource competition. In addition, nutrient analysis of the plant samples showed that none of our plants were deficient of essential elements (Mahler, 2004), further indicating that host plant quality was not a contributing factor to aphid fecundity in this experiment.

There was also no support for my third hypothesis that there would be a significant interaction between CO_2 level and *H. defensa*; the presence of *H. defensa* on aphid fecundity was not dependent on the CO_2 level. The host aphid relies on the obligate symbiont *Buchnera* to produce amino acids that cannot be found in the aphid's diet, however it has been reported that this obligate symbiont also produces amino acids for the facultative symbiont, *H. defensa* (Degnan et al., 2009). Since there was no significant difference in growth rate and reproduction between CO_2 treatments without *H. defensa*, this may indicate that *Buchnera* is producing adequate amounts of amino acids necessary for both the pea aphid and *H. defensa*. Additionally, *H. defensa* may have nutritional benefits to its pea aphid host. It has been found that the growth rate of whiteflies increases when they have H. defensa and are suffering nutritional stress (Su et al., 2014), so perhaps this same relationship can explain why aphids with this symbiont have a higher fecundity than those without.

One important next step would be to examine if symbiont/CO_2 interactions influence higher-order trophic levels, both physiologically and behaviorally. For example, is *H. defensa* still as effective at protecting pea aphids against parasitism in elevated CO_2 compared to ambient CO_2? A study focusing on parasitism may help determine whether certain biological control methods will become more or less efficacious in the future. If predators or parasitoids are less effective at locating or successfully parasitizing their prey in elevated CO_2 atmospheres, our pest management strategies would have to change going forward. In addition, studies should examine how aphids with other symbionts are affected by elevated CO_2. Investigating how aphids with other secondary symbionts reproduce in elevated atmospheric CO_2 may reveal which symbionts could become more prominent in aphid populations as CO_2 levels continue to rise. Not only would this information be interesting from a population genetics aspect, but it may also help inform agricultural producers of ways to limit the spread of diseases to crops by controlling the populations of the key vectors that spread those diseases.

CHAPTER 3

DOES THE SYMBIONT, *HAMILTONELLA DEFENSA*, ALTER PEA APHID, *ACYRTHOSIPHON PISUM*, BEHAVIOR IN ELEVATED CO2 ATMOSPHERES?

Introduction

Aphids have specialized structures on their abdomens called cornicles through which they secrete a fluid containing (E)-β-farnesene (Eβf), which functions as an alarm pheromone. As an aphid is being consumed, it often attempts to smear this secretion on a predator using its cornicles so that other aphids are aware of the predator's presence as it moves through the colony (Mondor, 2001). Pea aphids that are surrounded by identical clonemates are the most likely to produce cornicle secretions (Robertson et al., 1995), and pre-reproductive pea aphids that secrete cornicle droplets have shown a significant delay in offspring production, followed by an increase in fecundity compared to pre-reproductive aphids that did not expel cornicle droplets (Mondor & Roitberg, 2003).

Aphids respond to EBF in a number of ways to reduce the risk of predation; including long-term (i.e., transgenerational) responses such as increasing the proportion of winged offspring (Mondor et al., 2004) and short-term (i.e., immediate) responses such as kicking, moving, or dropping off their host plant (Keiser & Mondor, 2015). Of these behaviors, dropping off the plant is the costliest to the aphid's reproduction and survival (Nelson, 2007), and depends on a variety of factors such as predation risk, host plant quality, and the surrounding environment the aphid would be dropping into (Losey & Denno, 1998).

Although experiments have been conducted to see if responses to alarm pheromone differs between pea aphid biotypes that feed on different host plants (Ben-Ari et al., 2019), little is known about how endosymbiotic bacteria affects aphid-predator interactions. Pea aphids can have different types of endosymbiotic bacteria, which offer benefits in novel situations; e.g., surviving high temperatures, defense from parasitism, and protection from fungal infection (Oliver et al., 2010). Ensuring its host's survival is beneficial for a symbiont so that it can infect more individuals through vertical transmission of

the mother aphid to her daughters (Brownlie & Johnson, 2009).

While some symbionts are necessary for survival, like the obligate nutritional symbiont *Buchnera aphidicola*, aphids can also host a wide range of facultative symbionts. For example, pea aphids can harbor *Hamiltonella defensa,* a facultative symbiont that offers resistance to parasitoids (Oliver & Higashi, 2019). Aphid behavior has been shown to change with the presence of symbionts, as pea aphids harboring *Serratia symbiotica*, *Regiella insecticola,* and *Rickettsia* are less likely to drop off their host plants (Lavy et al., 2015), and aphids with *H. defensa* are less likely to display evasive behaviors in the presence of predators (Polin et al., 2014). Despite being more susceptible to predation, pea aphids with *H. defensa* increase reproduction after being exposed to their alarm pheromone compared to aphids without a facultative symbiont (Barribeau et al., 2010). It is possible that aphids harboring a secondary symbiont use an alternative response strategy compared to aphids without a secondary symbiont. Instead of dropping or fighting back, aphids with a secondary symbiont may try to stay on their host plant and increase their reproduction in response to alarm pheromone. This would benefit the symbiont and the aphid by increasing their fitness.

