Bitrophic Ecological Interactions in a Brassica System Provide Insights into Evolutionary Adaptations in Oviposition Decisions

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Abstract of Thesis

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Caterpillar pests pose a threat to global food security, costing producers billions of USD in lost yield and pest management costs annually. Understanding the factors affecting caterpillar pest population sizes, which include adult oviposition decisions and host-parasitoid interactions, can help mitigate their drastic effects on crop damage. With climate change and increasing temperatures threatening to change the agricultural field, the oviposition response of one of the most abundant pests, *Plutella xylostella,* was measured by counting the eggs oviposited on ambient-grown and temperature-treated lacinato kale (*Brassica oleracea* var. "Palmifolia": Brassicaceae) in choice tests (Chapter 1). Additionally, aspects of plant chemistry were measured to investigate the mechanisms behind *P. xylostella* oviposition decisions. My results showed that the female adult showed no oviposition preference between temperature treatments. Kale plants showed no difference across temperature treatments in percent carbon nor in water content but a difference was found in percent nitrogen. Our measure of nitrogen included glucosinolates (defense compounds) and proteins. However, a previous offspring performance study using our same protocol suggests that the adult female is not able to detect nutritional quality differences in the kale and the higher levels of nitrogen found in the temperature-treated kale is likely allocated toward glucosinolates and not protein. Parasitoids are important biocontrol agents of insect pest populations and understanding their evolutionary ecology is essential for pest management. During the summer of 2021, 360 caterpillars of the cabbage white butterfly (*Pieris rapae*) were collected biweekly at

different life stages from a local organic farm (Potomac Vegetable Farms in Vienna, VA, USA) and brought back to rear in the laboratory until butterfly emergence or parasitoid adult emergence. Rather than killing their hosts upon emergence, local populations *of P. rapae* keep their host alive after emergence, presumably to help protect parasitoid immature stages. This 'bodyguarding behavior' - a form of host manipulation - is poorly understood and likely entails fitness tradeoffs for the parasitoids exhibiting it. I explored the relationship between clutch size (the number of parasitoid eggs laid in a host) and bodyguard lifespan for the parasitoid, *Cotesia glomerata,* and found that increasing clutch size decreased bodyguard lifespan and reduced the proportion of immature parasitoids that successfully developed into adults (Chapter 2). These findings suggest that populations of *C. glomerata* that suffer high post-emergence mortality should have reduced clutch sizes to increase the effectiveness of their bodyguards.

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Chapter 1: Agricultural pest shows no oviposition preference for temperature-affected host plants

INTRODUCTION

Within the field of insect evolutionary ecology, the 'naïve adaptationist hypothesis' (Courtney & Kibota 1990) postulates natural selection should favor female insects that oviposit on the resources that provide their offspring with the highest fitness. The testing of oviposition preference-offspring performance relationships has allowed the identification of different key factors influencing the strength of this presumed relationship. Broadly, there have been three limiting constraints postulated to influence the strength and evolution of the preference-performance relationship: complexity, sensory, and searching constraints (Itami and Craig 2019).

There are multiple advantages to decreasing resource complexity in a species. When considering feeding behavior as a factor, a tight ecological linkage between oviposition preference and offspring performance can be found in species that are dietary specialists (Price 2003). This is supported by the neural limitation hypothesis, which posits that a specialist feeding pattern in developing offspring increases the preference-performance relationship strength as specialist female adults must process a relatively limited set of information when choosing a host for their offspring and, thus, can make more efficient decisions for oviposition than generalists that are faced with a wider suite of dietary options (Bernays 1998, 2001). These specialist feeding behaviors can produce relatively stable population dynamics, which in turn are predicted to strengthen the oviposition preference-offspring performance relationships (Price 2003).

Resource complexity during larval development can also impact the preference-performance relationship. A weakening of this ecological linkage is predicted when movements or changes in resources occur between the oviposition and offspring maturation stages, which can act to decouple adult decisions from offspring experiences with regard to these resources. Such movements are often necessary for offspring development, for example when immature insect herbivores must feed on multiple plant modules to meet their nutritional needs. Additionally, movements of offspring among host plant individuals and/or species can occur when there is differential mortality imposed by natural enemies on alternative hosts, for example when natural enemies concentrate their foraging activities on specific host insects (i.e., for parasitoids) or host plants (i.e., for herbivores) (Itami and Craig 2019). In either case, different patterns of host selection and use by parents vs. offspring can act to decouple the preference-performance relationship.

Oviposition preference is an important reproductive strategy for female insects in which they must choose the substrate, arthropod host, or host plant that will provide the best fitness for their offspring, but are further limited by not being able to actually taste and gain information on the nutritional profile of the tissues their offspring will be consuming. For this reason, other information about the host must be obtained. Mated females can obtain information about the physical aspects of the host, such as host size (Le Masurier 1987, 1991) or chemical cues. Additionally, gravid females almost always face searching constraints posed by their typically short life spans (Itami and Craig 2019). From the moment they successfully mate, adult females are racing against time to deposit their eggs prior to death. Most models of searching constraints predict that the

'choosiness' of gravid females decreases with age, often resulting in older females depositing eggs on hosts with a wider range of host quality for her offspring. These constraints also are thought to weaken selection on the preference-performance relationship.

Plant-herbivore interactions are mainly influenced by available plant cues and the ability of herbivores to recognize these cues to distinguish the resources that are best for offspring development. Also supported by the neural limitation hypothesis, sensory biases impact the relationship if the female has a poor ability to predict resource quality at the time of host plant selection (Craig et al. 1999). Drastic differences in plant quality between the oviposition and maturation period reduce the ability to detect a higher quality resource at the time of oviposition and this difference can be exacerbated with longer offspring development times as it allows for the plant or leaves to change nutritional quality with age (Ehrlich and Raven 1964; Craig et al. 1999). Females must invest resources into searching for a suitable host plant and this searching window of time can be shortened if the host plant species has a low spatial and temporal distribution and has highly variable quality, thus strengthening the preference-performance relationship (Itami and Craig 2019). Besides having variable nutritional quality, plants can directly affect larval development time if variable concentrations of defenses are present (Strong et al. 1984). There are ongoing studies to find which leaf surface infochemicals help mated females optimize behavioral host plant decisions.

A decisive factor in a female herbivore's choice of host plant is the release of secondary metabolites, such as terpenoids, known as volatile organic compounds (VOCs). The function of VOCs in nature is extremely diverse and continues to be

investigated but a consensus has been reached that VOCs are essential in interspecies communication. Biogenic VOCs represent a major source of atmospheric reactive carbon (Guenther et al. 2012). For the adult female herbivores ovipositing primarily on leaves, the leaf VOCs will be crucial in determining their host plant. In contrast to floral volatiles, which mainly evolved for sexual reproduction, leaf VOCs have evolved for plant survival (Schiestl 2014). This is accomplished through direct defense, deterring herbivore feeding and/or oviposition, or as an indirect defense by attracting its herbivores' natural enemies, such as parasitoids, after herbivore feeding or contact with a host plant (Turlings et al. 1995; Dicke 2009; Kessler and Baldwin 2001). Levels of VOCs can be altered in response to biotic and abiotic stresses, which plants use to communicate with other species in their vicinity (Holopainen and Gershenzon 2010). Generally, abiotic or biotic stress, such as age or sunlight, can be said to increase plant VOC emission, impacting phytophagous insects (Holopainen and Gershenzon 2010). The herbivore induced plant volatiles (HIPVs) emitted as a result of biotic stress from herbivore attacks contain a diverse array of mono- and sesquiterpenes (Gosset et al. 2009; Laothawornkitkul et al. 2008), which are central to both above- (Kessler and Baldwin 2001; Degenhardt et al. 2009; Kappers et al. 2005) and below-ground plant signaling (Hiltpold et al. 2011).

Plant chemical stimulation & impacts on oviposition are quite complex. There's no general consensus because in some plant species, like *Brassica rapa,* there is no evidence for chemical defense signaling and plant volatiles and glucosinolates (a non-volatile chemical defense) are uncorrelated (Hamilton et al. 2005). However, it has been found that for the dietary specialist *Plutella xylostella,* glucosinolates present in their host

plant *Brassica oleracea* significantly stimulate oviposition and feeding (Hopkins et al. 2009). The secondary metabolites used for defense, like glucosinolates, are typically toxic to generalists whereas specialists can sometimes be adapted to them by sequestering the chemicals in their own defense or metabolizing and excreting the compounds (Bowers 2022).

It is important to bear in mind that plant secondary defensive compounds aren't the only cues governing oviposition choices, which is why conflicting evidence may be found between chemical stimuli and herbivore oviposition preference. Other components such as the concentrations of carbon and nitrogen (and the C:N ratio) are crucial to herbivore development and can be used in decision-making by mated females. Higher carbon and nitrogen levels indicate an overall healthier plant, as higher carbon typically indicates a higher assimilation of carbon dioxide to form carbohydrates and enhance plant growth and nitrogen can be positively correlated to protein levels. High levels of intraspecific changes in mature leaves should select for generalist herbivores with a wide diet breadth. Conversely, canalizing defense compounds and investing resources into young leaves should select for the specialization of herbivores.

An abiotic stressor that has been shown to alter plant quality and attractiveness to herbivores is increased temperature, thus impacting the oviposition preference of herbivores and the subsequent development of its offspring (Pereira et al. 2002; Hunter and McNeil 1997). Anthropogenic greenhouse gas emissions, driven mainly by population growth, have seen a spike since the industrial revolution of the 1750s and global climatic conditions have changed as a result. The warming that has been observed in the late half of the 20th century has led to a hypothesized projection of an increase in

global mean temperatures of 1.5 - 4 ℃ by the year 2100 (IPCC 2014). As these changes occur, the effects of temperature on plant chemical profiles should also be considered.

