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A Comparative Study of Eastern and Western North American Populations of *Hippodamia convergens* (Coleoptera: Coccinellidae)

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A COMPARATIVE STUDY OF EASTERN AND WESTERN NORTH AMERICAN POPULATIONS OF *HIPPODAMIA CONVERGENS* (COLEOPTERA: COCCINELLIDAE)

THESIS

A thesis in partial fulfillment of the requirements for the degree of Master of Science in Entomology in the College of Agriculture, Food, and Environment at the University of Kentucky

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ABSTRACT OF THESIS

A COMPARATIVE STUDY OF EASTERN AND WESTERN NORTH AMERICAN POPULATIONS OF *HIPPODAMIA CONVERGENS* (COLEOPTERA: COCCINELLIDAE)

Hippodamia convergens is a widely distributed insect predator in the United States and parts of Canada and Mexico. Several insectaries collect overwintering adults from aggregation sites in the Rocky Mountains during their winter dormancy. Collected beetles are then sold throughout the United States for augmentative biological control. This practice could have negative impacts on local populations of *Hippodamia convergens* in the Eastern United States. Intra-specific variation among *H. convergens* populations was examined for two characteristics of adults: photoperiodic induction of diapause and the presence of three known male-killing endosymbiont bacteria; *Wolbachia, Spiroplasma,* and *Rickettsia*. Four populations of *H. convergens* were examined; two populations were collected in Kentucky and Illinois, and two populations were purchased from biocontrol companies in Arizona and California. No differences were observed among populations in their responses to diapause inducing photoperiods. Also, no evidence was found to indicate that the three endosymbiotic bacteria exist within the four *H. convergens* populations. The results from these experiments indicate that there are no differences in response to diapause inducing photoperiods, meaning that it is not likely to affect timing of diapause induction. The lack of endosymbionts would indicate that there are no reproductive barriers to intra-population matings.

KEYWORDS: *Hippodamia convergens*, Coccinellidae, Biological control, Endosymbionts, Intra-specific variation

Joshua Sean McCord

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DEDICATION

I dedicate this thesis to Nancy J. Woeste. It all started with a butterfly.

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Section 1

Introduction

A. Population Genetics

Species adapt to their native geographic range, often over millions of years of evolution. These ranges are limited by factors that include geographic barriers, natural predators, and food supply (Diehl & Bush, 1984). Geological events such as plate tectonics or glaciation can create conditions in which populations diverge, and become isolated from each other. When these events lead to reproductive isolation, they allow for genetic divergence to occur (Hoskin et al., 2005).Genetic divergence is a process that, over time, allows for reproductively isolated populations to accumulate different mutations or adaptations and can lead to a complete inability to breed between the populations if they are reintroduced to each other.

Reintroduction of previously separated populations is a more common occurrence now due to both conservation efforts and accidental introduction from increased transportation from human movement. One notable example of this is the Monarch butterfly, *Danaus plexippus* (Lepidoptera: Nymphalidae). Monarch butterflies have two populations on either side of the Rocky Mountains (Brower et al., 1995). In the 1980s and 1990s experiments were conducted that involved moving adults across this geographic barrier, for example from Nebraska to Oregon, for the purpose of increasing population numbers. Brower et al. (1995) and Aardema et al. (2011) discuss the issues that may arise due to these population introductions. They discuss several problems

that can arise due to the susceptibility of each population to insect pathogens and the loss of data that could help understand the Monarch's more basic biological features, such as timing of migration patterns. Altizer et al. (2000) studied the impact that the protozoan parasite *Ophryocystis elektroscirrha* had on populations of Monarchs with different migratory patterns. Their study showed that the levels of infection among Southern Florida (>70%), Western USA (~30%), and Eastern USA populations (<10%) varied. If individuals from one population, such as the Southern Florida population, were to be transferred to another region, they could increase the presence of the parasitic protozoan and potentially increase mortality of Monarch populations.

Reintroduction of populations is not always purposeful. The movement of products across the country allows some species of insects to travel on plants and other goods (Austin et al., 2011; Maki & Galatowitsch, 2004). Some of these species become pests in the newly introduced area. To control these pests, a purposeful introduction of a natural enemy can be made. These introductions are termed importation biological control.

B. Biological Control

Pedigo (2009) broadly defines biological control as "the employment of any biological agent for control of a pest." Pedigo's definition covers the basic idea of biological control, which is the use of herbivores, predators, parasitoids, and pathogens from both native and exotic locations to suppress a pest species. Such pest species can be an introduced species, originating from a different part of the world, or it can be a native species that is experiencing an outbreak and causing higher than usual damage.

There are three main types of biological control that can be utilized to reduce a pest population's numbers: importation, conservation, and augmentative (Huffaker & Messenger, 1976).

Classical (importation) biological control was the first form of biological control used in North America in 1889 (Caltagirone, 1989; DeBach & Rosen, 1991). This type of biological control is used when the pest is exotic to the area it is causing problems in. Attempts at controlling the pest are made with predators and parasitoids found in the pest's native range. Conservation biological control is the use of native, local enemies to control a pest problem. The environment is made more suitable by increasing the attractiveness or density of the food sources in the area, increasing shelter availability, or creating breeding or oviposition sites.

The type of biological control most pertinent to my research is augmentative biological control, which was first used by Doutt and Hagen (1949) with green lacewings. Similar to importation, augmentative biological control involves the release of control agents that are selected due to their ability to control the specified pest (Collier & Van Steenwyk, 2004). It differs in that the control agent can be a native species, or from an area that the pest is not from. The released control agent is also not expected to establish in the released area, and will only provide control for the season. Several companies in North America rear and sell pathogens, predators, and parasitoids to be used for augmentative biological control (White and Johnson, http://www2.ca.uky.edu/entomology/entfacts/ef125.asp). Augmentative biological

control also tends to be limited to a few specific areas when it is used, such as greenhouses (van Lenteren, 1988; van Lenteren et al., 1997).

There are two types of augmentative biological control that can be performed. Inundative control involves releasing large numbers of the control agent. This is meant to overwhelm the pest population and provide fast reduction of the pest population numbers. Generally, with natural enemies used for inundative control, only one life stage is attacking the pest. Inoculative control involves releasing a smaller number of the control agent at critical times to help prevent the pest population from ever reaching high enough numbers to cause damage. These control agents usually have both adult and larval stages that feed on the pest. An example of this type of biological control agent would be *Encarsia formosa*, a parasitoid used to control whiteflies in greenhouses (Birkett et al., 2003; Liu et al., 2014; Speyer, 1927; Vet et al., 1980; Woets, 1800).

Several studies have examined the quality of purchased augmentative biological control agents (Bjornson, 2008; O'Neil et al., 1998). Bjornson (2008) found that of the 22 shipments of *Hippodamia convergens* (Coleoptera: Coccinellidae) received, 13 contained microsporidia (*Nosema hippodamiae, N. tracheophila, and N. coccinellae*), and all 22 had adult *H. convergens* that were parasitized by a Braconid parasitoid (*Dinocampus coccinellae*). These results suggest that the screening practices, if any, of collected beetles are inefficient at detecting parasitoids and other infections or fungi that may be present in individual beetles, and could result in the spread of parasitoids and pathogens of biological control agents or other insects.