Aphid responses to alarm pheromone can also change depending on abiotic conditions. For example, aphids have shown increased movement responses to increasing temperature (Ma & Ma, 2012). While very little is known about aphid behavior in response to altered atmospheric conditions, it is a critical issue that must be addressed as the effects of climate change rapidly progress. It has been discovered that in elevated atmospheric CO_2, compared to ambient CO_2, adult aphids disperse less in response to alarm pheromone (Mondor et al., 2004). In addition, aphids produce less alarm pheromone when reared in elevated CO_2 (Boullis et al., 2017). Understanding how aphid defensive behavior is altered by the presence of a specific symbiont and altered atmospheric conditions can help us predict how the prevalence of certain symbionts in aphid populations may change in future environments.

In this experiment, I hypothesized that aphids with the secondary symbiont *H. defensa* will disperse less often than aphids without a secondary symbiont. I would expect to see aphids with *H. defensa* dispersing less often to maximize their feeding and take a risk on what natural enemy is present,

since if a parasitoid was attacking the colony, those aphids with *H. defensa* would be protected. Second, I hypothesized that aphids raised in elevated CO_2 would show less dispersal when exposed to alarm pheromone compared to aphids raised in ambient CO_2. I predict elevated CO_2 to interfere with the aphids' ability to react to their alarm signal, and since aphids produce less of their alarm pheromone in elevated CO_2, their dispersal should be much lower than aphids in ambient CO_2 Lastly, I hypothesized that there would be a significant, compounding effect between the presence of *H. defensa* and CO_2 level when assessing dispersal rates in response to alarm pheromone. That is, aphids would almost never respond to their alarm pheromone if they possessed *H. defensa* and were reared in elevated CO_2. Since I predict both of these factors to have a negative effect on dispersal separately, I expect to see a very large impact on aphid dispersal when both of these factors are present.

Methods

Four, second-instar aphids were placed on a young Broad bean plant, *Vicia faba* cv. Broad Windsor, and reared in either ambient CO_2 (400 ppm) or elevated CO_2 (900 ppm) conditions, via a machine called the Atmosim 2100. The Atmosim 2100 is made up of eight aquariums, with four aquariums at ambient CO_2 levels and four at elevated CO_2 levels. Each aquarium has its own tube that supplies it with CO_2 from a single CO_2 tank to ensure that each aquarium receives the correct amount of CO_2. Four plants, each in an individual pot, were kept in each aquarium. Plants and aphids were kept in individual mesh bags to prevent aphids from moving among plants. Aphids with the bacterial endosymbiont, *H. defensa*, were placed on plants in two aquariums of each CO_2 treatment (i.e., ambient vs. elevated), while aphids without symbionts were placed on plants in the other two aquariums for each CO_2 treatment. This resulted in a fully crossed experiment: aphids with *H. defensa* reared in ambient CO_2, aphids with *H. defensa* reared in elevated CO_2, aphids without a symbiont reared in ambient CO_2, and aphids without a symbiont reared in elevated CO_2.

After the aphids developed and reproduced for 9-10 days, aphids were exposed to 1 uL of a solution containing either their alarm pheromone (EBF), or the carrier for the alarm pheromone (control - hexane).

The Eβf treatment solution was made by mixing 0.6 µL concentrated Eβf with 10 mL of hexane, resulting in a solution of approximately 50 ng/uL (Keiser & Mondor, 2015). Since there were four plants in each aquarium, two were randomly selected to receive the EBF treatment and the other two were chosen to receive the control treatment. After a plant was randomly assigned a treatment, a cluster of aphids was located on the plant and the aphids in the cluster were counted before a treatment was applied. One microliter of the experimental treatment or the control was placed on a small piece of filter paper, and forceps were used to hold the filter paper near the cluster of aphids. The filter paper was presented to the cluster of aphids for 30 seconds and the number of aphids that dispersed (i.e., walked, ran, or dropped off the plant) out of the total number of aphids in the cluster was recorded. For each trial, a cellphone was used to record the aphid behavior so that the number of aphids that dispersed could be verified.

After the data was collected, I calculated the percentage of aphids per cluster that dispersed since there were different numbers of aphids in each cluster. A four-way nested analysis of covariance was performed using JMP Pro 16. Independent variables were: Pheromone treatment (EBF vs. control), CO_2 (ambient vs. elevated), Symbiont (absent vs. present), Pheromone treatment x CO_2, Pheromone treatment x Symbiont, CO_2 x Symbiont, Pheromone x CO_2 x Symbiont, Aquarium (nested within CO_2 and Symbiont), and Trial (1-4) as a covariate. Aphids per cluster was introduced as a weighting variable, as there is higher confidence in the proportions obtained from clusters with higher numbers of aphids. The dependent variable, proportion of aphids dispersing, was transformed using arcsin square root to better fit the assumptions of normality, prior to analysis. Post-hoc tests, when required, were Tukey's HSD tests. Statistics are presented on the transformed values, while figures are presented using the raw values.