Previous studies have examined diamondback moth oviposition preferences (Jafary-Jahed et al. 2018; Hamilton et al. 2005) and offspring performance on differing Brassicaceous crop plants (Hamilton et al. 2005), as well as ranges of temperatures where they are successful (Bahar 2014). It has been shown that adult females differentially lay eggs on different host plants, as well as different cultivars of *Brassica oleracea*, but what's not known is whether they respond to intraspecific variation in quality associated with temperature differences during the growing season. Preliminary data has demonstrated that when *P. xylostella* caterpillars develop on *Brassica oleracea* varieties such as green cabbage (*Brassica oleracea* var. "Capitata"), lacinato kale (*Brassica oleracea* var. "Palmifolia") and sprouting broccoli (*Brassica oleracea* var. "Italica"), the average pupal weight (a well-known measurement of immature performance) was found to be lower in hotter temperature treatments across all host plants, with a more drastic difference found on lacinato kale (Spahn, unpublished data); thus, hotter temperatures are likely to reduce Brassicaceous host plant quality. In these studies, caterpillars developed on the crops as temperature treatments were applied, mimicking a similar scenario to what will be seen in the future with climate change.

However, we currently lack an understanding of whether increasing plant temperatures are likely to influence adult oviposition and whether adult females are capable of assessing temperature-mediated changes in plant quality likely to impact offspring performance. It is complex to determine how all the factors involved in the preference-performance relationship will change with temperature and how big of an

influence these will have on oviposition preference. Therefore, experiments that directly test and isolate the effect of temperature on plant quality and that quantify oviposition responses are needed. In this study, the oviposition preference of diamondback moths (*Plutella xylostella*) offered kale plants (*Brassica oleracea* var. "Palmifolia") grown under ambient vs. increased temperature treatments (+ 5°C; temperature-treated plants hereafter) was quantified and multiple aspects of plant chemistry and nutritional quality were measured to address the following questions: 1) Do mated female *P. xylostella* prefer ambient-grown plants over temperature-treated plants for oviposition when offered a choice?; 2) Do females presented with temperature-treated plants in a no-choice test lay fewer total eggs than females presented with control plants? 3) Does foliar chemical composition differ between ambient grown and temperature-treated plants? Based on the literature on how thermal stress alters plant chemistry, oviposition preference, and offspring performance, I hypothesized that if *P. xylostella* are under selection to optimize offspring performance, females would lay fewer eggs on temperature-treated kale in both choice and non-choice tests.

METHODS

Study System

Lacinato kale (*Brassica oleracea* var. "Palmifolia": Brassicaceae) is a cultivated crop in the family *Brassicaceae*. Lacinato kale grows as a biennial plant, meaning it requires two years to successfully reproduce and only needs to be sown every two years. However, due to cruciferous crops, like lacinato kale, being popular vegetables for human consumption, they can commonly be found perennially in great amounts on

agricultural fields. Brassicas are typically grown in North American agricultural fields during spring and summer months with overwintering occurring in the colder months.

Glucosinolates are specific defense compounds characteristic of Brassicaceae that are used as a host plant recognition signal by specialist herbivores; these compounds are also reported to stimulate larval feeding and oviposition for a variety of specialist insect species (Hopkins et al. 2009; Hamilton et al. 2005; van Loon et al. 2002; Li et al. 2000; Qiu et al. 1998). While little is known about how glucosinolate concentrations change over plant ontogeny in *B. oleracea*, 12-week-old plants of two different varieties had higher water content and lower protein, carbohydrate and fiber content than 8-week-old plants but did not differ in lipid content (Adelanwa & Medugu 2015).

The introduced diamondback moth (DBM), *Plutella xylostella* (Lepidoptera: Plutellidae), goes through four larval instars in approximately ten days. Pupae spin a loose silk cocoon and emerge as adults eight to ten days later. Adult female DBMs can oviposit eggs singly or in small groups, typically in the foliage depressions, and can lay up to approximately 300 eggs in their lifetime. Larvae eclose from eggs after about five days. Multiple generations, ranging from eight to twelve depending on the latitude, can occur annually, with the moth overwintering as an adult. The resilient species possesses relatively stable, predictable population dynamics dependent on climatic conditions and seasonal phenology, and has demonstrated a tolerance to a wide range of temperatures, suggesting that temperature alone may not be a limiting factor in the development of *P. xylostella* (Bahar et al. 2014; Golizadeh et al. 2007; Spahn and Lill 2022).

Thought to have originated in Europe, the DBM now has a global distribution that includes Americas, Europe, Southeast Asia, Australia, and New Zealand (Talekar and

Shelton 1993). The DBM is a specialist of wild and cultivated brassicas and the first recorded observation of *P. xylostella* (Lepidoptera: Plutellidae) in North America was in 1854, quickly spreading across the continent and now being found anywhere where brassicas grow.

Because *P. xylostella* (Lepidoptera: Plutellidae) feeds on virtually all of the known cultivars of *B. oleracea*, this oligophagous insect is ideal for studying oviposition preferences. As a host plant specialist, *P. xylostella* has relatively low resource complexity constraints, and generally only feeds on the leaves of one individual plant over its development, which in theory should strengthen the preference-performance link (Sarfraz et al. 2010; Muimba-Kankolongo 2018). However, a factor that may weaken the preference-performance relationship of the DBM is the consideration of its primary parasitoid, *Diadegma insulare* (Hymenoptera: Ichneumonidae). At this higher trophic level, resource complexity is added as the ovipositing female DBM must contend with potential differences among cultivars in the susceptibility of its offspring to the parasitoid.

Like most Lepidoptera, DBM females possess tarsal chemoreceptors and antennal and ovipositor sensilla that allow them to detect host plant quality-related signals (e.g., HIPVs and/or other secondary metabolites, such as glucosinolates; Qiu et al. 1998; Justus and Mitchell 1996). The aforementioned nutritional change that occurs with age in brassicaceous plants is unlikely to affect the caterpillars, which have a relatively standard observed development time of about two to three weeks, during which the host plant nutritional content does not radically change (Hamilton et al. 2005, Jafary-Jahed et al. 2018).

Research Methods.

At the end of May 2022, we collected diamondback moth (DBM), *Plutella xylostella,* caterpillars at different life stages feeding on lacinato kale (*Brassica oleracea* var. "Palmifolia") at an organic farm located at Potomac Vegetable Farms in Vienna, VA, USA. The *P. xylostella* caterpillars were brought back to the lab and reared in separate deli containers until they emerged as adults. To provide a humid environment in the rearing containers for the *P. xylostella* caterpillars, we placed filter paper at the bottom of each container, moistening it with water as needed.

Once adult DBMs emerged, individuals of both sexes were placed in square 12 in. × 12 in. × 12 in. cages for mating to occur and to establish a colony. No more than 30 individuals were kept in a cage at a time. We set a lacinato kale leaf (*Brassica oleracea* var. "Palmifolia") in a lidless deli container upon moistened filter paper in the cage for the mated *P. xylostella* females to oviposit on. A cotton ball moistened with 10% honey water solution was set in a hole in the lid of another deli container and placed inside this mating cage for adult moth feeding. I then collected the eggs laid by the moths collected in May 2022 from the field to establish a colony of diamondback moths in June 2022 (starting with N= 10 females). In March 2023, 100 mixed sex pupae were ordered from Benzon Research rearing laboratories in Carlisle, PA, USA. These pupae were used to establish a colony in May 2023. The colony was reared in an environmental chamber set to a diurnal cycle (13L : 11D) with a temperature of 27°C.

I divided clutches of the *P. xylostella* caterpillars based on oviposition date into separate containers and reared individuals to adulthood in an environmental chamber (27°C). All caterpillars were fed an ambient grown lacinato kale (*Brassica oleracea* var.

"Palmifolia") diet as caterpillars and all moths in the mating cages for the colony were given ambient grown lacinato kale oviposition leaves.

The DBM colony established in June 2022 lasted until September 2022; the DBM colony established in May 2023 lasted through the end of June 2023.

Oviposition Trials

All lacinato kale plants (*Brassica oleracea* var. "Palmifolia") developed in the GW Harlan Greenhouse under ambient temperature for a minimum time of 6 weeks prior to applying any temperature treatment. Once the plants reached the 6-week age, lacinato kale plants were placed in square 12 in. × 12 in. × 12 in. cages in the GW Harlan Greenhouse under two different temperature treatments (control vs. hot) for 7 days. The control lacinato kale treatment consisted of lacinato kale plants grown under the ambient temperature of the greenhouse (27°C). The hot treatment consisted of lacinato kale plants grown under a Berlese funnel lamp (120 V / 25 W incandescent light bulbs) placed on top of the cage to achieve the hotter temperature treatment (~5 °C temperature increase from the unheated cages). This 5 °C temperature increase represents the higher end of the predicted climate change temperature that is to come by the end of the 21st century. Plants under both treatments were watered daily as needed.

Moths were sexed as caterpillars once they reached the third or fourth instar when the gonads were visible and were separated into deli containers with larvae of the same sex and age. Female DBMs that emerged 3 to 5 days before the completion of the lacinato kale temperature treatments were placed in mating cages separated by their emergence date to allow for the tracking of their age for the oviposition trials. Females that emerged on the same day were placed into a cage with at least 2 males per female

and were kept in their mating cages until the female's placement in choice tests to increase the likelihood of mating. A deli container with a cotton ball moistened with 10% honey water solution was set in a hole in the lid and placed inside oviposition trial mating cages; no lacinato kale leaves were placed inside mating cages to prevent oviposition and ensure that the females remained naïve to the kale. To facilitate the matings for the oviposition trials, a cage of only males was kept during the 7 days of the lacinato kale temperature treatment period. When both sexes were placed into a mating cage, caterpillar sex ratios were tracked to help with adult sexing as sexual dimorphism in DBMs is not easily distinguishable. Females were then separated from males in the oviposition trial mating cages once the lacinato kale completed its treatment time.

Individual mated female DBMs were placed into individual cages in the greenhouse under either a no choice or choice test. The no choice test entailed situating one lacinato kale plant (either ambient or temperature-treated) for 48 hours in the cage with the mated female. The choice test consisted of placing both an ambient and temperature-treated lacinato kale plant simultaneously in the cage for 48 hours with a mated female. Kale plants in the choice tests were matched by size. During the 48-hour oviposition trial, all heat lamps were removed to avoid any confounding temperature effects on moth behavior.