C. *Hippodamia convergens*

Hippodamia convergens, commonly called the convergent lady beetle, is native to the United States of America, parts of Canada, and was also introduced to parts of South America for biological control (Gordon, 1985; Hagen, 1962). In California, overwintering adults aggregate in the crevices of mountains until spring, and emerge from their overwintering locations to return back to the valleys and farms to feed and mate. Adults release a pheromone when they head to their overwintering locations (Wheeler & Cardé, 2013). This pheromone is composed of three main compounds: 2-Isobutyl-3-methoxypyrazine, 2-sec-butyl-3-methoxypyrazine, and 2-isopropyl-3-methoxypyrazine. Together, these compounds make up the aggregation pheromone that is used to attract other individual beetles to a single location so that they may overwinter together in a large mass. Aggregation sites have been shown to be reused over several years when the current generation detects trace amounts of the pheromone left behind by previous generations (Wheeler & Cardé, 2014). In many areas of the United States east of the Rocky Mountains, beetles have also been found to aggregate on hills and mountains. Douglass (1930), Stewart et al. (1967), and Yanes et al. (1982) reported the presence of *H. convergens* in the mountains of New Mexico, Arkansas, and Oklahoma. Both Sherman (1938) and Thomas (1932) noted that *H. convergens* are found in the mountains of the Carolinas during winter months, and Throne (1935) found adults in hills in Michigan. Denemark and Losey (2010) found aggregations of Coccinellidae along the Finger Lakes Region of New York. Farther north, Turnock and Wise (2004) found evidence of overwintering beetles in the leaf litter surrounding Lake Manitoba, in Canada. Lee

(1980) also found that *H. convergens* adults would form brief 2-3 week aggregations along the shores of lakes in the northern mid-west of the United States. This could indicate that the overwintering location of *H. convergens* in other parts of the continent when mountains are not available is in leaf litter and along shore lines.

Dormancy in *H. convergens* adults during the winter months is defined as a type of hibernation by Hagen (1962). Hibernation is a type of reproductive dormancy and occurs during the cold months, during which the beetles live off accumulations of fat-bodies that they have built up. It is possible that during summer months, when temperatures are undesirable, the beetles enter a form of dormancy known as estivation (Michaud & Qureshi, 2006). After diapause ends in early spring, beetles emerge and mate (Hagen, 1962; Michaud & Qureshi, 2005). Females will oviposit on a variety of plant life, and eggs take between two and four days to hatch, depending on temperature (dos Santos et al., 2013). The larvae and adults are aphidophagous, and have been shown to eat a wide variety of aphid species that are native, introduced, and invasive to the U.S. (Hodek & Honek, 1996; Rutledge et al., 2004).

Hippodamia convergens plays a large role as an inundative biological control agent, and has been in use since around 1910 (Carnes, 1912). Currently, several insectaries based in the Western U.S.A. collect aggregating adults from the mountains in winter and sell them throughout the USA and Canada for use in greenhouses, small personal gardens, and larger crop fields (White and Johnson http://www2.ca.uky.edu/entomology/entfacts/ef125.asp). They also are purchased for release at weddings and other events. The collected individuals are kept at low

temperatures until they are shipped to buyers. In 2012, there were 62 commercial sellers of biological control agents. Of these, 28 (45%) sell *H. convergens* as a control agent (White and Johnson, http://www2.ca.uky.edu/entomology/entfacts/ef125.asp)

The spread of pathogens and parasitoids, such as the Braconid endoparasitoid, *Dinocampus coccinellae,* are some of the risks that are encountered in augmentative biological control programs using *H. convergens*. However, there may be other risks that are overlooked. The geographic barriers that separate populations can lead to local populations adapting to local conditions of their environment. When reintroducing these populations to each other, unforeseen problems may occur. One of the largest barriers that exist in the U.S. is the Rocky Mountain range, which spans from Canada to the Mexican border. This range separates populations of many insects between the western and eastern portions of the country. However, due to biological control practices shipping large numbers of *H. convergens* from the west side of the mountain range to the eastern region of the U.S., potentially differentiated populations are being moved across this geographic barrier.

Movement of populations across geographic barriers may be problematic due to differences in each population's physiology and environmental adaptations. A recent study by Sethuraman et al. (2015) showed high levels of inter-population genetic diversity in eleven populations of *H. convergens.* This means that genetic differences are observed among populations. The populations used in their study came from several locations across the U.S.A. including Arizona, California, and Kentucky, and Illinois. This genetic diversity could indicate that there may also be some phenotypic differences

among the populations. These may be expressed as differences in diapause induction conditions, bacterial symbiont relationships, the ability to develop on certain prey items only found in one part of the continent, or the response to the chemical blend of overwintering pheromones. My research examines two of these factors to determine if intraspecific variation in phenotypically important traits does exist.

D. Objectives

The objectives of my research were to determine if intraspecific variation exists among populations of *H. convergens* that are separated by the geographic barrier of the Rocky Mountains. Two specific character traits were examined.

1. The photoperiodic induction of diapause.

2. The presence of selected endosymbionts among populations.

Section 2

Photoperiodic induction of diapause

Introduction

Dormancy is a state of reduced or ceased development or reproduction. Many insects enter a state of dormancy prior to when environmental conditions become unfavorable for long periods of time (Hagen, 1962; Nechols et al., 1999). During this time the insects stop reproducing and reduce activity. Some insects can continue to mate, but the females do not oviposit. *Hippodamia convergens* has been shown to enter dormancy at two different times during its life cycle. During the summer, when the temperatures get too hot for the beetles and when prey numbers decrease, *H. convergens* in Kansas have been shown to enter a state of dormancy called estivation to prevent starvation (Michaud & Qureshi, 2005; 2006). When this happens, beetles aggregate on flowers from which they consume plant fluids such as sap. They stay in a state of reproductive diapause until temperatures drop to more tolerable levels and prey numbers increase again (Michaud & Qureshi, 2005).

The second state of dormancy is called diapause, observed during the winter months. During this time, *H. convergens* adults will leave the fields that they are feeding and reproducing in (Hagen, 1962). The adults will fly to aggregation sites, which can be hundreds of miles away, and form groups that can contain thousands of individual beetles. During this state of diapause, the beetles will not mate and no oviposition

occurs. When temperatures increase and day lengths increases, the beetles leave the aggregation sites, undergo long-distance flight behaviors, and then search for food.

Photoperiod plays an important role in some insect species' ability to enter and exit diapause at the appropriate times (Paolucci et al., 2013; Tauber & Tauber, 1972; 1973), although other factors such as longitude, altitude, and genetic plasticity are also important (Tauber & Tauber, 1982). Photoperiods are a regularly occurring environmental factor, and as day lengths shorten, temperature decreases, food availability is reduced, or a combination of these factors occurs, some insect species respond by preparing for and entering diapause (Nechols et al., 1999). As the day lengths increase, the insects will emerge and resume activity if photoperiod is an indicator of diapause termination. Moving populations to an area that they are not adapted to could alter the response to photoperiods.

This set of experiments was designed to examine the effect of photoperiods on *H. convergens* populations from different latitudes, specifically to determine if differences exist between populations found farther south compared to populations found farther north. That is, I hypothesized that populations from different latitudes would have different photoperiods that induced diapause. The basis of this set of experiments is that populations from different latitudes may show variation in the photoperiod that induces diapause. These experiments examined differences in diapause induction between the northern populations, represented by Illinois and Kentucky *H. convergens*, and southern populations of *H. convergens*, represented by California and Arizona populations. Any differences that may exist among populations

would be useful in finding areas that *H. convergens* can be sent to and from for biological control purposes. These areas may fall within certain latitudes and have similar seasonal patterns, which *H. convergens* being moved around will be able to easily adapt to.

Materials and Methods

The methods for this experiment were based on those outlined by Obrycki et al. (1983) and Tauber and Tauber (1973) in previous studies of predatory lacewings and lady beetles *Hippodamia convergens* adults were purchased from two companies in the Western United States. Rincon-Vitova is based in southern California, which is assumed to be the approximate latitude where their populations are collected. The latitude for their collection locations is estimated to be at 34.2°N. This population was referred to as the California population. Arbico Organics is based in Tucson, Arizona, and it is estimated that their adult beetles are collected from the surrounding mountains at latitude of 32.2°N. This population was referred to as the Arizona population. Additional populations of adult *H. convergens* were collected from Lexington, Kentucky at latitude 37.1°N, which was called the Kentucky population, and the Richardson Wildlife Foundation near Amboy, Illinois at latitude 39.7°N, referred to as the Illinois population. Within each population, adults were separated into mating pairs and kept in 100 mm diameter petri dishes at 22°C at a 15:9 light-dark cycle. The pairs were given a wet cotton ball for moisture and fed *ad libitum* pea aphids, *Acyrthosiphon pisum*, daily. A total of 20 females, 5 per population, served as sources for the eggs used throughout the experiment. Eggs collected from each mating pair were systematically assigned to

three photoperiods; 15:9, 13:11, and 11:13 at 22°C. These photoperiods were adapted from Obrycki et al. (1983), using information gathered from weathspark.com (Diebel & Norda, 2014). Weatherspark.com (Diebel & Norda, 2014) provided the longest and shortest day length at each collection location for the year. None of the collection locations has a day length longer than 15 hours, thus the 15:9 L:D cycle simulated conditions during the summer reproductive period. All populations experience the 13:11 and 11:13 L:D cycles in their natural environments at two points during the year. It was hypothesized that the shortest photoperiods should induce diapause in all populations. The photoperiods were maintained in Percival biological cabinets controlled by the Intellus Control Systems.