Results

There was a significant difference in the proportion of aphids that dispersed in response to EBF vs. the hexane control, with an average of 33% of aphids moving when exposed to Eβf and an average of 2% of aphids moving when exposed to the control treatment ($F_{1, 39} = 13.29$, $p = 0.0008$) (Fig. 3.1). This is not surprising as this response has been documented numerous times, but it does confirm that the

experimental protocol in this study was effective. There was also a significant difference in the number of aphids that dispersed when infected vs. not infected with *H. defensa*, with 6% of uninfected aphids dispersing but 29% of infected aphids dispersing ($F_{1, 39} = 7.14$, $p = 0.011$) (Fig. 3.2). Level of CO_2 did not significantly influence the number of aphids dispersing ($F_{1, 39} = 1.01$, $p = 0.32$) (Fig. 3.3). There was, however, a significant interaction between presence of a symbiont and CO_2 level ($F_{1, 39} = 4.55$, $p = 0.039$) (Fig. 3.4). Using a Tukey's HSD test, it was discovered that the presence of a symbiont had large effects on dispersal under ambient CO_2 levels (no symbiont = 1%, symbiont = 41%), but not under elevated CO_2 levels (no symbiont = 11%, symbiont = 16%). There were no other significant differences in the analyses.

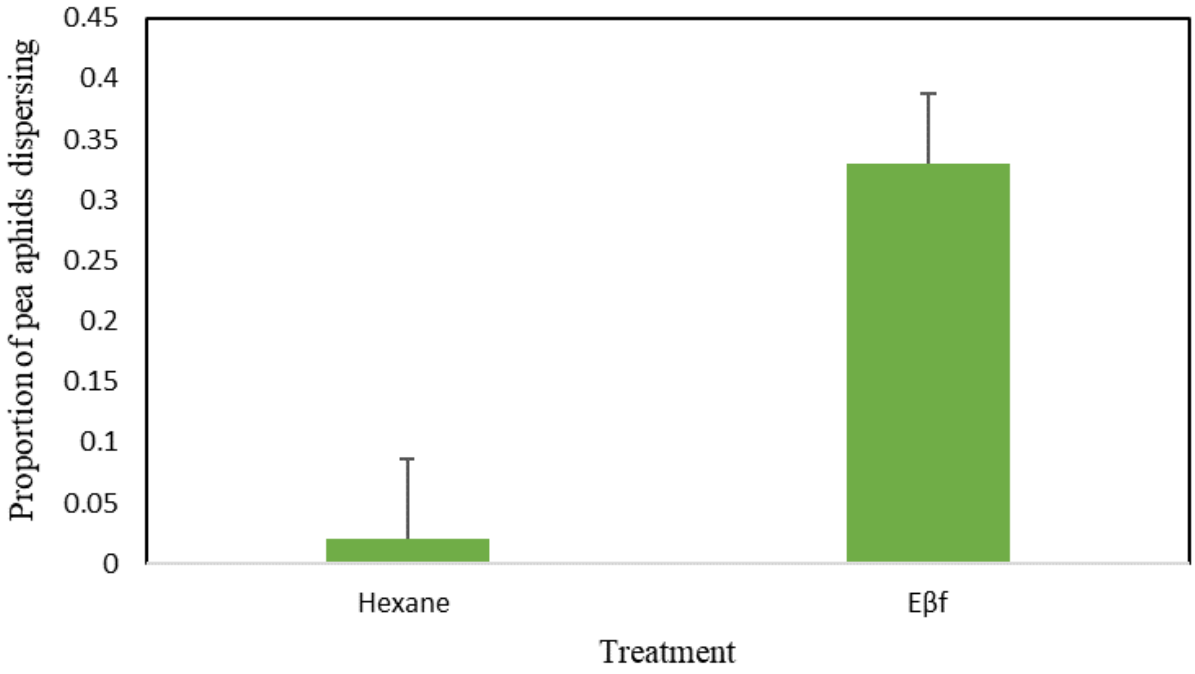

Figure 3.1. Proportion of Pea aphids dispersing when exposed to hexane (control) vs. alarm pheromone (E-β-farnesene) ($F_{1, 39} = 13.29$, $p = 0.0008$)