After 24 hours of the mated *P. xylostella* female being in the cage with the lacinato kale plants of their respective treatments, I collected the lacinato kale plants and placed the mated female into a deli container while I counted the number of eggs. This first 24-hour block of the oviposition trial is denoted 0-24. The number of eggs laid per leaf was recorded, keeping track of the leaves by numbering them from youngest to

oldest (the youngest leaf received the number one). After counting the eggs, I placed the female DBM back into its respective treatment cage to complete the 48 hour oviposition trial. After 48 hours and the end of the oviposition trial (denoted 0-48), I removed the mated female DBM from the treatment cage and counted all *P. xylostella* eggs oviposited on each lacinato kale leaf, recording the amount and summed the total number of eggs oviposited. The second 24-hour block is denoted 24-48. Mated females who completed oviposition trials were placed back into the colony cage. A total of 60 trials were conducted as shown in **Table 1**.

Table 1. Breakdown of the choice tests used for oviposition trials.

Test	Treatment/Kale Given	Number of Trials
Choice	Control & Hot	30
No Choice	Control	15
No Choice	Hot	15

Temperature Treated Lacinato Kale Chemistry

For the elemental analysis, all the leaves from 22 control and 19 temperature-treated plants were rinsed and dried to remove any soil or other residue from the leaves to prevent erroneous readings. The stem and midvein of every leaf was removed as the *P. xylostella* caterpillars do not feed on these plant tissues. All the leaves from a single plant were placed in coin envelopes to then dry in an isotemp incubator for a week at approximately 85℃. Once all the leaves were dry, the leaf samples were placed in 2.0 ml microcentrifuge tubes and ground to a fine powder using a ball mixer mill at a frequency

of 30 cycles per second for two minutes. About 250 mg of the ground leaf material was carefully weighed on a Metler-Toledo microbalance and packaged to run through the elemental analyzer (Leco 828 series carbon/nitrogen analyzer). The carbon-nitrogen content could not be analyzed for individual leaves on a plant because of the minimum sample weights, which required that a bulk sample be taken from the plant including leaves of all ages.

A total of three mechanical hole punches were taken out of leaves from 11 control and 8 hot plants right after the oviposition trials ended to capture the water content the ovipositing female responds to and the water content the offspring would be consuming. One hole punch was taken from the oldest, middle and youngest leaf, being careful to avoid the leaf midvein. If the plant had an odd number of leaves then I rounded the half to the next whole number, and took a hole punch from the leaf at that position. The same leaf numbering used to count eggs in the oviposition trials was used. The wet weight of all three hole punches was then measured in milligrams. The three hole punches for each plant were placed in coin envelopes and dried in the isotemp incubator for a week. Afterward, the dry weight was measured in milligrams.

Data Analysis

Paired t-tests were conducted to compare the number of eggs oviposited on the plants under choice tests between the two 24-hour blocks, and between treatments. Unpaired t-tests were performed to compare the number of eggs laid on the plants in the no-choice tests between the two 24-hour blocks, between treatments. Additionally, because every kale plant used in the oviposition trials (both choice and no-choice) had a minimum of five leaves, a chi-square test was performed to compare the total eggs from

the 48-hour oviposition trials found on leaves one through five and the distribution was plotted in a barplot. The carbon to nitrogen (C:N) ratio was calculated by dividing percent carbon by percent nitrogen. The weights of the hole punches were used to calculate percent water content, using the formula: (wet weight - dry weight)/wet weight × 100. Unpaired t-tests were conducted to test for differences between treatments in percent carbon, percent nitrogen, the carbon to nitrogen ratio, and water content.

RESULTS

Oviposition Trials

Oviposition data was collected for a total of 63 female DBMs, but analyzed oviposition data excluded 3 females that died before the 48-hour oviposition period ended. The number of eggs laid in the first 24-hour, the second 48-hour block and the full 48-hour oviposition trial were recorded for 60 *Plutella xylostella* females under 30 choice and 30 no-choice tests, as outlined in **Table 1**.

We were concerned that the females may lay a different number of eggs in the two days of the oviposition trial so paired t-tests were performed to compare the two 24 hour-blocks across treatments within choice tests. Independent two sample t-tests assuming unequal variances were performed to compare treatment means in both choice and across no/choice tests. When comparing the first 24-hour block (0-24) to the second 24-hour block (24-48) within and across treatments, no differences were found in the total number of eggs laid; therefore, we conducted all the analyses on the total number of eggs oviposited in the full 48-hour period.

The total number of eggs on the control plants under the choice tests ranged from 0 to 75 with a mean of 24.8 ± 4.04 (SEM) eggs, while the number of eggs on hot plants

under choice tests varied from 0 to 81 with a mean of 19.4 ± 3.52 (SEM) eggs. No differences were observed between treatments in the total number of eggs laid in choice tests (paired t = 1.12, p = 0.14, df = 29; **Figure 1**). For the no-choice tests, the number of eggs on control plants varied from 29 to 77 with an average of 51.4 ± 4.12 (SEM) eggs, and the hot plants had a total number of eggs ranging from 2 to 101 with an average of 48 ± 4.90 (SEM) eggs. An independent two sample t-test showed no difference among treatments (t = 0.35, p = 0.36, df = 20; **Figure 2**).

Most of the kale plants had older leaves with an average of 11 plants per plant category of 6 leaves through 13 leaves (**Figure 3**). As aforementioned, every kale plant for the oviposition trials had a minimum of 5 leaves so the range and mean number of eggs oviposited in the 0–48 hour period on each leaf age (1-5) was compared. The total number of eggs on leaves one through five differed from random (assuming equal distribution across leaf ages) (X^2 = 581.10, df = 4, $p < 0.0001$; **Figure 4**). Paired t-tests were conducted comparing the average number of eggs laid on young and middle leaves, whereas two-sample t-tests assuming unequal variances were performed to compare young and middle-aged leaves to old leaves (**Figure 5**). The average number of eggs on young leaves was significantly lower than those on middle-aged leaves, with middle-aged leaves receiving more than three times the eggs of young leaves, on average (t = 5.48, p < 0.0001, df = 89). Similarly, the average number of eggs on young and old leaves was significantly different, with old leaves having a 3.29-fold higher mean (t=4.78, p < 0.0001, df = 76). The average number of eggs on middle leaves was not significantly different from the average number of eggs on old leaves (t = 0.04, p = 0.96, df = 143).

Carbon (C) and Nitrogen (N) Ratio

Carbon and nitrogen data were obtained for 22 control-treated kale plants and 19 hot-treated kale plants. In control kale, carbon varied from 31.04% to 41.41% with a mean of 37.24% ± 0.46% (SEM), nitrogen varied from 1.47% to 4.88% with a mean of 2.55% ± 0.21% (SEM), and the C:N ratio ranged from 8.85 to 21.17 with an average of 16.63 ± 1.24 (SEM). The ranges for hot kale were 33.61% to 38.61% for carbon with an average of 36.49% ± 0.33% (SEM), 1.96% to 5.20% for nitrogen with an average of 3.7% ± 0.22% (SEM), and 8.51 to 19.20 for the C:N ratio with a mean of 10.74 ± 0.87 (SEM).

Our hypothesis that the carbon and nitrogen levels would be different between kale plants of both treatments was partially supported. Percent carbon did not differ between treatments ($t = 2.03$, $p = 0.19$, $df = 37$; **Figure 6A**), but percent nitrogen did, with hot-treated kale having 68.83% higher average levels of nitrogen ($t = 2.02$, $p = 0.0006$, $df = 39$; **Figure 6B**). Similarly, the C:N ratio was significantly higher on control than hot treated kale ($t = 2.03$, $p = 0.0004$, $df = 36$, **Figure 6C**).

Water Content

Water content was obtained for 11 control treated kale crops and 8 hot treated kale plants. Water content for control plants ranged from 2.59% to 5.00% with a mean of 3.64% ± 0.19% (SEM), whereas water content for hot plants varied from 2.32% to 4.59% with an average of 3.54% ± 0.52% (SEM). Our hypothesis that hot treated plants would have a lower water content than control kale plants due to water loss under hot temperatures was rejected, with no difference found between the two ($t = 0.32$, $p = 0.38$, $df = 14$; **Figure 6D**).

DISCUSSION

No difference in the average number of eggs was found between treatments in either the choice tests or the no-choice tests, rejecting our hypothesis that temperature-treated plants would be less preferred as an oviposition host (**Figure 1 and 2**). Previous work in this same system (Spahn 2023, dissertation) found that *P. xylostella* reared on temperature-treated kale plants (using an identical protocol) had reduced pupal weights relative to controls, suggesting that the host plants are changing in response to temperature and negatively impacting offspring fitness during larval development. However, the results of this study clearly indicate that *Plutella xylostella* females are either not able to detect those nutritional quality differences or that the differences are insufficient to elicit an oviposition avoidance response (i.e., below a putative 'avoidance threshold' for oviposition). It may also be that female DBMs lack the sensory ability to detect relatively subtle temperature-induced changes in nutritional and/or defensive profiles of the plants. Because the plants were only exposed to the temperature treatments for 7 days prior to testing, resulting chemical/nutritional changes in foliage quality may have been subtle.

When considering the spatial distribution of eggs on plants, it is important to take into account that leaves, even with the same numbering, were of different ages and sizes as plants varied in the total number of leaves, which could have had an influence on the amount of eggs oviposited. Despite not showing plant-level oviposition preferences, I found that female moths showed a non-random distribution of eggs among leaves of different ages. On average, the number of eggs increased with leaf age over the first five leaves. Furthermore, although not every plant in the oviposition trials had greater than

five leaves, for the subset of larger plans, leaves 10 and 11 had the highest means (**Figure 3**). Although younger leaves typically have more nitrogen and more water, theoretically being able to provide better nutrition for offspring, older leaves received more eggs on average, demonstrating that DBMs like to lay eggs lower down in the plant; middle-aged leaves were the most preferred, overall (**Figure 3 and 5**). A possible reason for which *Plutella xylostella* females did not oviposit as much on the newer leaves could be that younger leaves have more glucosinolates. Brassicaceous plants often defend the younger leaves more than older leaves because damage to younger leaves is integrated over the leaf's lifespan (Bowers 2022). Therefore, in terms of fitness to the plant, it is better to have damage to later leaves as older leaves will fall sooner too. Additionally, eggs and larvae on younger leaves may be more susceptible to desiccation and predation as the top leaves can experience more heat and are more exposed.