The F1 eggs hatched. Individual larvae were separated and placed in Fisher Brand Shell Type 1 glass vials (21x70 mm, 4 Dram) with one individual larva per vial. Between 20 and 53 larvae from each population were reared at each of the three photoperiods (15:9, 13:11, and 11:13) at 22° C. Larvae were fed *ad libitum* pea aphids. Each day, the larvae were examined for molting or death. Data were recorded for developmental times, sex ratio, and survival for each population at each photoperiod.

Following pupation and emergence, adults were paired within their designated photoperiod into non-sibling mating pairs and allowed to mate. These pairs were observed under their assigned photoperiod for diapause behavior. Obrycki et al. (1983) determined that adult female *Adalia bipunctata* (Coleoptera: Coccinellidae) maintained at 22°C that oviposited within 14 days of emergence were not in diapause. However, due to differences among species of predatory lady beetles and variation in pre-

oviposition period, a period of 31 days was used in this experiment as the division between diapausing and non-diapausing females. This was an arbitrary number that was selected at the end of the experiment and based off of the oviposition data that was collected. Females were determined to be in diapause if a female failed to oviposit within the first 31 days following eclosion. A 90 day period was selected as the end point for the experiment. The 90 days began on the day that the individual beetles eclosed. This was an arbitrary time frame that was selected because it allowed enough time for the females to be mated and to oviposit in response to the photoperiodic conditions. At the end of the 90 day period, to determine if the ovaries of a female beetle contained eggs, females were dissected. This was done by killing each female in 95% ethanol, and then placing her in a plastic petri dish with water. To remove the reproductive system of the beetle, each beetle was held at the end of the abdomen and on the thorax. The last few segments were then pulled on, which resulted in the end of the beetle pulling apart from the rest of the thorax and taking the reproductive system with it. The ovaries were then examined using a Jenco USA GL7-290 microscope at 40x magnification. Ovaries that had fertile eggs in them showed large, yellow, oval shaped masses inside. Female beetles that were in a state of reproductive diapause had small, translucent grey spheres inside of the ovaries instead of the larger, yellow ovals. These are eggs that have not developed. They show that the female is capable of producing eggs, but that the female is not putting resources into the eggs to allow them to continue to develop. Unmated females oviposit egg masses that have not been fertilized, although the eggs are not viable. This occurs when the female has been well fed.

The percentage of females that entered diapause and the number of days until first oviposition were collected. The duration of diapause was calculated for each individual female beetle that did not oviposit within 30 days in the experiment. This was determined by calculating the day that each female eclosed to either the first day that they oviposited, which signals an end of diapause, the date that the female beetle died, or the end of the experiment at the predetermined 90 day mark. Data from each individual was gathered and compared with the other individuals from the same population, as well as among the three populations. Sex ratios were examined using Chi-squared analysis. Chi-squared analysis was also used to examine the number of diapausing vs. nondiapausing females in the northern (Illinois and Kentucky) versus southern (California and Arizona) populations. A Kaplan-Meier survival analysis was also performed using the SPSS program to examine the northern vs. southern populations for differences in time spent in diapause. A Two-way ANOVA was used to determine the variation in the developmental times from egg to adult among the populations at the three photoperiods. This was done using JMP 10. The Two-way ANOVA was run with a type III SS, and the population (4 factor levels) and photoperiods (3 factor levels) as factors. This gives the user a large array of calculations derived from the data. Included in these results are the ANOVA results, as well as the Effect Tests results.

Results

Developmental Time

The average number of days for all individuals to develop from the four populations from oviposition to adult was 26.2 days in the 11:13 photoperiod, 23.6 days

in the 13:11 photoperiod, and 25.2 days in the 15:9 photoperiod (Figure 1 and Table 1). The average development time among the four populations reared at the three photoperiods were biologically similar, in that they all fell within a range that has been observed in previous studies. However, development times among the four populations were statistically different, which was determined by running a two way ANOVA in which the population effect ($F_{2, 348} = 74.22$, $p<0.001$, degrees of freedom.=2) the photoperiod effect ($F_{3, 348} = 3.826$, $P<0.001$) and the population x photoperiod interaction ($F_{6, 348} = 2.13$, $P = 0.0498$) were all significant. Average development time for all four populations across the three photoperiods ranged from 22.6 to 27.5 days.

Sex Ratio

In total, 360 individual beetles were used in this experiment. Of those, 180 were male and 180 were female. For the entire experiment, there was a 0.50 female to male sex ratio. Chi squared analysis of sex ratios showed no significant differences among treatments when all 12 treatments were considered separately ($\chi^2= 18.27$, p-value = 0.075, df = 11).. A χ^2 was then performed comparing the total number of males and females in each of the three photoperiods. The 11:13 photoperiod had a ratio of 0.50 females to males ($\chi^2 = 0.009$, $P = 0.93$), the 13:11 photoperiod had a ratio of 0.50 ($\chi^2 = 0.009$, $P = 0.93$), and the 15:9 photoperiod had a ratio of 0.51 ($\chi^2 = 0.03$, $P = 0.86$). None of the photoperiods deviated from the 0.50 female to male sex ratio. Last, each population was run through a Chi Square test on its own (Table 2). The California ($\chi^2= 3.56$, $P = 0.059$), Illinois ($\chi^2= 1.14$, $P = 0.286$), and Kentucky ($\chi^2= 3.45$, $P = 0.063$) all failed to reject the null hypothesis. The Arizona population, however, rejected the null

hypothesis (χ^2= 5.081, P = 0.024). The Arizona population deviated towards a male biased population. The Illinois population also showed a slight bias towards males, while the California and Kentucky populations showed slight biases towards females.

Pre-imaginal Mortality

Most of the mortality that occurred in all of the populations was observed before the pupal stage, with only 4 deaths occurred as pupa. For the other 45 deaths, 8 occurred at the 1st instar, 11 at the 2nd instar, 7 at the 3rd instar, and 19 at the 4th instar (Table 3).

Diapause Induction

For the Arizona (N=15, total number of individual females), California (N=4), and Kentucky (N=11) populations, 100% of the beetles that were reared in the 11:13 were determined to be in diapause by failure to oviposit within 31 days (Figure 1). This was also observed in the 13:11 photoperiod (Arizona N= 11, California N= 7, Kentucky N=7). In the 15:9 photoperiod, 80% of the Kentucky (N=5) and California (N=5) populations were in diapause, while 67% of the Arizona (N=15) population was in diapause. These three populations followed a similar pattern in that the two shortest photoperiods have 100% of the females in diapause. The fourth population, Illinois, followed a slightly different pattern. In the 11:13 photoperiod, only 80% of the females were in diapause. That number decreased slightly to 78% in the 13:11 photoperiod, and was reduced further to 33% in the 15:9 photoperiods. The Illinois population followed a pattern that had a constant decrease in proportion of females in diapause as the amount of light that they were exposed to increased.

A Chi squared analysis was run to compare the populations of the photoperiod experiment. Populations were grouped together into northern (Illinois and Kentucky) vs. southern (Arizona and California) populations, because numbers were too low to allow individual analysis. The χ^2 for the Southern (Arizona and California) populations across photoperiods was 12.41 with P = 0.002 and 2 degrees of freedom, while the χ^2 for the Northern (Illinois and Kentucky) populations was 8.01 with P = 0.018 and 2 degrees of freedom. This indicates that both of the groups were statistically significant, with p-values below .05, and rejected the null hypothesis. This means that the percentage of beetles in diapause varied as a function of photoperiod. Rejecting the null hypothesis in this case indicates that there was a difference in the percentage of females in diapause across the photoperiods.