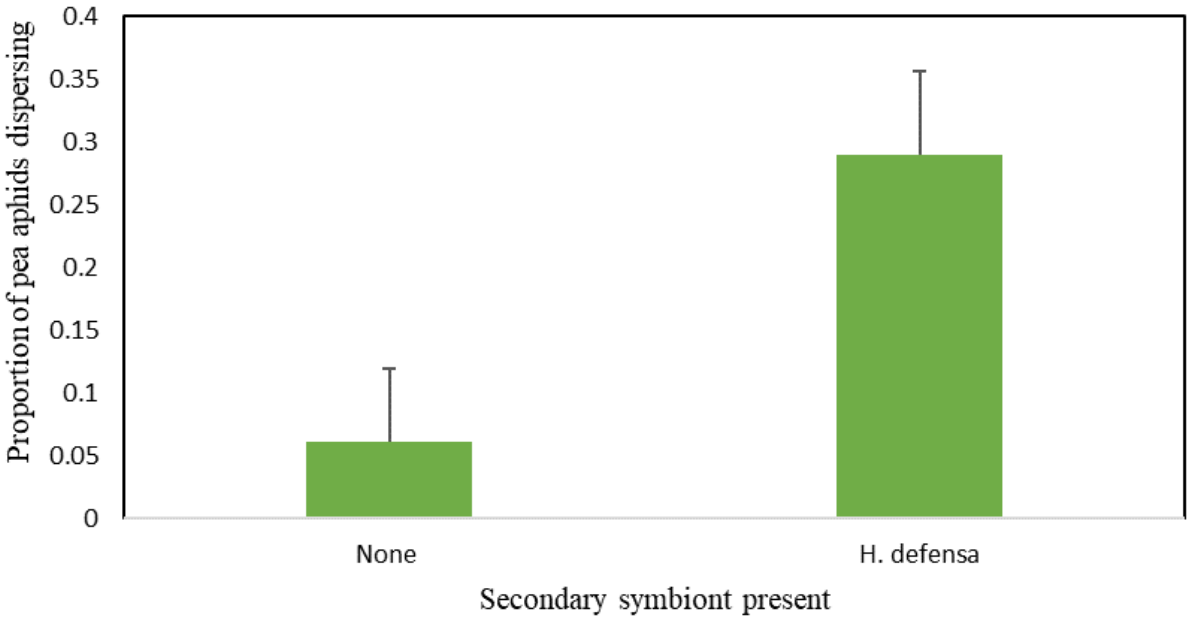

Figure 3.2. Proportion of Pea aphids dispersing with vs. without the symbiont *Hamiltonella defensa* ($F_{1, 39}$ = 7.14, p = 0.011)

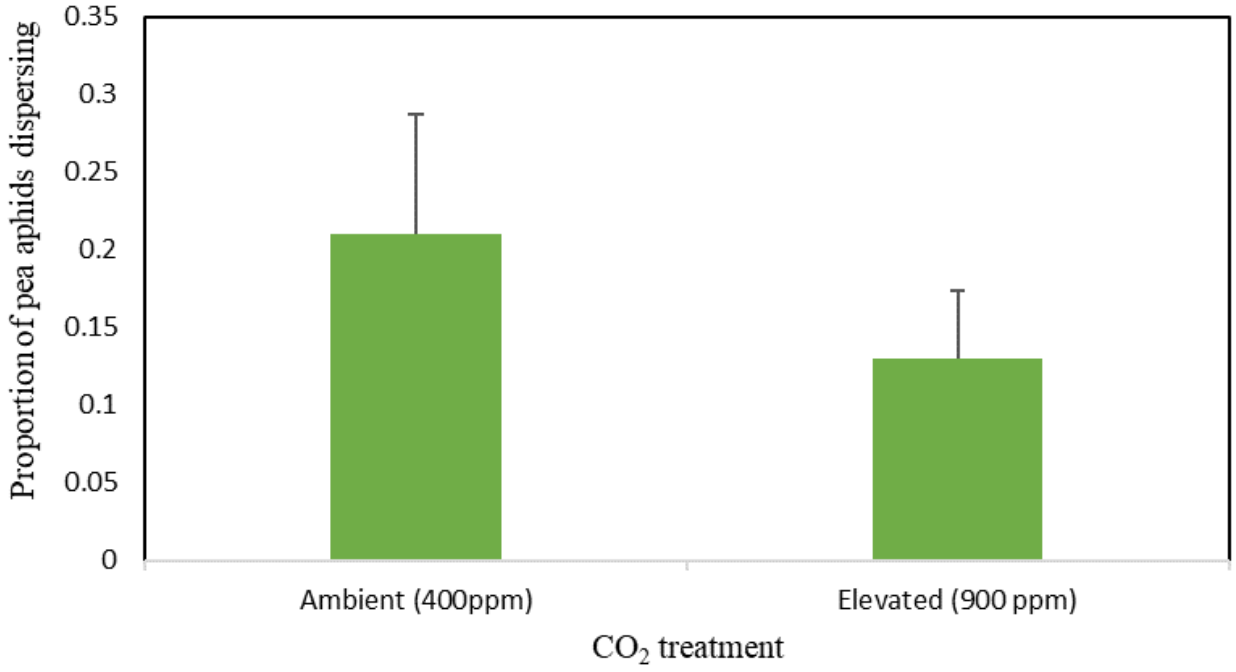

Figure 3.3. Proportion of Pea aphids dispersing in ambient vs. elevated CO_2 ($F_{1, 39} = 1.01$, $p = 0.32$)