The observation that percent carbon was not significantly different between treatments could result from the 5 ℃ difference in treatments not causing a drastic difference in carbon fixation rates (**Figure 6A**). On the other hand, hot-treated kale had a significantly higher percentage of nitrogen (**Figure 6B**). This measure of nitrogen includes a mixture of both glucosinolates (nitrogen-containing secondary defensive compounds) and proteins (primary compounds that are more accessible and nutritional for larvae). The significant difference in nitrogen could be due to either higher levels of protein or glucosinolates, but the decreased pupal weight data shown previously (Spahn 2023) suggests that the higher nitrogen content in temperature-treated kale is likely allocated to defensive compounds like glucosinolates under hotter temperatures compared to ambient grown kale. If the higher percent nitrogen was attributed to higher

protein levels, then the larvae could potentially gain more weight, but that was not the case. Due to the nitrogen being higher in temperature-treated kale, the C:N ratio was also significantly lower in temperature-treated kale (**Figure 6C**). Future research should include measurements of foliar glucosinolate concentrations in temperature-treated plants, which was beyond the analytical capabilities of our lab, to help determine whether there is an optimum production level for the kale plants under which glucosinolates provide a strong enough oviposition cue but the glucosinolate levels are not too high to deter larval development.

Additionally, conducting similar experiments with kale damaged by con- and heterospecifics instead would be of great use as HIPVs can have a great influence on oviposition decisions and may change under temperature changes. When considering the susceptibility of offspring to natural enemies, the DBM prefers to oviposit on host plants damaged by heterospecifics even if the plants may be nutritionally suboptimal for their offspring, and on conspecific-damaged crops compared to undamaged host plants, (Shiojiri et al. 2002; Uematsu and Sakanoshita 1993).

SIGNIFICANCE

The diamondback moth, *Plutella xylostella,* is known as one of the world's most damaging and most widely distributed pests of cruciferous crops, costing approximately 4 to 5 billion USD annually in crop damage and pest management globally (Zalucki et al., 2012). The difficulty with managing DBM populations in areas with crucifers comes partially from the resistance the species has developed to pyrethroid insecticides since before its confirmation in the 1980s. Further resistance to other insecticides developed, leading to the cost in billions of this pest management. With climate change and

increasing temperatures threatening to change the agricultural field, the negative fitness effect seen in the lower pupal weight when offspring develop on hot-treated kale and the lack of sensory detection of nutritional quality in kale by the female should be beneficial for biocontrol. The lower fitness reduces the likelihood of survival and reproductive success. On organic farms, such as the one we collected *P. xylostella* larvae from, that do not use insecticide that can kill pests and beneficial insects, the survival of parasitoids can be favored leading to possible solutions to further minimize the pest's impacts on the agricultural industry.

CHAPTER 1 FIGURES

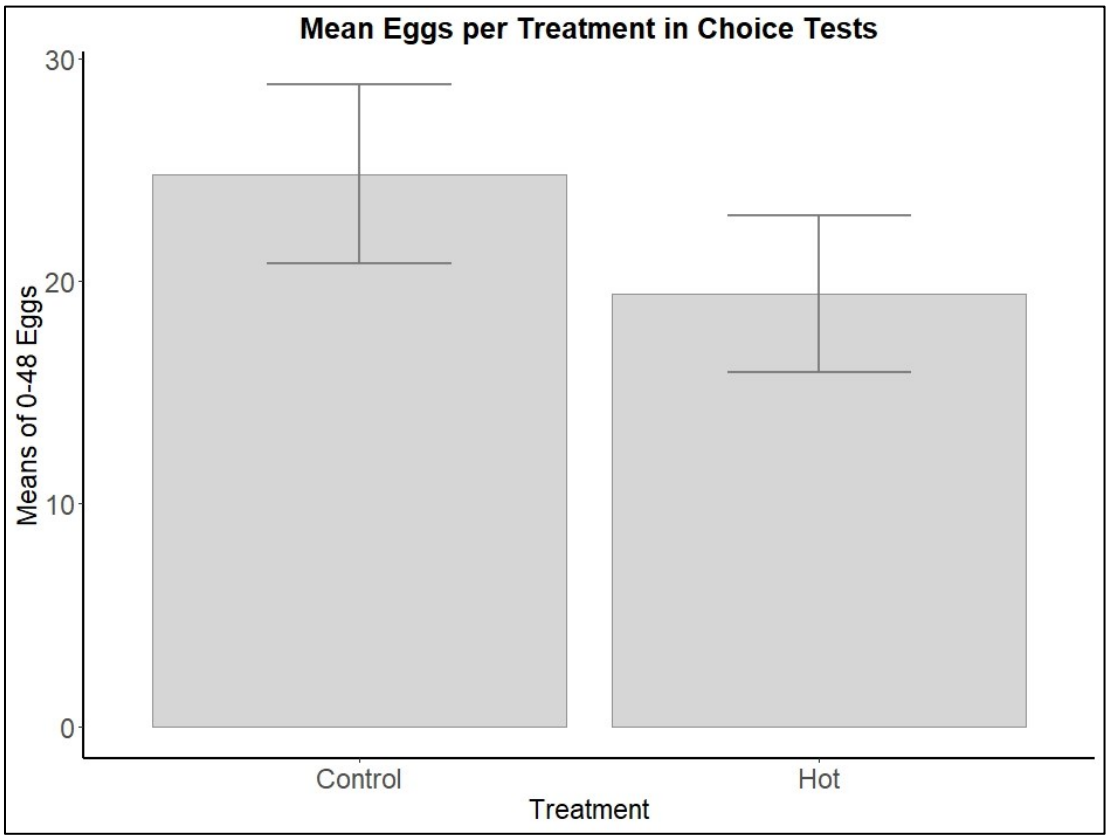

Figure 1. Mean number of eggs oviposited in the 48-hour oviposition trial for control and hot treated kale plants under a choice test.

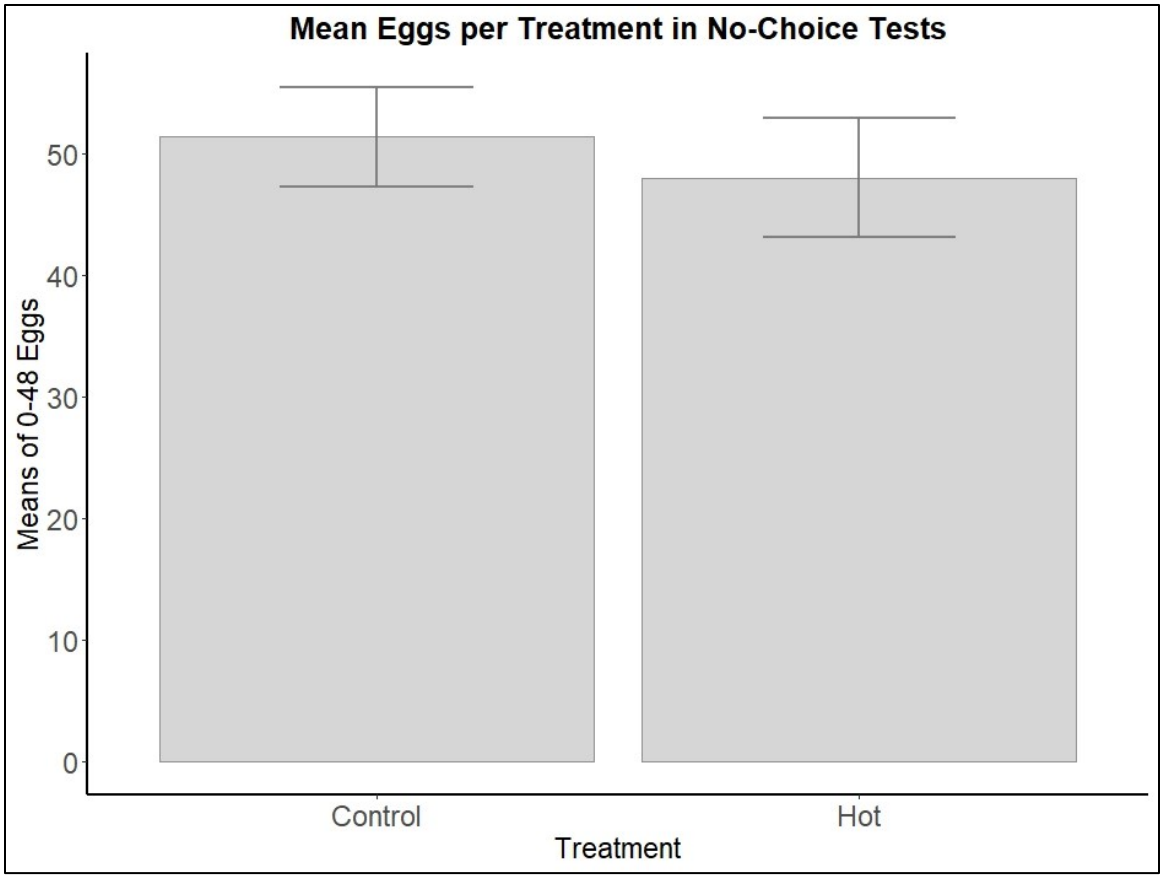

Figure 2. Mean number of eggs oviposited in the 48-hour oviposition trial for control and hot treated kale plants under a no-choice test.