To determine if there was a difference in the percentage of females in diapause between the northern and southern regions, a Fishers Exact test was run. This test was only run on the females in the 15:9 photoperiod. The Fishers Exact test determined that the northern and southern populations do not vary in their photoperiodic induction of diapause (Fishers Exact test = 0.46, $p > 0.05$).

The survival analysis was performed to determine if there was any difference in diapause induction between northern and southern populations (Table 4, Figure 3). For each photoperiod, the 95% confidence interval for the Northern and Southern populations coincided (Table 4). This indicates that no significant difference was found between the northern and southern populations.

Duration of Diapause

Females in the experiment were found to be in diapause based on the number of days it took them to oviposit, failure to oviposit within 90 days, or their death before oviposition. The duration of diapause in each of the four populations within each of the three photoperiods was examined using the mean, median, and range in the number of days for beetles that were determined to be in diapause (Table 4). The mean of each population within each photoperiod gives the average number of days that the females of the population were determined to be in diapause. This number ranged from 43.1 days to 80.9 days. With three of the populations, the California, Arizona, and Kentucky populations, the mean number of days for the duration of diapause decreased as the day length increased. With the fourth populations, the Illinois population, the mean decreased from the 11:13 photoperiod (60.6 days) to the 13:11 photoperiod (49.4 days), but then increased again at the 15:9 photoperiod (72.0 days). This result is likely due to the low number of individuals within each photoperiod who were determined to be in diapause.

The median of each population within each photoperiod also varied. This value indicated the center of the dataset of each population within each photoperiod. The medians ranged from 38.5 days to 90 days among populations and photoperiods. Three of the calculated medians were 90 days, which is the highest number of days that a beetle could be in diapause in this experiment. One of these 90 day median values was observed in the Arizona populations in the 11:13 photoperiod, the second 90 day median was for the Kentucky population in the 11:13 photoperiod, and the last 90 day

median was for the Illinois population in the 15:9 photoperiod. There was only a slightly noticeable pattern between the medians of diapause and the photoperiods. The shortest photoperiod, 11:13, had the highest values for median duration of diapause, while the longest photoperiod, 15:9, had some of the shortest with the exception of the Illinois population.

The range in the number of days in diapause for each population in each photoperiod was the last calculation done for this data. The range is the difference between the largest and smallest values in the dataset. These values fell between 40 days and 79 days. This indicated that the spread of the datasets was broad, and that there was a wide variation in responses, as none of the calculated ranges were 90 days, which would be the largest range that could be achieved in this study.

Discussion

For this experiment, populations were grouped into northern and southern populations, instead of eastern and western. This was done due to the use of latitude determining the photoperiod each population would see in their local environments. The hypothesis for this experiment was that beetles from more southern locations would react differently to the photoperiods compared to beetles from the northern locations. It was also predicted that as the day length the beetles were exposed to increased, the number of beetles in diapause would decrease. However, this second prediction was not consistently observed (See Figure 2). With the California, Arizona, and Kentucky populations, the number of beetles in diapause remained the same at the 11:13 and 13:11 photoperiods. These populations only showed a decrease in the

number of females in diapause between the 13:11 and 15:9 photoperiods. Only the Illinois population showed the pattern that was predicted, with a decrease in the number of females in diapause seen as day lengths got longer.

The average development times of the populations were similar to those observed in other studies. Observations reported by Hagen (1962), who recorded development times between 11 and 29 days at an unknown temperature, depending on the origin of the *H. convergens* population, were somewhat similar to those seen in this study. This is also noted in other studies which looked at the development time of *H. convergens* at or close to 22°C. Obrycki and Tauber (1982) observed a 24.9±1.6 days development time for a population of *H. convergens* from Ithaca, NY that were laboratory reared at a 16:8 L:D cycle under 21.1°C. Miller (1992) observed development times of two populations of *H. convergens*, one from Corvallis, Oregon and the other from Tucson, Arizona, to be 28.8±0.2 days and 29.9±0.5 days, respectively, when reared under a 16:8 L:D and 21°C. Also, Michels and Behle (1991) observed a development period of 29 days for *H. convergens* collected from Bushland, Texas and reared at 20°C.

The percentage of developing larvae and pupae that died during the experiment was relatively low when compared to other studies of *H. convergens* (Obrycki et al., 2001), with the exception of the Kentucky population in the 15:9 photoperiod (Table 2). This population had 50% of the population die before reaching the adult stage. The deaths were observed from the 1st instar through pupation, one individual died while attempting to eclose. In total, 1 larva died in 1st instar, 2 in 2nd, 3 in 3rd, 6 in 4th, 2 as pupae, and 1 during eclosion. There was noticeably higher mortality at the later instars,

which also require more food. It is possible they were not fed enough, although other larvae being run through the experiment at the same time and in the same developmental stage were able to develop with no issues.

Several explanations are possible to explain the high number of females in diapause, which did not support the initial predictions made before the experiment began. It is possible that the overall results seen in this photoperiod inducing diapause experiment were due to an experimental design flaw. *H. convergens* enter diapause and aggregate, which leads to a production of an aggregation pheromone. This pheromone attracts other beetles and allows for the creation of a large aggregation mass. The design of the experiment placed all of the experimental beetles in one of three Percival biological cabinets together, creating a lack of independence. If one beetle were to enter diapause, the pheromones could be circulated throughout the cabinet, possibly leading to other beetles entering diapause. This may have happened despite the beetles being kept in dishes containing lids, as the dishes were not air tight. However, this experimental design is commonplace and has been used previously to study lady beetle responses to photoperiods (Obrycki & Tauber, 1982). This would lead to the conclusion that having multiple beetles together in a single cabinet is unlikely to have affected the results of the study in a significant way.

Another explanation is that the low numbers of females that were mated were not sufficient to accurately represent the population responses. Many females were not mated due to an uneven number of males and females at the time of eclosion, or due to a high rate of mortality. Also, at the beginning of the experiment, sibling mating was

avoided. However, a month into the experiment, crossing among siblings started to utilize as many females as were available. Since females were mated within a week of eclosion, any remaining females went unmated. There was also an issue having males and females from different parental pairs ready to be mated at the same time. This meant that several females for both the Arizona and California populations in all three photoperiods went unmated. In the future, one might consider moving males among the females once mating has been observed.

Another explanation that may explain the results could be related to the life stage of *H. convergens* that is sensitive to short day lengths. During the set up of the experiment, it was assumed that the early stages of the life cycle, the egg to the pupae, are sensitive to short day lengths. As such, all adult beetles that acted as a parental generation for the experimental beetles were kept in the longest photoperiod (15:9) while reproducing. If, instead of the early life stages, it is the adults that are sensitive to the day length, the experiment was set up in a way that might explain the unexpected results that were observed. This is based on the assumption that *H. convergens* females maintained at L:D 15:9 would produce a high percentage of offspring that would enter diapause. This explanation would mean that adults are primed towards the photoperiod that they are residing and reproducing in.

The data from this experiment had one interesting result that will be addressed in the endosymbiont experiment (Section 3). The Chi squared analysis for the California population showed a deviation from the standard 50/50 sex ratio. This population seemed to favor males more heavily than females. However when a Chi squared

analysis was done on all of the populations in all of the photoperiods, the result was not statistically significant and failed to reject the null hypothesis. Since the larger Chi squared analysis failed to reject the null hypothesis while the California population Chi squared alone did reject the null hypothesis, it can be assumed that the Arizona, Kentucky, and Illinois populations have a slight female bias that is not statistically significant. This could be an indication that another organism is acting on this populations, affecting the sex ratio. A skewed sex ratio can be a sign of a bacterial endosymbiont within the population, skewing the population in favor of symbiont transmission.

Table 2.1: Development time (days ± standard error) for four populations of *H. convergens* from oviposition to adult eclosion at three L:D photoperiods (15:9, 13:11, 11:13) at 22°C. Individuals were fed ad libitum pea aphids daily.