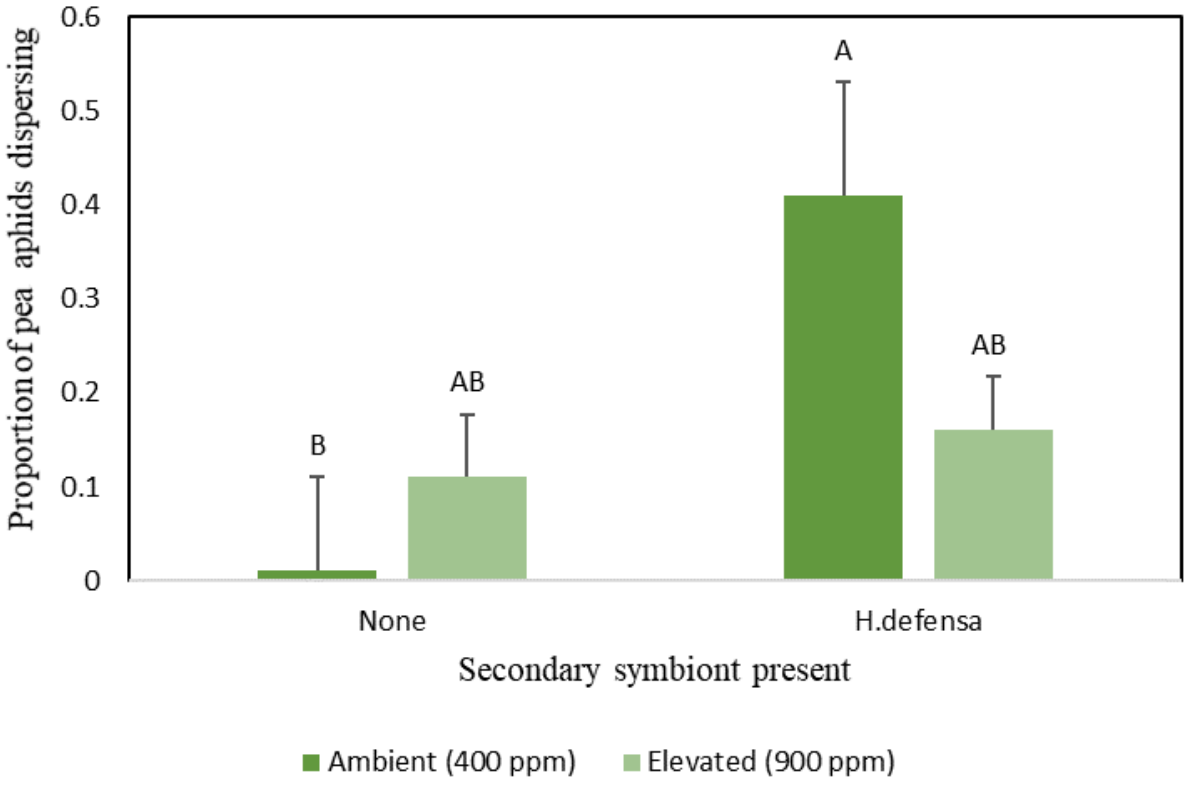

Figure 3.4. Proportion of Pea aphids dispersing with vs. without the symbiont *Hamiltonella defensa* in ambient vs. elevated CO_2 ($F_{1, 39} = 4.55$, $p = 0.039$)

Effect Tests					
Source	**Nparm**	**DF**	**Sum of Squares**	**F ratio**	**Prob > F**
CO_2	1	1	0.942411	1.0127	0.3205
Symbiont	1	1	6.642459	7.1376	0.011
Treatment	1	1	12.366317	13.2881	0.0008
CO_2*Symbiont	1	1	4.235703	4.5514	0.0392
Treatment*CO_2	1	1	0.053292	0.0573	0.8121
Symbiont*Treatment	1	1	2.063219	2.217	0.1445
Symbiont*Treatment*CO_2	1	1	0.993054	1.0671	0.308
Aquarium#[CO_2, Symbiont, Treatment]	8	8	9.546974	1.2823	0.2808
Trial	1	1	1.630121	1.7516	0.1934

Table 3.1 The results of all effect tests for the analysis of covariance with aphid dispersal as the dependent variable

Discussion

Based on previous research and the literature (Mondor, 2001), I expected aphids to respond to alarm pheromone more than to the control treatment (hexane). This turned out to be correct, thereby verifying previous studies, but also confirming that our EBF solution was biologically active and our experimental protocol was appropriate.

My hypothesis that aphids would be less likely to disperse when infected with *H. defensa* was rejected. In fact, aphids with *H. defensa* were far more likely to disperse than aphids without the facultative symbiont. It has been suggested that resistance to one type of natural enemy via symbiont leads to a tradeoff of being more vulnerable to other natural enemies (Ferrari et al. 2001), and this may be due to how H. defensa mediates an alarm pheromone response. Even though aphids with *H. defensa* produce less alarm pheromone overall (Boullis et al., 2017), they are likely just as sensitive to alarm pheromone when they receive that signal. This would allow aphids with *H. defensa* to react to their alarm pheromone on a case-by-case basis, where they are unlikely to disperse until a certain threshold is reached, in which case they become much more likely to disperse than aphids without the secondary symbiont. This would be very beneficial to the aphid as well as the symbiont, as if a small amount of alarm pheromone was detected, perhaps indicative of a parasitoid or a distant threat, aphids with *H. defensa* would be able to continue to feed and reproduce efficiently. Alternatively, if a large amount of alarm pheromone is detected, perhaps indicating a large predator is nearby, *H. defensa* triggers a severe dispersal response in its host to ensure it is able to survive and infect the next generation of aphids. Another potential reason for these results could be the way alarm pheromone was presented to the aphids, or the level of pheromone the aphids experience. Studies that showed aphids with *H. defensa* move less than aphids without it used live predators (Polin et al., 2014) and parasitoids (Dion et al., 2011), whereas this study used a standardized Eβf solution on filter paper. Aphids may respond differently to an actively searching natural enemy as opposed to a solitary EBF emission, as extreme defensive behaviors are costly to aphid fitness (Nelson, 2007). In addition, perhaps the amount of pheromone I used in my experiments was perceived as a larger threat by the cluster of aphids compared to the amount generated by a single

natural enemy moving in one direction through a colony. This study used about five times the amount produced by one aphid (Mondor et al., 2000), which could indicate a large attack as opposed to just a single natural enemy like other studies have used. This result raises many additional questions regarding how aphids respond to natural enemies and perceive different levels of increased predation risk threats.