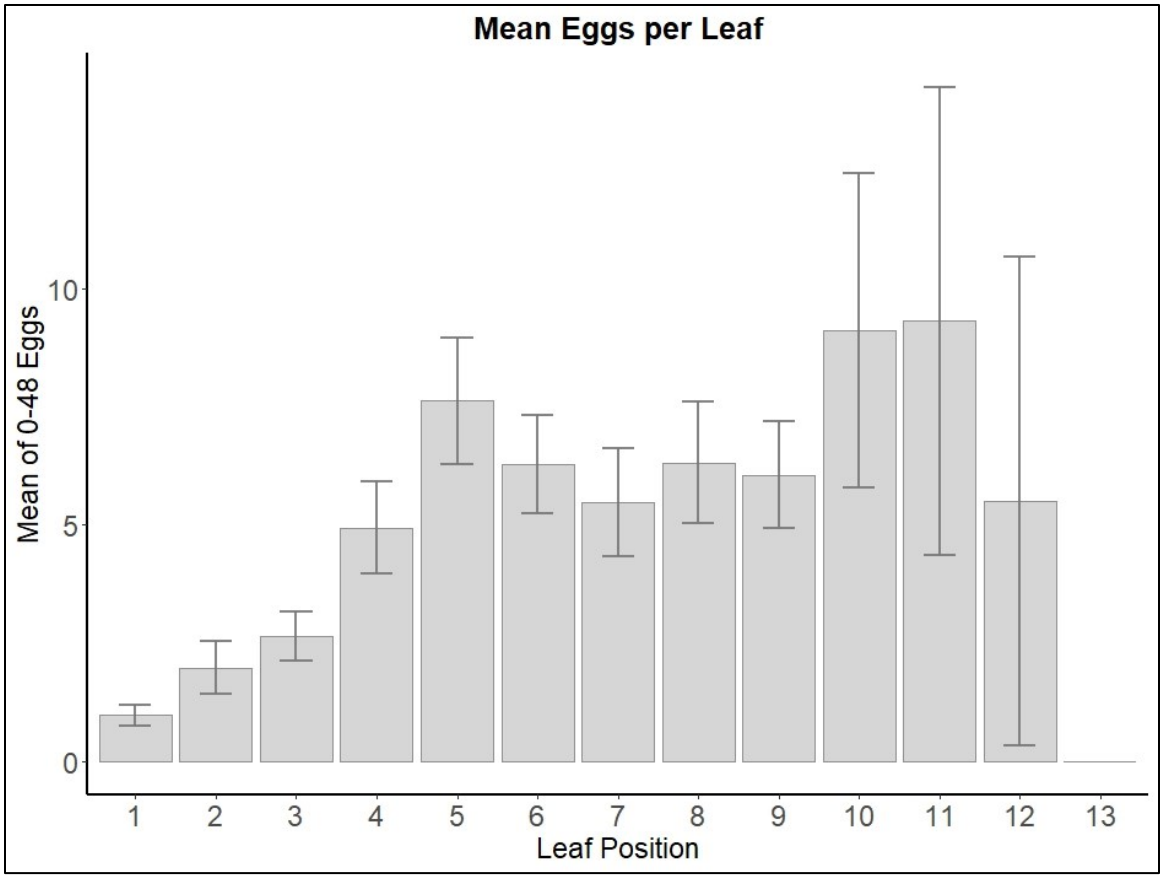

Figure 3. Mean number of total eggs oviposited during the 48-hour oviposition trial per leaf, leaves 1 through 13 with standard error of the mean bars.

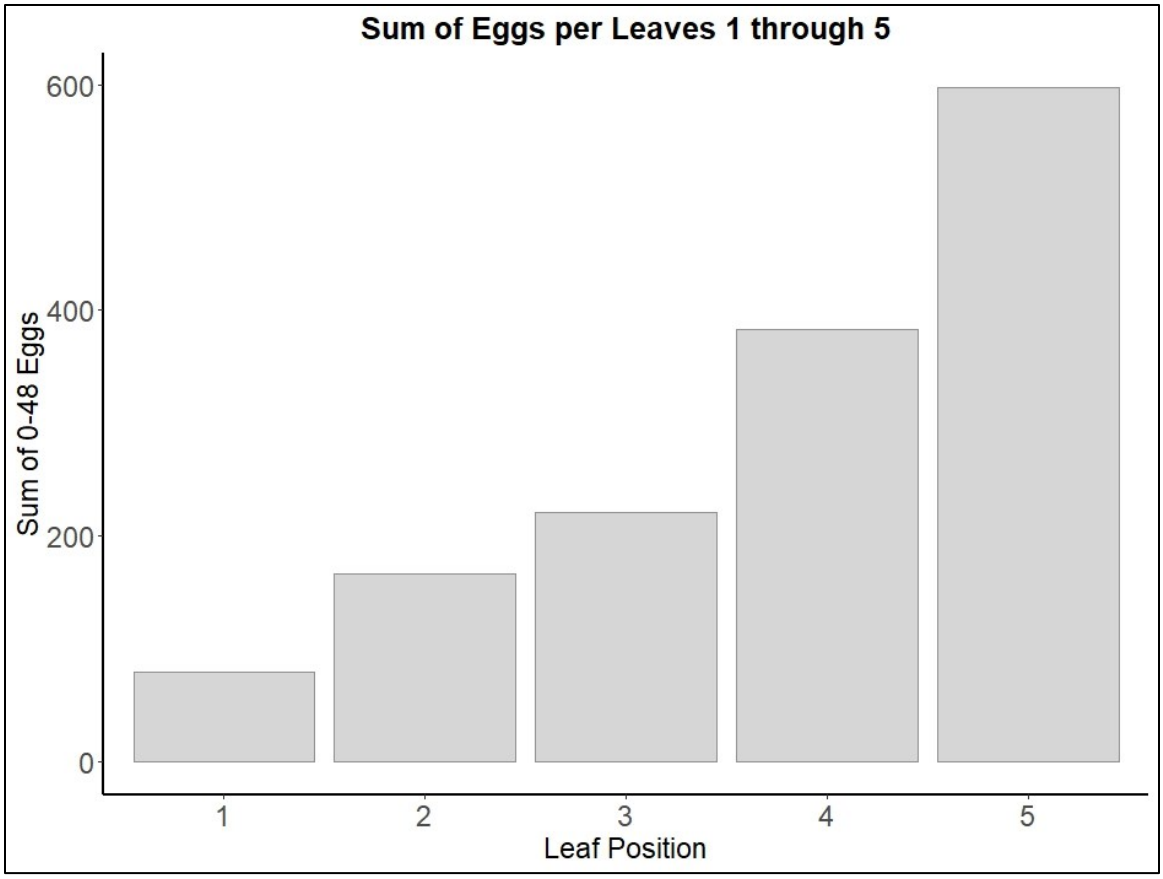

Figure 4. Sum of eggs oviposited in the 48-hour oviposition trial for leaves 1 through 5.

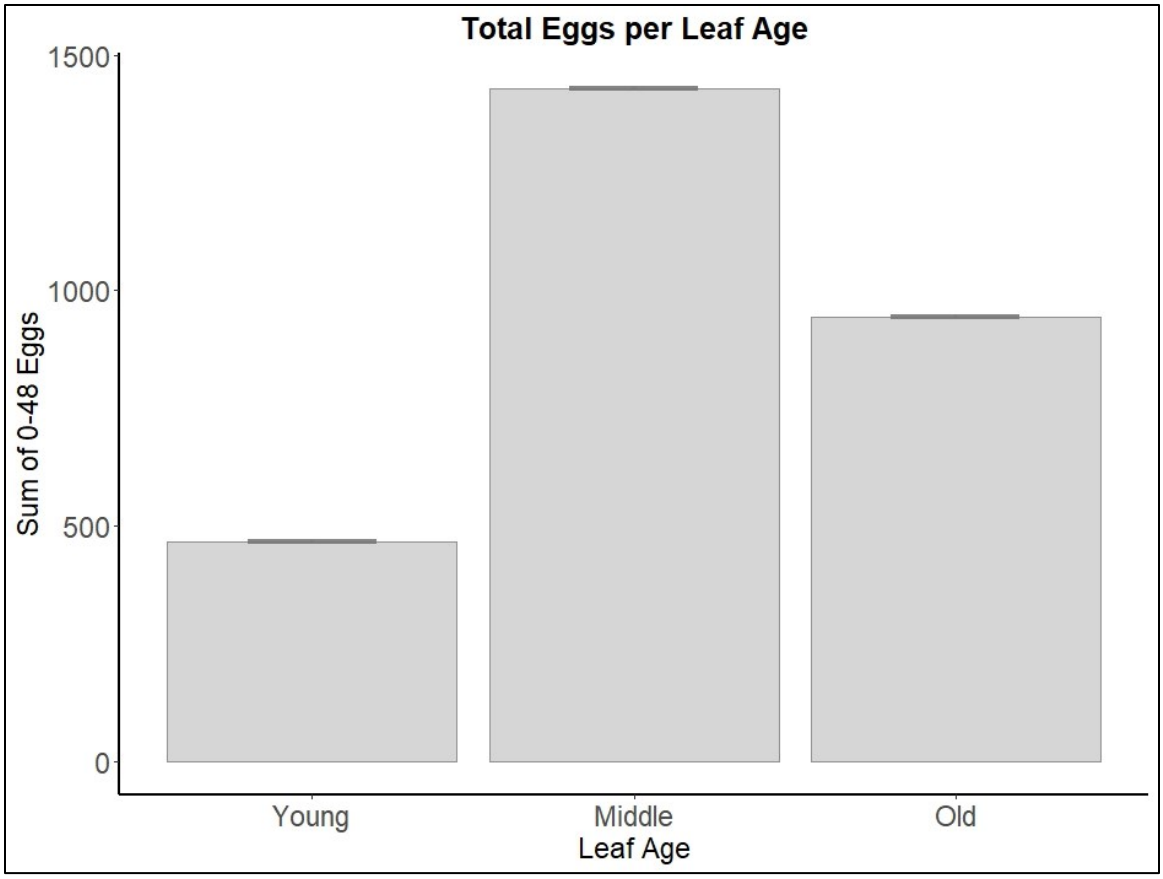

Figure 5. Sum of the total number of eggs oviposited in the 48-hour oviposition trial for young leaves (Leaf 1 through Leaf 3), middle leaves (Leaf 4 through Leaf 6), and old leaves (Leaf 7 through Leaf 13).