	15:9	13:11	11:13
Arizona	25.8±1.4 (N=48)	23.9±1.7 (N=30)	26.7±2.4 (N=40)
California	25.6±1.1 (N=27)	25.3±1.3 (N=23)	27.5±2.5 (N=22)
Illinois	23.9±1.0 (N=27)	22.6±1.3 (N=24)	25.6±1.3 (N=20)
Kentucky	25.3±1.5 (N=30)	22.6±1.1 (N=33)	24.9±1.4 (N=31)
Total	25.2±0.21 (N=132)	23.6±0.16 (N=110)	25.2±0.14 (N=113)

Table 2.2: Sex ratios (number of females/total number of adults) of four populations of *H. convergens* reared at 22°C. Individuals were fed ad libitum pea aphids daily.

Population	N	Sex ratio (F:M)	χ^2	P
Arizona	123	0.39	5.081	0.024
California	72	0.61	3.56	0.059
Illinois	71	0.43	1.14	0.286
Kentucky	94	0.59	3.45	0.063

Table 2.3: The number of dead individuals and percentage of pre-imaginal mortality of *H. convergens* for each of the four populations at three L:D photoperiods (15:9, 13:11, 11:13) in 22°C in the three photoperiods.

	Treatments		
	15:9	13:11	11:13
Arizona	5/48 (10.4%)	5/30 (16.7%)	5/41 (12.2%)
California	2/27 (7.4%)	2/23 (8.7%)	5/22 (22.7%)
Illinois	3/27 (11.1%)	1/24 (4.2%)	0/20 (0%)
Kentucky	15/30 (50%)	2/33 (6.1%)	4/31 (12.9%)

Table 2.4: Survival Analysis of Southern Vs. Northern populations across three Light: Dark Photoperiods (11:13, 13:11, 15:9)

N vs. S	Photoperiod	Mean days in diapause			
		Estimate	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
South	11:13	84.294	3.796	76.855	91.734
	13:11	61.422	7.398	46.922	75.923
	15:9	41.300	7.555	26.492	56.108
	Overall	63.131	4.453	54.403	71.859
North	11:13	66.495	6.343	54.063	78.927
	13:11	51.645	6.948	38.027	65.263
	15:9	41.615	9.114	23.753	59.478
	Overall	54.136	4.547	45.223	63.049
Overall	Overall	58.922	3.221	52.609	65.234

Table 2.5: The duration of diapause in days for the females of four populations of *Hippodamia convergens* while maintained in three photoperiods (15:9, 13:11, 11:13). The average, median, and range (Highest value – lowest value) of the photoperiods are given.

	15:9	13:11	11:13
Arizona	Mean: 43.1 Median: 38.5 Range: 77 (90 = highest, 13 = lowest) N = 10	Mean: 56.5 Median: 60 Range: 79 (90 = highest, 11 = lowest) N = 11	Mean: 80.9 Median: 90 Range: 48 (90 = highest, 42 = lowest) N = 15
California	Mean: 57.8 Median: 50 Range: 49 (90 = highest, 41 = lowest) N = 4	Mean: 62.7 Median: 62 Range: 54 (90 = highest, 36 = lowest) N = 7	Mean: 64.0 Median: 69.5 Range: 63 (90 = highest, 36 = lowest) N = 4
Illinois	Mean: 72.0 Median: 90 Range: 54 (90 = highest, 36 = lowest) N = 3	Mean: 49.4 Median: 52 Range: 67 (90 = highest, 23 = lowest) N = 7	Mean: 60.6 Median: 61 Range: 63 (90 = highest, 27 = lowest) N = 4
Kentucky	Mean: 50.3 Median: 40.5 Range: 60 (90 = highest, 30 = lowest) N = 4	Mean: 61.6 Median: 60 Range: 48 (90 = highest, 42 = lowest) N = 7	Mean: 74.6 Median: 90 Range: 40 (90 = highest, 50 = lowest) N = 11

Figure 2.1: Development time (days ± standard error) for four populations of *H. convergens* from oviposition to adult eclosion at three photoperiods (15:9, 13:11, 11:13) at 22°C. Individuals were fed ad libitum pea aphids daily.

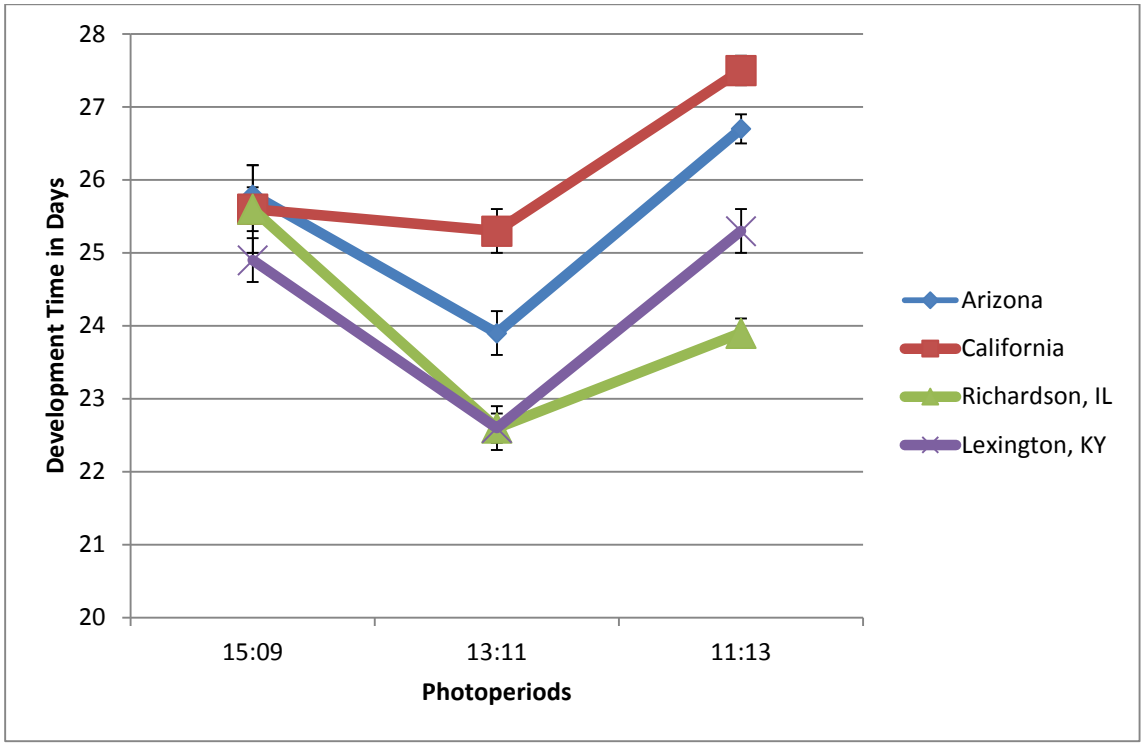


Figure 2.2: The percentage of female *H. convergens* that were found to be in diapause, indicated by a lack of oviposition after 31 days, or dissection of the females at the end of the 90 day experiment. The four populations were maintained in three photoperiods (15:9, 13:11, 11:13) at 22°C and fed *ad libitum* pea aphids daily. 1.A (Arizona), 1.B (California), 1.C (Illinois), 1.D (Kentucky)