I also hypothesized that aphids would exhibit a greater dispersal response in ambient CO_2 as opposed to elevated CO_2. This hypothesis was rejected as there was no difference in the number of aphids that dispersed in ambient vs. elevated CO_2 environments. Previous studies have shown that aphids are more likely to disperse when feeding on low quality host plants (Dill et al., 1990), but the plants used in this experiment showed no signs of being nutrient deficient (Table A.1), which indicates that in this study, CO_2 level was not indirectly influencing aphid behavior via host plant. Like other behavioral studies (Mondor et al., 2004), I did find that less aphids dispersed in elevated CO_2, however the lack of a significant difference suggests that something else may be at play in this interaction. Perhaps instead of aphids being less receptive to alarm pheromone, studies in the past have shown less dispersal because the aphids are producing less alarm pheromone in elevated CO_2 (Boullis et al., 2017). This study used a constant amount of alarm pheromone rather than getting aphids to release their own alarm pheromone to initiate a dispersal response in the colony, which could be why the dispersal was not significantly different between the CO_2 treatments.

Lastly, I hypothesized that there would be a significant interaction between the presence of a secondary symbiont and CO_2. This hypothesis was confirmed, as there was a large difference in dispersal rates between infected vs. uninfected aphids under ambient CO_2 levels, but similar rates of dispersal in infected vs. uninfected individuals under elevated CO_2 levels. Other studies have shown that elevated CO_2 lowers dispersal in aphids (Mondor et al., 2004), however my results show this is much more apparent in aphids that have a symbiont. This result is very interesting from an ecological point of view, as it indicates that predator and prey dynamics may be very different in elevated CO_2. If elevated CO_2 alters dispersal of aphids with *H. defensa* or other symbionts more than aphids without symbionts, it could heavily alter the symbiont composition in populations, as those aphids would be more susceptible

to predation. This result raises the question of other symbionts and their interactions with CO_2 level.

Additional experiments should be conducted to give further insight into how aphid behavior may play out in future atmospheric conditions. In this experiment there was limited space in the aquariums, so the plants with aphids on them had to be taken out of their respective CO_2 environment to perform the experiment. Exposing aphids to alarm pheromone in the actual elevated CO_2 environment may show a different effect than when they were taken out of their CO_2 treatment prior to exposure. Future studies should also consider more extensive testing of host plant quality and how that impacts aphid behavior in elevated CO_2. Aphids are less likely to display defensive responses in scenarios of food depravation (Villagra et al., 2002), so looking at behaviors under other stressful scenarios like poor host plant quality in elevated CO_2 may determine what factors can influence aphid dispersal. Additionally, documenting the instar of aphids that disperse may provide additional information, as different age groups show different degrees of defense responses (Gish & Inbar, 2006).

Experiments in the laboratory setting may not be truly indicative of the responses of aphids in wild populations, as high symbiont prevalence is easier to retain in a lab setting than in wild populations (Oliver et al., 2005). Additional research is required to discover more about insect behavior in an elevated CO_2 environment. Since these results and other studies have shown aphids are not as reactive to their alarm pheromone in elevated CO_2 (Sun et al., 2010), a logical next step would be to determine if increased CO_2 alters the efficacy of predators in biological control programs, when aphids harbor facultative symbionts.

CONCLUSION

The goal of this thesis was to untangle some of the complex interactions that pea aphids have with their facultative symbiont, *H. defensa*, and elevated CO_2 levels associated with global climate change. These types of studies will become increasingly important as CO_2 levels continue to rise and affect the crops we grow and the insects that prey on those plants.

In Chapter 2, I found that pea aphids harboring *H. defensa* had a much higher fecundity than those without, and elevated CO_2 conditions did not affect this interaction. *H.defensa* may have some type of nutritional benefit as seen in other insects (Su et al., 2014), leading to an increased fecundity. However, this still raises questions about why H. defensa does not have a higher prevalence in wild populations (Dykstra et al., 2014). In addition, I suspect that I did not see a difference in fecundity between CO_2 levels because the plant analysis revealed that all plants were healthy despite being raised in different CO_2 conditions.

In Chapter 3, I found that symbiont presence had a significant effect on aphid dispersal, while CO_2 level did not. However, I did see a significant interaction between *H. defensa* presence and CO_2 level, indicating that these variables may have an impact on aphid populations in the future. While some of my results, like aphids with symbionts being more responsive to alarm pheromone, go against some previous studies (Dion et al., 2011; Polin et al., 2014), this raises new questions about how aphid symbionts affect their interactions with predators. Perhaps the amount of alarm pheromone present triggers different responses in aphids, and further studies should test how much alarm pheromone triggers specific responses like moving vs. dropping off of the plant.