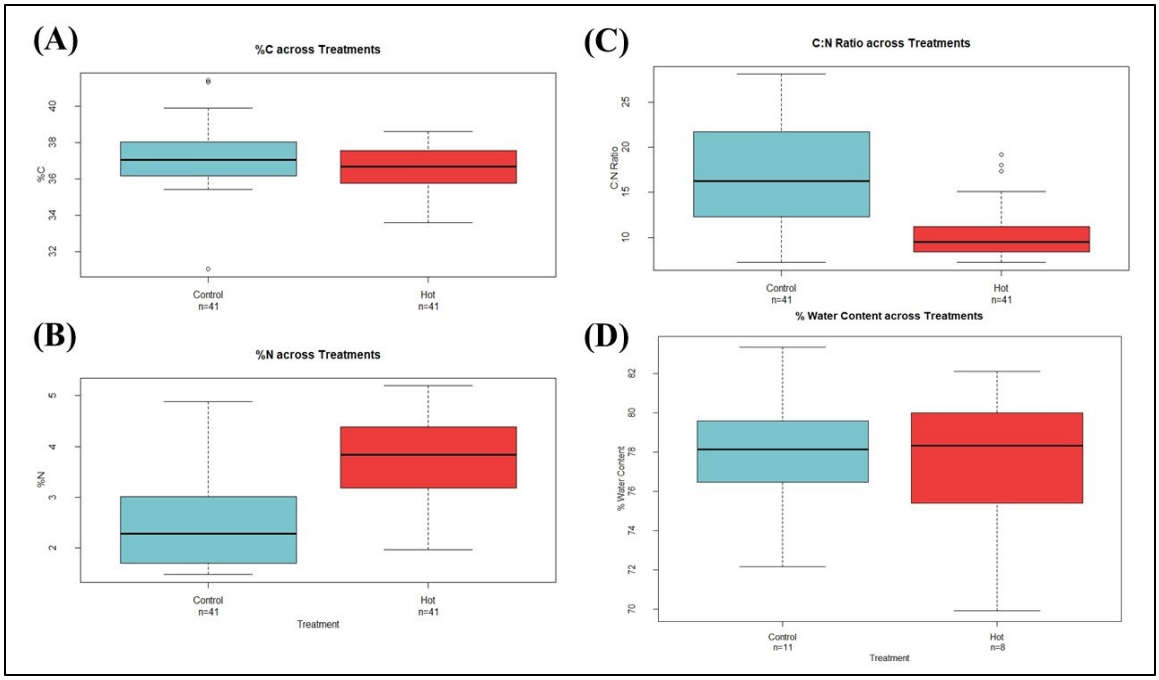

Figure 6. Boxplots of the differences in plant chemistry of control and hot grown kale. From the bottom to the top, the start of the line represents the minimum value in the data. The bottom of the box represents the first quartile or the 25th percentile. The bolded line in the middle of the box represents the median of the data. The top of the box represents the third quartile or the 75th percentile. The top end of the line represents the maximum value in the data. The circles represent potential outliers. **(A)** Percent carbon measurements. **(B)** Percent nitrogen measurements. **(C)** Carbon to nitrogen ratio recorded. **(D)** Percent water content measurements.

Chapter 2: Bodyguard caterpillar lifespan decreases with clutch size in a gregarious parasitoid

INTRODUCTION

Breeding animals are faced with crucial decisions affecting fitness from the time they mate to when their offspring are born (and beyond for species that have parental care; Kölliker et al., 2012). One main question parents face is how many offspring they should produce in a single reproductive event to ensure their genes are passed to future generations, also called the optimal clutch or litter size (Godfray 1994). The generally applied and known concept of Lack clutch size was developed, based on a European robin (*Erithacus rubecula*) clutch size study, to take into account the many ecological and evolutionary considerations that influence clutch size decisions. The Lack clutch size concept aims to determine what clutch size maximizes both offspring fitness and the female's reproductive success during the lifetime of both the mother and her offspring (Lack 1947). These relationships typically involve fitness tradeoffs – in attempting to maximize one aspect of fitness (e.g., offspring number), another aspect of fitness, such as adult survival, is negatively impacted. The literature concerning clutch size clearly demonstrates that tradeoffs govern clutch size, with the most pervasive tradeoff occurring between offspring number (clutch size) and offspring size, since maternal resources are finite.

Parental clutch size decisions not only determine offspring number, but also affect the properties of individual offspring. An important dynamic trade-off exists between clutch size and offspring size and this relationship is affected by sibling competition (Charnov & Skinner 1984; Godfray 1994). In animal species with parental care,

competition can be regulated by the parents (Parker & Macnair 1979; Godfray 1991), but in species without parental care, mothers typically reduce the clutch size to mitigate the anticipated negative effects of sibling competition (Godfray & Parker 1991, 1992). The larger the clutch size, the more intraspecific competition typically occurs between the developing offspring, and the smaller in size the successful adults will be (Stearns 1992; Takagi 1985; Hardy et al. 1992; Vet et al. 1994). The resulting offspring's smaller adult body size, which is often a strong fitness correlate in both males and females, can provide a disadvantage when competing with heterospecific and conspecific organisms for limited resources (Martin 1991; Lozano 1994; Schneider & Lubin 1997; Renison et al. 2002; Petersen & Hardy 1996).

Insect parasitoids, organisms whose larval life stage feeds on an arthropod host, eventually killing it, have emerged as an excellent model for studying the evolutionary dynamics of clutch size because the host represents a discrete resource unit for immature parasitoid development (Godfray 1994). In addition, clutch size and offspring survival are relatively easy to document, and the host-parasitoid system can be experimentally manipulated (Godfray 1994; Wilson & Lessells 1994; Mayhew & Hardy 1998). While all parasitoids ultimately kill their host, the most speciose groups of parasitoids are wasps or flies that allow their hosts to continue developing after oviposition. Known as 'koinobionts,' these insects undergo larval development by exclusively consuming another arthropod's tissues while avoiding damage to critical organs to ensure host survival and feeding for the span of parasitoid development (Godfray 1994; Harvey et al. 2013). Once the parasitoid larva pupates and the adult emerges, parasitoid adults are then free-living and disassociated from their larval host. Parasitoids can be solitary if the

female oviposits one egg per host or gregarious if the female oviposits multiple eggs per host (Godfray 1994). Depending on their life cycle, parasitoids can be endo- or ectoparasitoids and can attack their hosts at different life stages, varying from ovipositing into eggs to ovipositing into the last instar or even into pupae or adult insects. Endoparasitoids hatch inside the host from eggs oviposited by an adult female and continue their development within the parasitized host, whereas ectoparasitoids develop externally on the host while consuming bodily fluids through the host cuticle. The host is the only source of nutrition for the parasitoids during their immature stages. Because koinobiont parasitoids allow their hosts to continue developing whereas 'idiobionts' paralyze their host, curtailing further development, some species are able to attack one life stage (e.g., the host larva) but emerge from another life stage (e.g., the pupa). The advantage to the koinobiont lifestyle is that by allowing their host to continue developing, the parasitoid can ultimately gain access to greater food resources, since older hosts typically have greater biomass.

Intrinsic constraints on clutch size in parasitoids

Parasitoid reproductive strategies, particularly the distribution of eggs among hosts in time and space, are thought to be under strong selection due a variety of intrinsic factors governed primarily by parasitoid physiology and behavior. Prior to oviposition, the major constraints that affect clutch size decisions are costs of reproduction, the female's physiology, and host quality. The *marginal value theorem* predicts that the optimal clutch size is greatly affected by host searching time and the handling time required to prepare the host for oviposition, including subduing a host (often involving injection of paralytic venom) and piercing through a tough cuticle during oviposition

(Charnov & Skinner 1984; Skinner 1985). The vast majority of parasitoid species are koinobiont and inject venom into their host to temporarily paralyze them, minimizing host handling time. When the host encounter rate is low and/or the time to prepare a host for oviposition is high, larger broods are predicted, which secondarily results in greater intraspecific competition (Charnov & Skinner 1984; Skinner 1985; found to be supported in *Cotesia glomerata* in Ikawa & Okabe 1985). Host scarcity can also encourage female parasitoids to return to a previous host and oviposit additional eggs – known as 'self-superparasitism'. In Japanese populations of *Cotesia glomerata,* a parasitoid of *Pieris rapae*, it was observed that when presented with a series of hosts, the female oviposited the largest clutch in the first caterpillar and smaller broods in the successive hosts (Ikawa & Suzuki 1982). The authors demonstrated that the parasitoid exhibits an adaptive response by reducing clutch size to avoid egg exhaustion in the presence of abundant hosts. Additionally, ovipositing high numbers of offspring can be costly in time that could be better used to search for new, high-quality hosts so it may not be favorable for the parent to produce the Lack clutch size. The fitness gain per egg added to the brood can vary between parasitoid species but typically a female has the greatest fitness gain when ovipositing the first egg with successively smaller gains for each additional egg approaching Lack clutch size (Godfray 1994).

In addition to the costs of reproduction, producing large clutches can also waste eggs that could provide a higher fitness gain if oviposited in smaller clutches on other hosts. When female parasitoids are not limited by time but instead by their finite egg supply, the parasitoid will be selected to use their eggs to produce new clutches on hosts where her offspring will achieve greater fitness returns, when there is a short supply of

eggs. Constrained optimization or dynamic programming models hypothesize that female parasitoids should modify their brood sizes according to current egg reserves, specifically reducing clutch size when egg supplies are low (Mangel 1987; Mangel & Clark 1988). Whether this is true will depend upon the parasitoid species' life history. Proovigenic parasitoids, adults emerging with a set complement of mature eggs, should see clutch size decrease as the parasitoid ages if there is no age-specific mortality (Heimpel & Rosenheim 1998). However, because the risk of mortality often increases with a female parasitoid's age, older parasitoids might produce larger broods to minimize the risk of dying with unlaid eggs. Charnov and Skinner focused on synovigenic parasitoids, adults that continue to develop eggs throughout their lifetime, and found that, if the host encounter rate is fixed, synovigenic parasitoids should always deposit the mature eggs currently available into a host and search for a new host afterward (1988). Another aspect of the female parasitoid's physiology to take into account is her ability to lay a precise amount of eggs. In gregarious parasitoids who quickly lay a high number of eggs in a given host, this may not be possible. If the parasitoid is penalized more for overshooting the Lack clutch size than for undershooting, smaller clutches are selected for; if the consequences are more dire when undershooting the Lack clutch size, larger clutches are selected for (Godfray 1994).