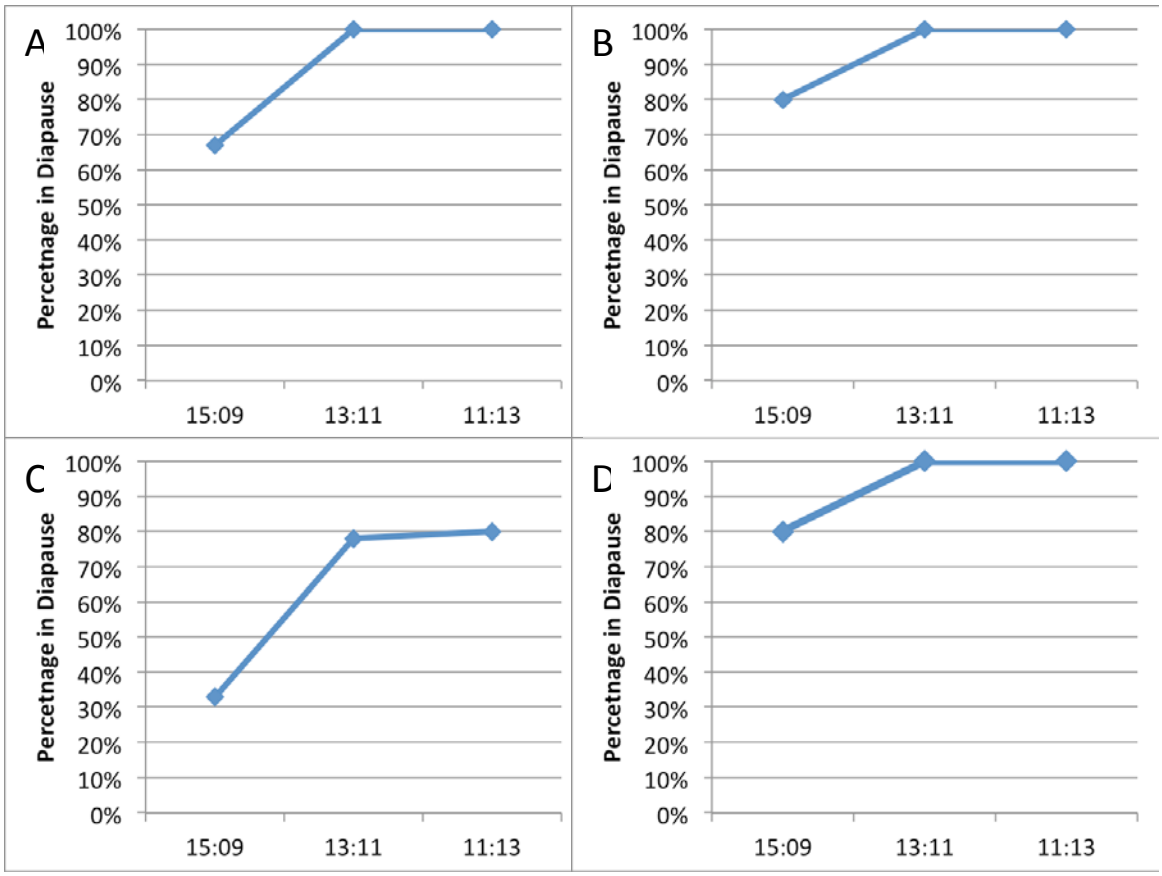
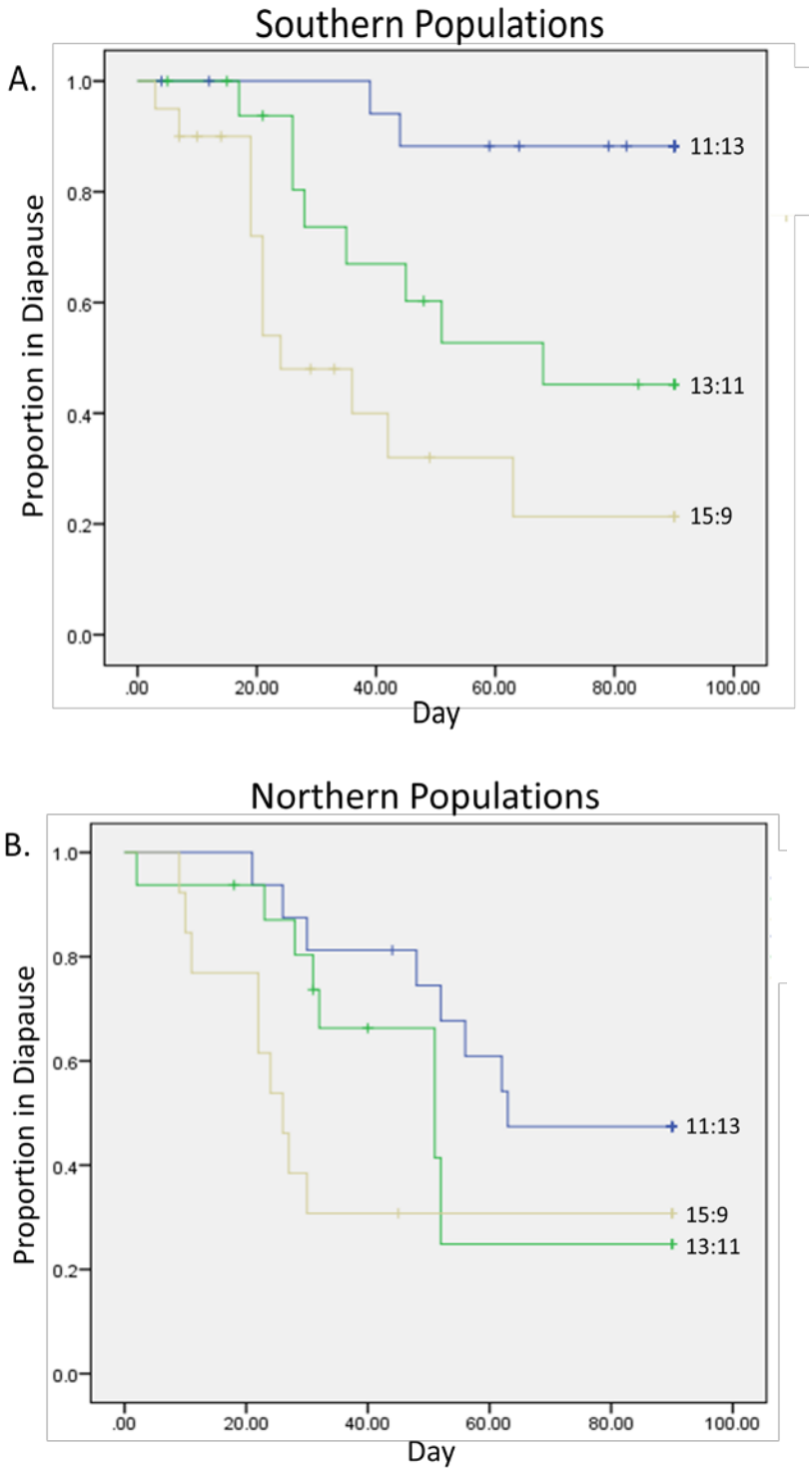


Figure 2.3: Survival Analysis of Southern (A) and Northern (B) populations with each photoeperiod measured in the proportion in diapause through time measured in days.



Section 3

Presence of three genera of endosymbiont bacteria

Introduction

Endosymbionts are maternally inherited bacteria that commonly occur in insects (Nakamura et al., 2005). They are passed from mother to offspring via vertical transmission and have a shared fate with their insect host. In the event that the host dies, so will the endosymbiont, so many endosymbionts aid their host in some way. In many cases, multiple endosymbionts have been reported in a single insect population (Brady & White, 2013; Groenenboom & Hogeweg, 2002). Endosymbionts are capable of filling a variety of roles within insects. In some cases, endosymbionts aid in the breakdown of food, as is seen with termites (Ohkuma, 2003), while in other instances, endosymbionts can encase parasitoid eggs and protect the host as part of the immune system (Brady & White, 2013; Martinez et al., 2014; Oliver et al., 2014; Xie et al., 2014). Both of these instances show a mutualistic relationship between the insect host and the endosymbiont. However, it is not always obvious when an endosymbiont is aiding its host.

There are also endosymbionts that are capable of distorting an insect population's sex ratio. The reproductive manipulations promote vertical transmission of the symbionts, which are passed maternally in the cytoplasm (Werren et al., 1994). There are four major types of reproductive manipulations; male killing, feminization, parthenogenesis, cytoplasmic incompatibility. These endosymbionts will stop their host from mating with individuals who lack the symbiont, or will kill off any hosts that can't

carry the endosymbionts. Cytoplasmic incompatibility, a situation in which the eggs and sperm cannot form viable offspring when a female that lacks an endosymbiont mates with a male who is infected with an endosymbiont, is a common occurrence. However, for Coccinellidae, male killing is the most common form of reproductive manipulation that is observed.