Novel abiotic conditions, resulting from elevated CO_2, will undoubtedly change the prevalence of certain symbionts as they respond to these unknown pressures (i.e., hidden reaction norms). Future studies should look at different combinations of abiotic and biotic factors (e.g, greenhouse gases, facultative symbionts) as the more we learn about how aphid fitness and behavior change due to those variables, the greater the efficacy of our pest control strategies and our knowledge of ecosystem functioning.

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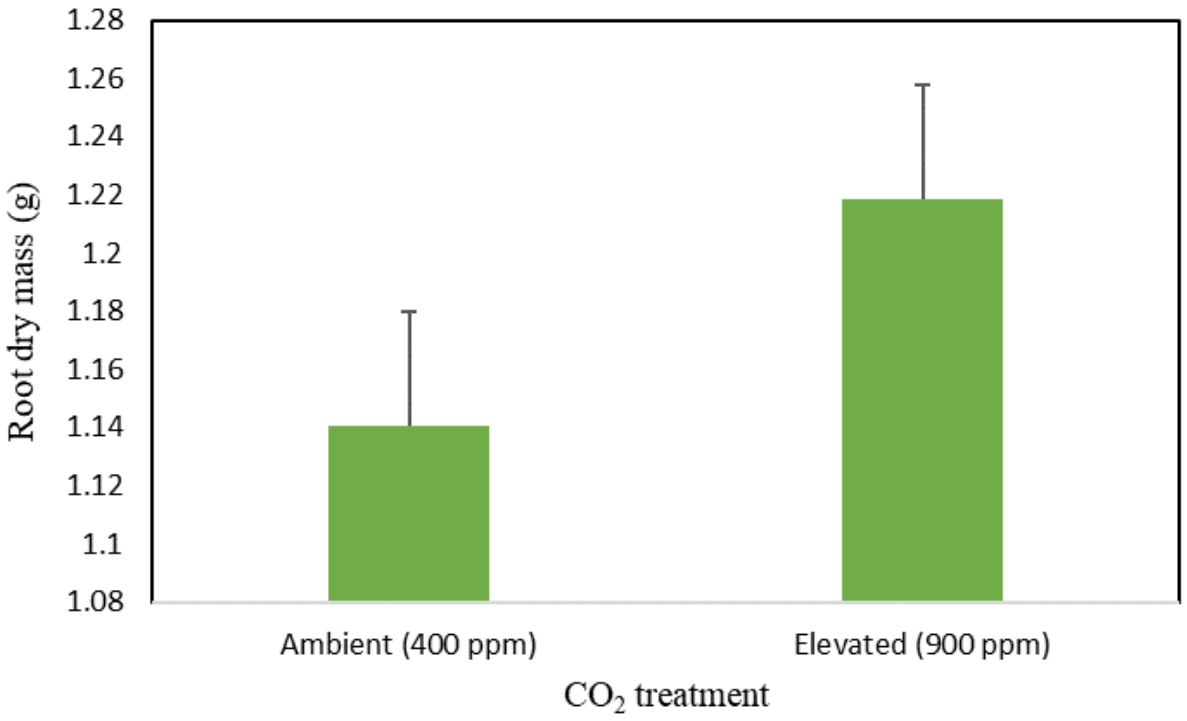
APPENDIX