In addition to female physiology and sibling competition, parasitoid clutch size has been shown to depend greatly on host quality. A strong correlation exists for females laying larger broods on bigger hosts, whether those of later instars of a single host species or a different species with a larger body compared to the rest of the available species hosts (Godfray 1987; Zaviezo 2000; Hardy et al. 1992; Le Masurier 1987). Research

suggests that parasitoid clutch size decisions are generally made during the initial external examination of a host prior to piercing with its ovipositor, which can be done by the female parasitoid walking on the host while continuously drumming with her antennae (Klomp and Teerink 1962). Idiobiont parasitoids arrest their host development so the total resources available to their offspring is known by the female at the moment they oviposit into their host. Koinobiont parasitoids, on the other hand, oviposit into their host without completely knowing the amount of resources available to their offspring as their offspring grow with the host. Higher risks and trade-offs are to be accounted for by koinobiont parasitoids. In parasitoid species who target arthropod eggs, oviposition preference will be given to younger eggs as older eggs provide fewer resources for parasitoid larval development and generally support a smaller clutch size (Pak 1986). Host quality can also impact egg maturation time. Better quality hosts can provide better nutrition to the larvae and allow a quicker development, which minimizes the probability of the host dying prior to parasitoid maturation. With all the aforementioned clutch size constraints that can work in combination or individually, observed parasitoid clutch sizes are commonly recorded as being below the estimated Lack clutch size for that species (Charnov & Skinner 1984; Waage & Ng 1984; Takagi 1985; Dijkstra 1986; Hardy et al. 1992; Vet et al. 1994). One explanation for this phenomenon is that selection is biased toward maximizing the female's reproductive success rather than offspring fitness due to the aforementioned costs of reproduction (Charnov & Skinner 1984; Godfray 1994).

After oviposition, offspring can still modify the 'realized clutch size' through intraspecific competition. In smaller broods, larval competition within a single clutch is reduced as there are more resources available for each individual and less advantages to

being competitive. Therefore, smaller clutches can be selected for since it increases offspring fitness even though it reduces offspring number. The trade-off between total offspring fitness and the number of larvae in a host is referred to as the "larval competition curve" (Credland et al. 1986; Wilson 1994) or the "number fitness relationship" (Skinner 1985). Reduced sibling competition can be observed in broods of genetically identical individuals which result from polyembryonic parasitoids (several offspring can develop from a single egg) or from parasitoids that lay single-sex broods (Godfray 1994). Clutch size of polyembryonic species are affected by both the number of eggs oviposited by the female and the number of asexual divisions by oviposited eggs.

Extrinsic constraints on clutch size in parasitoids

Parasitoids employ their host not only as a source of nutrition but also as a means for survival. Because the parasitoid larvae depend entirely on their host for development, the immature parasitoid(s) will die along with the host if the host dies before the parasitoid completes its development. Parasitized hosts, along with their parasitoid larvae, are frequently attacked by arthropods and other predators, other heterospecific primary parasitoids, or hyperparasitoids (that lay eggs in the developing parasitoids). Collectively, the risk of attack by these natural enemies can also be an important selective force on parasitoid oviposition behavior. Primary hyperparasitoids oviposit into primary parasitoids located inside a parasitized host (Sullivan & Völkl 1999). Secondary hyperparasitoids attack the exposed, later stages of parasitoids such as the prepupal and pupal stages found in cocoons (Harvey 2008a). Thus, manipulation of host biology and ecology, prior to or even following parasitoid larval emergence, to reduce mortality from

predators and hyperparasitoids could be highly adaptive for parasitoids, and may present a strong selection force (Fritz 1982).

Pre-emergence host manipulation by parasitoids is thought to be largely hormonal. Prior to emerging, parasitoids can use hormonal levels to their advantage in two ways, by being physiological conformers or physiological regulators (Lawrence 1986). Physiological conformers are parasitoids that adapt their own hormones to synchronize with the host's development and utilize the host's hormones as stimuli to molt. On the other hand, physiological regulators actively manipulate their host's endocrine system or secrete hormones themselves. For example, some parasitoids attacking adult female arthropods disrupt host hormones involved in the production and accumulation of yolk (vitellogenesis), effectively halting oviposition to use the nutrients intended for egg production for parasitoid larvae development instead (Beckage 1985). Parasitoids attacking hosts at the larval stage often use hormones to prolong the host's larval feeding stage, increase the host's feeding rate or redirect the uptake of nutrients from host hemolymph to obtain more resources for the parasitoid larvae (Beckage and Riddiford 1982; Vinson & lwantsch 1980a, 1980b). This can be done by interfering with the host's juvenile hormone (JH, that maintains juvenile characters) and ecdysone (the hormone promoting molting) or by interfering with their metabolism. Prolongation of host development was seen more often in large clutches of *Cotesia congregata* than small clutches (Beckage and Riddiford 1983). Large clutches of parasitoids require more nutrients so delaying molting for parasitized larvae would be evolutionarily advantageous, especially for gregarious parasitoids. However, redirecting host development heightens the risk of predation or hyperparasitism especially if the host is

larger in size, thus more visually obvious, and it increases parasitoid generation time, negatively affecting rapidly growing parasitoid populations. When starved or developing on low quality hosts (Abarca et al. 2020), unparasitized larvae can go through supernumerary instars so parasitized hosts that remain in the feeding stage longer can also just be attributed to trauma from parasitoid attack (Godfray 1994). Another strategy employed by parasitoids of gregratious hosts (e.g., caterpillars that feed together in groups) is to delay or initiate host pupation and manipulate the host to wander away - a behavior associated with lepidopteran pupation - from their colony to avoid being an easy target for hyperparasitoids that might forage in a density-dependent manner (Stamp 1981; Brodeur & McNeil 1989). Host maturation can likewise be accelerated and is more common with solitary endoparasitoids. When only one egg is oviposited, the parasitized larva contains an excess of resources for parasitoid development which increases the risk of infection for the exposed parasitoid pupae by the putrefaction of excess host tissue (Godfray 1994). Accelerated host maturation prevents the host from reaching full body size and decreases generation time and predation risk for parasitoids.

For endoparasitoid larvae that manage to survive within-host predation and attack from hyperparasitoids, they must ultimately pierce through the host's cuticle to emerge from their host and pupate. Parasitoid larva egression typically results in the death of insect hosts. However, a variety of species of parasitoids (mostly in the hyperdiverse family Braconidae) emerge without killing their host, and proceed to pupate on, beside or under it (Harvey & Strand 2002; Grosman et al. 2008; Harvey et al. 2008a, b). This phenomenon is still poorly understood and requires the parasitoids to forgo feeding on essential host tissues in order to allow the moribund host to persist post-emergence. This

strategy of leaving unconsumed tissues behind in order to permit their moribund host to remain animated as a post-emergence 'zombie' presumably imposes a nutritional constraint on parasitoids that is likely to impact clutch size decisions. The amount of time the host is able to live after parasitoid larval emergence varies among species. In the case of the gregarious braconid, *Apanteles kariyai*, its caterpillar host is manipulated to travel to open areas and dies shortly after crawling away from the newly formed parasitoid pupae (Sato et al. 1983). The parasitoid can induce this behavior so as to prevent the host from dying nearby and putrefying (increasing the risk of disease for pupae) or to minimize attracting predators and hyperparasitoids to the vulnerable post-emergence parasitoid larvae and pupae. Ectoparasitoids can also influence their host to modify their habitat structures, such as spider webs with additional layers of silk, to make them more resistant to destruction and protect parasitoid cocoons (Matsumoto 2009, Gonzaga 2010).

Host bodyguards

A subset of post-emergence caterpillar hosts are termed "bodyguards" because they exhibit behaviors that appear to provide a protective function to the parasitoids, including acting aggressively when disturbed (e.g., thrashing violently and/or regurgitating) and laying down silk on top of emergent parasitoid cocoons (Tanaka & Ohsaki 2006, 2009; Grosman et al. 2008; Harvey et al. 2008a, b; Janssen et al. 2010). There are two non-mutually exclusive hypotheses that have been put forth to explain how this bodyguarding behavior has evolved: 1) the bodyguard does in fact protect post-emergence parasitoid larvae and/or pupae against natural enemies; and 2) the animated bodyguard provides an alternate food item or general distraction to generalist

predators, enhancing survival of the parasitoids. These hypotheses differ in which enemies are likely to be most important. The "usurpation hypothesis," for which evidence has only been found in a few studies, suggests that the parasitoids are actively manipulating the dying host as defense against their own natural enemies (Harvey et al. 2011). The protection provided by the bodyguard's aggressive behavior will be more important if hyperparasitism is the dominant threat to the primary parasitoid. The second hypothesis is that bodyguards can also serve as an alternative meal for potential generalist predators that preferentially consume the bodyguard over the parasitoid larvae/pupae (Harvey et al. 2013). In one of the few tests of the effectiveness of caterpillar bodyguards, researchers found that in a greenhouse study, the rate of hyperparasitism of a primary parasitoid that uses cabbage white (*Pieris brassicae*) caterpillars as bodyguards did not differ from those that did not have a bodyguard present (i.e., the bodyguard did not appear to provide a protective function) (Harvey et al. 2008b).

While the ultimate selective pressures governing the evolution of bodyguarding as an 'extended phenotype' (Dawkins 1982) are still under considerable debate, there has been very little empirical work examining how adding this additional layer to an already complex host-parasitoid interaction might influence parasitoid clutch size decisions. In particular, to our knowledge, there are no studies to-date examining the relationship between parasitoid clutch size and the bodyguarding phenotype. In this study, I collected parasitized larvae of a common pierid butterfly (*Pieris rapae*) from a local organic farm and reared them in the lab to address the three main questions: 1) What is the distribution of parasitoid clutch sizes within caterpillar hosts?; 2) does the likelihood of parasitoid

development and survival to adult emergence vary with clutch size?; and 3) How does parasitoid clutch size influence the lifespan of *P. rapae* bodyguard caterpillars?

METHODS

Study System

The bodyguard caterpillar, *Pieris rapae*

The cosmopolitan cabbage white butterfly, *Pieris rapae* (Lepidoptera: Pieridae), is a specialist of cruciferous crops (*Brassicaceae*). Host plants can include all vegetables of the cabbage and mustard families such as broccoli, collards, kohlrabi, among others. The butterfly's life history is solitary, not gregarious like its British counterpart, *Pieris brassicae*. *P. rapae* can have multiple generations per year, ranging from 2 to 8 depending on the region and its weather, and undergo 5 instars throughout its larval development (Capinera 2014). During the year, overwintered pupae will usually emerge at the end of May as adults, and reach an egg-laying peak in mid July (Dempster 1967). During its brief adult lifespan, *P. rapae* can lay from 300 to 400 eggs, which are oviposited singly on the underside of host leaves. The first and second larval instars feed on the leaf they hatch on. Afterwards, the larva moves along the plant to feed on other leafy foliage until maturation (Dempster 1967). Immature *P. rapae* develop for approximately 15 days before emerging as adult butterflies and complete metamorphosis can range from 11 days to a few weeks. In seasonal environments, *P. rapae* enter diapause and overwinter as pupae in response to shortening day lengths and declining temperatures. Overwintering will occur for the larvae that pupate late in the year and the cycle repeats.