The formation of relationships with some endosymbionts could be detrimental to a biological control agent like *H. convergens*. Male-killing endosymbionts can lead to a more female biased population, although it has been shown that in some species the percentage of the total population infected by a male-killing symbiont is generally low (Jiggins et al., 2001). A female biased population may not necessarily be problematic, and, in fact, could benefit a biological control agent like *H. convergens* by creating more mating opportunities for each male in the population. On the other hand, presence of cytoplasmic incompatibity could be particularly problematic in an augmentative biological control agent, because relocations could bring differentially infected populations in contact with one another, and induce incompatibilities.

There are four genera of endosymbionts that have been described to act as male killers in species of predatory Coccinellidae: *Wolbachia*, *Rickettsia*, *Spiroplasma*, and several Flavobacteria species (Johnstone & Hurst, 1996; Weinert et al., 2007). Several investigators have tested Coccinellidae species for these four male-killing endosymbionts. Majerus and Majerus (2010) identified *Rickettsia* with an infection rate of 31.6% in the Japanese species *Propylea japonica*, and Majerus et al. (1999) identified that a *Spiroplasma* caused female biased populations of *Harmonia axyridis* that had

been observed by Matsuka et al. (1975), whom noted a 10% infection rate. Hurst et al. (1999a; 1996; 1997) found evidence of Flavobacteria in *Adonia variegate* and *Coleomagilla maculata,* and *Wolbachia* (Hurst et al., 1999b), *Spiroplasma*, and *Rickettsia* (Hurst et al., 1999c) in *Adalia bipunctata*. Werren et al. (1994) also found *Rickettsia* in *A. bipunctata*. Recently, Elnagdy et al. (2014) found related strains of *Flavobacterium* in *Coccinula crotchi* and *Coccinula sinensis.*

Coccinellids that contain male-killing endosymbionts tend to show several uniform characteristics. These include ovipositing tightly clustered egg masses, cannibalism, and a preference for aphids as a food source (Majerus & Majerus, 2012). These characteristics are not a result of a relationship with an endosymbiont, but do make it easier for the beetles to form relationships with endosymbionts. Tightly clustered egg masses and cannibalism of siblings provide easier and quicker meals for newly hatched female 1st instars. Female Coccinellids that contains a male killing endosymbiont will oviposit their egg clusters, which are close to a 1:1 sex ratio. However, the male eggs will not develop and these “dead” eggs give the newly hatched female larvae a large first meal that does not need to be searched for. After this, the female first instars are larger and more capable of survival than other individuals who do not have a male killing endosymbiont and did not get their first “dead brother” meal.

Although it has been hypothesized that *Hippodamia convergens* could have male killing endosymbionts based on their egg laying strategy, willingness to consume siblings in both the field and in captivity, and their primary food choice of aphids (Majerus & Majerus, 2012), few *H. convergens* adults have been examined for endosymbionts and

no symbionts have been identified. Weeks et al. (2003) tested 3 individual *H. convergens*, along with 29 other Coleopteran species, from California for both *Wolbachia* and a different symbiont, *Cardinium* (referred to as CLO in the study), but found no evidence of either. Aside from Weeks et al. (2003), no other records are available discussing the screening of *H. convergens* for endosymbionts. The present study aimed to change that by looking for *Wolbachia, Spiroplasma,* and *Rickettsia* in *H. convergens* beetles from populations from California, Arizona, Kentucky, and Illinois.

Materials and Methods

Four populations of *H. convergens* were examined for the presence of selected endosymbionts to determine if variation in infection frequency exists among populations which could potentially influence mating success. Twenty females each from the Arizona, California, and Illinois populations and thirteen adults from the Kentucky population were examined for endosymbionts. Each individual adult was surface sterilized by placing adults in a 5% bleach solution for one minute, followed by a dip in a 95% ethanol solution for one minute. The ethanol rinse was repeated two more times, for a total of three washes. Finally, the beetles were placed in deionized water for one minute.

The wings, head, and thorax of each beetle were removed, leaving only the abdomen. The abdomens underwent DNA extraction using the Qiagen DNEasy kit following the manufacturer's instructions. The abdomens were used because many endosymbionts that manipulate host reproduction are localized in the host's

reproductive organs (Werren et al., 2008). Following DNA extraction, each sample was run through a PCR process using diagnostic primers that detect a desired symbiont (Table 5).

The reaction mixes for each endosymbiont varied, but all reactions totaled 10 µL. For *Wolbachia*, the reaction mix contained: 2.0 µl of DNA template, 3.5 µl of double distilled H_2O, 1 µl of 10X buffer, 0.8 µl of dNTPs, 0.6 µl of 25 mM $MgCl_2$, 1 µl of 5.0 pmole/µl of both the forward and reverse primers, and 0.1 µl of 5 U/ µl NEB Taq. The reaction mix for *Rickettsia* was similar to the mix for *Wolbachia*, however there were some differences. The *Rickettsia* mix used 1.1 µl more of double distilled H_2O (4.6 µL), 0.1 µl less 25 mM $MgCl_2$ (0.5 µL), and 0.5 µl less of 5.0 pmole/µl of both the forward and reverse primers (0.5 µL). The *Spiroplasma* reaction mix differed from the other two. It contained 2.0 µL of DNA template, 3.32 µL of double distilled H_2O, 1 µL of 10X buffer, 1 µL of dNTPs, 1 µL of 25 mM $MgCl_2$, 0.8 µL of 5.0 pmole µL of both the forward and reverse primers, and 0.08 ml of 5 U/ µL NEB Taq. A positive control was again loaded with each PCR to confirm that the PCR worked properly.

PCR reactions were mixed in a Thermo Scientific 1300 Series A2 biological safety cabinet. PCR were run in a BioRad C1000 Thermal Cycler using a predetermined program for time and temperature (Table 5). Afterwards, the reactions were loaded onto an agarose gel (1 g agarose to 100 mL of 1 TAE Buffer) stained with GelRed, and run at 85 volts for 45 minutes in a gel rig using a VWR Power Source. Gels were then placed in a UVP High Performance UV Transilluminator to view the gel bands.

In the event that positive bands appeared and indicated a potential presence of a symbiont, a gel extraction was performed and the extracted material was sent to Beckman-Coulter (Danvers, MA) for Sanger sequencing. To do this, individual beetles that appeared to have a positive gel band for a selected endosymbiont were rerun in a PCR with double the reagents (20µl total volume per reaction instead of 10 µl). Once the gel had undergone gel electrophoresis, bands were removed by using a heated blade to cut out small sections of the gel with the desired DNA and placing them in a 1.5 mL centrifuge tube. The tube with the gel contents in it was then weighed. The *GenCatch* kit by Epoch Gel Extraction Kit was used at this point, following the manufacturer's instructions, to elute the DNA. This final product was stored at -20° C until sequencing. Resulting sequences were examined and manually edited in Geneious V6.0.6 (AgMatters, Auckland, NZ). Microbe identity was determined by comparing sequences to the NCBI database using the Megablast algorithm.

Results

PCR results showed 9 possible gel bands for *Wolbachia, Rickettsia,* and *Spiroplasma* in the Arizona, California, and Illinois populations. The Kentucky population did not show any bands that would signify symbiont presence. The individual beetles that had the clearest bands were selected and rerun in the larger PCR described above, and gel extracted for sequencing. In total, 8 samples were sent for sequencing with Beckham-Coulter. For *Wolbachia,* two Arizona beetles and one Illinois beetle were sequenced. These sequence results were very low quality when examined in Geneious v6.0.6, had no usable information, and could not be blasted. For *Spiroplasma,* two

California beetles were sequenced. These sequences were identical to one another. After manually trimming the sequences and comparing them to the NCBI database, it was determined that the PCR had amplified DNA from the bacteria *Staphylococcus sciuri*. This bacteria has been identified in several insects and is known to cause health issues in humans (Washington et al., 2015). For *Rickettsia,* three beetles from Arizona were sequenced. These sequence results, when compared to the NCBI database, showed a high percentage (80%) match to *Dendroctonus ponderosae*, the mountain pine beetle. This leads to the conclusion that the primers amplified a piece of beetle DNA, presumably from *H. convergens* itself.

Discussion

This study did not support the hypothesis that the male-killing endosymbionts *Wolbachia, Spiroplasma,* or *Rickettsia* exist within the four populations of *H. convergens.* Given that these beetles show all of the characteristics noted in other Coccinellids that are infected with endosymbionts, it was predicted that an endosymbiont infection would exist. Endosymbionts have been found in many other Coccinellids in recent studies, although few have examined *H. convergens* for them.