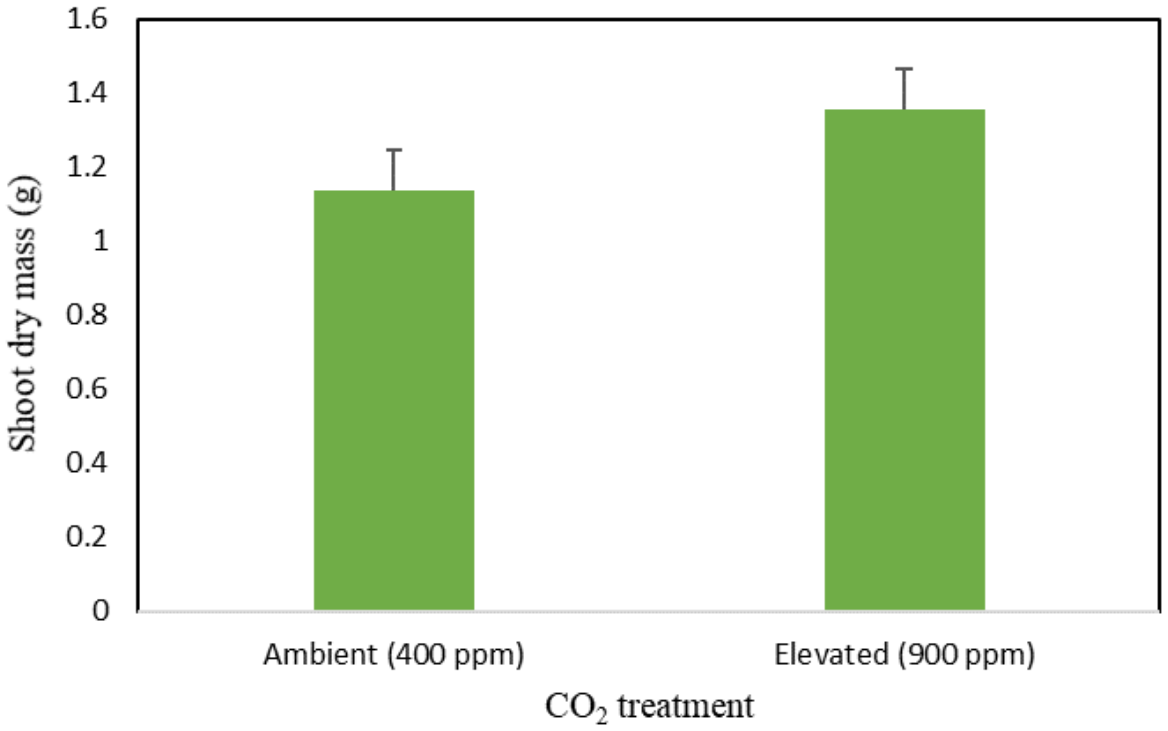
PLANT NUTRIENTS AND PHYSIOLOGY

		% (percent)					
Sample	**CO_2 level**	**Ca** calcium	**K** potassium	**Mg** magnesium	**P** phosphorus	**N** nitrogen	**S** sulfur
1	Ambient	0.68	4.77	0.81	0.56	6.69	0.38
2	Elevated	0.68	4.63	0.80	0.41	5.63	0.34
3	Ambient	0.80	2.57	0.96	0.31	4.40	0.37
4	Elevated	1.26	2.19	1.31	0.26	4.16	0.27
5	Ambient	0.63	5.10	0.70	0.77	4.99	0.44
6	Elevated	0.57	4.86	0.65	0.78	5.28	0.38

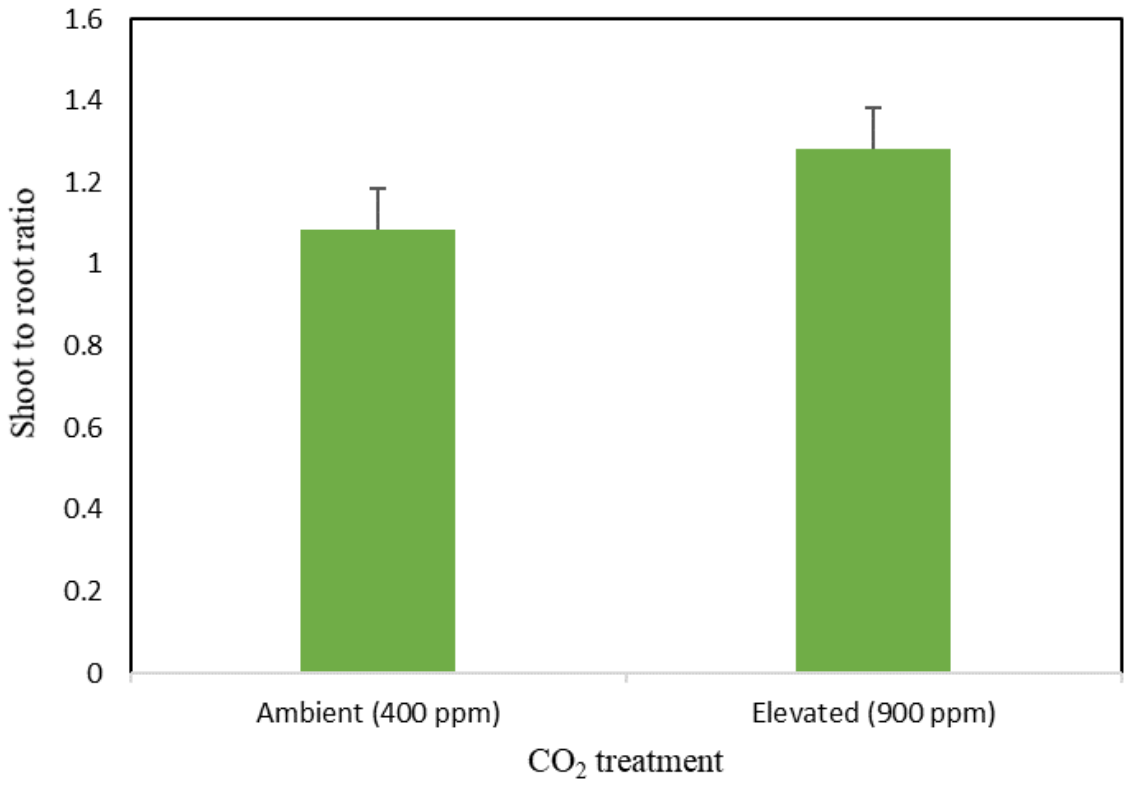
Percent of six essential nutrients in Broad bean, *Vicia faba*, plants grown in ambient vs. elevated CO_2



Average root biomass of Broad bean, *Vicia faba*, plants grown in ambient vs. elevated CO_2 ($F_{1, 91} = 0.86$, $p = 0.36$).



Average shoot biomass of Broad bean, *Vicia faba*, plants grown in ambient vs. elevated CO_2 ($F_{1, 91} = 9.33$, $p = 0.0030$).



Average shoot to root ratio of Broad bean, *Vicia faba*, plants grown in ambient vs. elevated CO_2 ($F_{1, 91}$ = 4.65, p = 0.034).