The most obvious reason for this study not observing any endosymbiont infections is that these endosymbionts may not exist within *H. convergens*. The endosymbionts tested for were all known to be reproductive manipulators, which *H. convergens* may not have relationships with. However, this study is too preliminary to make this assertion with any certitude.

Reproductive manipulating endosymbionts can occur at a low rate, around 10%, in many recorded insect species (Jiggins et al., 2001). However, the infection rate of an endosymbiont varies from species to species and depends on the host and host population. It is possible that these endosymbionts could occur at that rate or lower in *H. convergens* and were missed during the study. To evaluate experimental power, a geometric distribution was calculated using the Probability Mass Function (PMF) (Stewart, 2009) and the sample size of the study, 73. The probability mass function gives the probability that a discrete random variable equals another variable. Using this equation, it was determined that the actual endosymbiont frequency based on the 73 beetles that were used in the study, was unlikely to be above .04, or 4% infection rate. This can be interpreted as saying that if any of the endosymbionts that were tested for do exist within *H. convergens*, their infection rate would be at or below 4% of the total population. The sample sizes in this experiment were larger than previous studies that have been done (Weeks et al., 2003) but still relatively low at only 13 or 20 individual females per population and only 73 total females. However, the present study's sample size could still have missed very low frequency infection rates.

It is also a possibility that the beetles have other strains of symbionts that would not have been picked up by the primers that were used for this experiment. The primers were designed for certain bacterial genera, and would not be effective at detecting unexpected bacteria genera, nor would they necessarily detect even the targeted strains, if mutations had occurred in the target DNA to prevent the primers from binding. In the absence of good binding sites, the primers may have been replicating a

large amount of “junk” DNA that was diluting the results of the PCR process. Future attempts at characterizing symbionts in *H. convergens* should include next-generation sequencing with non-specific primers, to detect a broader diversity of bacteria.

Very few investigators have attempted to screen *H. convergens* for endosymbiont presence and of those who have the sample sizes have been small. The information in the present study may cause others to use a larger sample and check for a broader array of endosymbionts within *H. convergens* populations.

Table 3: The forward and reverse primers used to detect three selected endosymbionts *Wolbachia, Spiroplasma,* and *Rickettsia* in four populations of *H. convergens.* All primers were adapted from White et al. (2015).

Target Symbiont	Target Gene	Primer Name	Primer Sequences 5' to 3'	Thermocycling Condition
Spiroplasma	16S	16SA1F TKSsSpr	AGAGTTTGATCMTGGCTCAGTA GCCGTGGCTTTCTGGTAA	94°C for 180 seconds followed by 35 repeated cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 360 seconds
Rickettsia	16S	16SA1F Rick16SR	AGAGTTTGATCMTGGCTCAGCA TCCATCAGCGATAAATCTTTC	95°C for 120 seconds followed by 35 repeating cycles of 92°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and 72°C for 360 seconds
Wolbachia	wsp	wsp_F1 wsp_R1	GTCCAATARSTGATGARGAAAC CYGCACCAAYAGYRCTRTAAA	94°C for 120 seconds followed by 36 repeating cycles of 94°C for 30 seconds, 59°C for 45 seconds, 72°C for 70 seconds, and 70°C for 600 seconds

Section 4

Conclusion

The goal of this study was to determine if there were any drawbacks to shipping adult *Hippodamia convergens* across the country for biological control. The two specific objectives were to determine if intraspecific variation existed in the photoperiod that induces diapause in four populations including two that are commercially sold (Arizona and California), and two that were field collected (Illinois and Kentucky) , and to determine if there were differences in the populations' infections by three male killing endosymbionts. While results from the experiments were not as hypothesized, the information gathered from them is still useful and insightful.

It was hypothesized that there would be a difference in photoperiod triggering diapause between northern and southern populations of *H. convergens*. The results were not what were hypothesized. It was observed that populations do not react differently to different photoperiods based on the results of the Chi squared and Survival Analysis. . However, it was observed that diapause starts and ends spontaneously, and that beetles that are constantly exposed to a photoperiod and a constant temperature during their entire life can enter and exit diapause without any change in the environment.

It could be hypothesized that these beetles have a genetic link to the photoperiod that they use to enter and exit diapause. However, this was not something that was tested for, as this study focused on correlations with genetic variation that was observed by Sethuraman et al. (2015). If this is true, then cross breeding beetles from

different populations may cause problems for the seasonal development of the offspring. Offspring may favor one parent's photoperiod inducing diapause over another, or they may favor a photoperiod between the two, or a completely nonrelated photoperiod. Individuals that are the result of a cross breed between a naturally occurring population and a population that is introduced for biological control could be less adapted to the environment that they are residing in, and may die off while attempting to overwinter. This could lead to a decline in the populations where *H. convergens* adults are being released for biological control purposes.

The endosymbiont study did not reveal any information that may lead to restrictions in human movement of H. convergens based on variation in the presence of endosymbionts. . However, the Probability Mass Function calculation showed that the sample sizes that were used in the experiment were too small to accurately state whether or not the endosymbionts exist within the population. With more beetles being tested per population, the results may have been different.

Hippodamia convergens has been utilized as a biological control agent for over one hundred years. Several studies have measured its effectiveness as a biological control agent. Randolph et al. (2002) examined the effectiveness of *H. convergens* for controlling the Russian wheat aphid. Their study found that there was no measureable economic benefit to releasing *H. convergens* for biological control purposes, as the plants increased their biomass at the same rate both with and without natural enemies.

Another study, by Flint and Dreistadt (2005), found that *H. convergens* reduced aphid populations in rose cultivars, but the number of individual beetles required per

bud for an uncaged rose was 100 beetles. It should also be noted that Flint and Dreistadt claim that the number of beetles required to control aphids effectively is around 2300 beetles per m^2. That number is vastly greater than the recommended number of beetles, 11-12 per m^2 that the sellers suggest. This study shows that *H. convergens* is capable of reducing aphid numbers in an outdoor environment, but only with a number of beetles that is far greater than insectaries suggest.

Hagler and Naranjo (2004) performed a study in Arizona that observed the movement of *H. convergens* released for biological control purposes on cantaloupe and cotton. The released beetles were marked with rabbit or chicken immunoglobulin before being released into their respective crop. After 15 days, fewer than 1% of the released beetles were recaptured for ELISA gut analysis. The other 99+% dispersed from the release site quickly.

Studies like this suggest that *H. convergens* is capable of acting as a reliable augmentative biological control agent when the number of beetles released is large compared to the area that is to be controlled. However, it has been noted that beetles purchased from some biological control companies are not ready to act as biological control agents upon arrival. Many companies ship the beetles while they are still in diapause and the beetles arrive to the buyers still in this state. If the beetles are not given time to exit the diapause state, upon release they will find a place to aggregate, possibly away from the intended area of control. This further increases risks like those examined in this study.

Although *H. convergens* is not a reliable biological control agent in some environments such as open fields, it is still sold widely in the United States for field releases. While those who purchase this insect for biological control are hoping to do good by not using insecticides thus there is an assumed benefit for the environment and use a natural method for controlling their pests, they may in fact be furthering a decline in *H. convergens'* ability to act as a biocontrol agent.

This study did shed some light on the overall hypothesis that differences exist between population's physiology and environmental adaptations, but it did not answer the questions completely. Translocation of the beetles across the country for augmentative biological control can cause disruptions in their photoperiodic responses, as imported beetles won't be adapted to the local environments they are released in. This can result in population decline both in the origin population due to a loss of beetles from being shipped across the country, and in the naturally occurring populations due to a loss of individuals from cross breeding, if the photoperiodic response is a genetically heritable trait. The relationship that these beetles have with endosymbionts is still unknown. It is possible that they don't contain any endosymbionts or that their relationship with endosymbionts is infrequent. This study provides the basis for others to determine if the practice of shipping *H. convergens* across the country should continue or if there should be restrictions put in place to limit or halt this augmentative biological control practice.

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