Pieris rapae is native to Europe, Asia and North Africa, with the divergence of subspecies in Europe and Asia coinciding with the diversification of brassicaceous crops and progress of human trade routes (~1200 years B. P.) (Ryan et al. 2019). It was introduced into Montreal in the 1860s and then spread to the rest of North America (Milne & Milne 1995). The cabbage white butterfly is currently one of the most abundant and successful invasive species, currently found in every continent except Antarctica and South America, posing a significant problem as a pest for agriculture (Ryan et al. 2019).

A study in Abbots Ripton, Huntingdonshire, Enlgand found that half of *P. rapae's* mortalities occurred during the first 2 instars due to predation by arthropods such as ground beetles and harvestmen (Dempster 1967). Birds were the main predators in the later 2 instars, consuming about 20% of the larvae with a preference for the larger caterpillars. The fungi and viruses present in the study had an insignificant effect on the overall mortality of the caterpillars. However, a study conducted in Merivale, Ontario observed that the main threat to later instars of *P. rapae* was the granulosis virus, killing 68% of the caterpillars on average (Harcourt 1966). Similarly, the parasitoid species present did not pose a significant threat to the larvae overall, but one species managed to kill roughly 20% of the fourth instar. *P. rapae* is parasitized at the first or second instar by its primary parasitoid, *Cotesia glomerata,* but relatively little research has been conducted on this host-parasitoid interaction, whereas extensive research has been conducted on its the similar species found in European congener, *Pieris brassicae*, and its bodyguarding behavior with parasitoid pupae. It is clear that different arthropod predators are important at distinct life stages of the caterpillar.

We have observed that local mid-Atlantic populations of *P. rapae* caterpillars often exhibit bodyguarding protective behaviors, such as thrashing (a behavior only observed when parasitized) and regurgitation, putatively to fend off parasitoid predators and/or hyperparasitoids while the parasitoids are in their pupal stage, similar to the behavior documented in *Pieris brassicae*. Populations of *P. rapae* in the western U.S. do not demonstrate this behavior when parasitized and die immediately after parasitoid emergence (P. Ode, personal communication) so there seems to be an interesting polymorphism in behavior that needs to be studied further. To our knowledge, our lab is the first to document this behavior in *P. rapae* and *C. glomerata* interactions (European *P. rapae* are not known to exhibit bodyguarding behaviors either). The host caterpillar feeds as the parasitoid larvae develop within its body, but after parasitoid larvae egression, the caterpillar bodyguard ceases to eat altogether. Immediately after parasitoid emergence from the host, parasitoid larvae start to spin their cocoon near the bodyguard caterpillar, which sometimes sits on top of the parasitoid cocoons and/or lays down a layer of silk over the parasitoids (personal observation).

Specialist primary parasitoid, *Cotesia glomerata*

The gregarious koinobiont endoparasitoid, *Cotesia glomerata* (Hymenoptera: Braconidae), specializes on pierid butterflies. As mentioned above, *C. glomerata* attacks the first or second larval instar of its host, ovipositing 20 to 30 eggs per host and does not provide parental care (Le Masurier 1991). The larvae egress from their host to pupate approximately 15 to 20 days after oviposition and spin individual cocoons, usually in clusters (**Figure 7**). After pupation, it then takes the parasitoid 7 to 10 days to become an adult and mating occurs immediately after adult emergence (Le Masurier 1991). Adults

feed on nectar and can lay multiple clutches throughout their lifetime. The entire life cycle duration typically ranges from 22 to 30 days and can vary with temperature. Information about the parasitoid's ovigeny is not currently available.

C. glomerata is also native to Europe, where it mostly parasitizes the large white butterfly, *Pieris brassicae* (Lepidoptera: Pieridae). In 1883, the wasp was introduced into the United States as potential biocontrol for populations of *Pieris rapae* (Swan 1964). A study collecting *C. glomerata* pupae from the field in the Netherlands, found 70% of the cocoons to be parasitized by one of its two main hyperparasitoids, *Acrolyta nens* and *Lysibia nana*, both of which are in the family Ichneumonidae (Harvey 2009). *C. glomerata* is mostly hyperparasitized in its larval and pupal stages.

Throughout the summer of 2021, 360 caterpillars of the cabbage white butterfly (*Pieris rapae*) were collected biweekly at different life stages from a local organic farm (Potomac Vegetable Farms in Vienna, VA, USA.) feeding on various *Brassica oleracea* host plant varieties: green cabbage (*Brassica oleracea* var. "Capitata"), curly kale (*Brassica oleracea* var. "Sabellica"), lacinato kale (*Brassica oleracea* var. "Palmifolia") and sprouting broccoli (*Brassica oleracea* var. "Italica"). The collected *P. rapae* caterpillars were brought to the laboratory to be reared individually in separate deli containers and each caterpillar's collection date was recorded. We placed filter paper at the bottom of each container, moistening it with water as needed, to prevent caterpillar desiccation and maintain host plant quality. *P. rapae* caterpillars were fed the host plant variety they were collected from until parasitoid larval emergence of gregarious braconid wasp parasitoid, *Cotesia glomerata* (Hymenoptera: Braconidae), or butterfly emergence (if the collected caterpillar was not parasitized). Caterpillars were fed and their containers

cleaned on a daily basis. Once parasitoid larval emergence occurred, we ceased the feeding of parasitized *P. rapae* caterpillars as they no longer eat and kept the deli container closed to allow for the full development of the parasitoids.

Laboratory Methods

Each caterpillar was placed on open shelves inside the laboratory as the visibility allowed for continuity of observation and recording of significant events. If the caterpillar was not parasitized, the pre-pupation, pupation, and butterfly emergence dates were recorded. For parasitized caterpillars, we recorded the dates of parasitoid larval emergence, bodyguard caterpillar death, and parasitoid adult emergence. The bodyguard was determined to be dead once the caterpillar no longer moved in response to stimulus mimicking predators, i.e. a brief stab with a paintbrush.

After parasitoid adults died, we counted all the parasitoid larvae, pupae and adults that emerged from parasitized caterpillars as measures of 'parasitoid load' (our proxy for clutch size, presuming a strong positive relationship between eggs deposited and the number of surviving larvae that emerge from the host). The number of parasitoid larvae was counted as the number of parasitoid cocoons plus the number of larvae that died prior to spinning a cocoon. The number of parasitoid pupae was determined by counting the cocoons, which are easily discernible. The number of parasitoid adults was determined by the number of parasitoid adults that had successfully emerged from their cocoons. If the parasitoid adult had completed development inside of the cocoon but had died trying to emerge from the cocoon, it was only counted as part of the parasitoid pupae data. A few clutches (N = 6) of post-emergence parasitoids were collected from the field (without attendant bodyguards) and reared; emergence dates and counts of larvae, pupae,

and adults from these collections were included in the clutch size-parasitoid demography data set.

DATA ANALYSIS

A histogram was created to visualize the distribution of bodyguard survival with data from 140 bodyguards. Linear regression models were used to examine the effect of the parasitoid load (number of emerging parasitoid larvae, parasitoid pupae and parasitoid adults) on the lifespan of 140 parasitized bodyguard caterpillars. The number of parasitoid larvae was recorded for a total of 133 bodyguards. The number of parasitoid pupae and the number of parasitoid adults was recorded for 139 parasitized *P. rapae* caterpillars, an amount which exceeds the information we collected for parasitoid larvae as 6 *C. glomerata* pupal masses were collected from the field. The data from these 6 pupal masses was only used in analyses of transition probabilities for demographic data of parasitoid pupae and parasitoid adults since these parasitoid clutches were collected without their bodyguards. Linear regressions were also used to relate the number of parasitoid adults to the number of parasitoid larvae and to the number of parasitoid pupae. To explore how the transition success from larvae to pupae (pupal transition success = no. of total pupae/emerging larvae) or from pupae to adult (adult transition success = number of adult parasitoids/number of pupae) varied with clutch size, regressions were also employed. The proportions were calculated under the assumption that the number of parasitoids from the previous life stage was larger or equal to the number of parasitoids of the next life stage. All of the above data analysis met normality assumptions except for the comparison between proportion of transition success from

larvae to pupae and larvae clutch size; an arcsine square root of pupal transition success transformation was made to meet normality.

RESULTS

From the 360 cabbage white butterfly caterpillars that were collected from the field, 44.44% were parasitized with *Cotesia glomerata*. The lifespan of bodyguards after parasitoid larval emergence varied from 0—12 days with a mean of 3 ± 2.1 (SEM) days (**Figure 8**). The number of parasitoid larvae per bodyguard varied from 2—71 with a mean of 25.6 ± 12.2 (SEM). The number of parasitoid pupae per bodyguard ranged from 2—68 with a mean of 25.3 ± 11.8 (SEM). The number of parasitoid adults that successfully emerged ranged from 0—57 adults with a mean of 19.8 ± 10.4 (SEM) adults.

When regressing bodyguard lifespan on parasitoid load, we found a significant negative relationship with the number of parasitoid larvae ($F_{1, 113} = 7.25$, $p = 0.0082$, adjusted $R^2 = 0.05$, **Figure 9**) and a significant negative relationship with the number of parasitoid pupae ($F_{1, 117} = 7.20$, $p = 0.0084$, adjusted $R^2 = 0.05$, **Figure 10**). When comparing the days of bodyguard survival to the number of parasitoid adults, no linear relationship was observed ($F_{1, 118} = 0.14$, $p = 0.71$, adjusted $R^2 = 0.007$, **Figure 11**). A significant positive relationship was found between the number of parasitoid adults and the number of parasitoid larvae ($F_{1, 131} = 61.04$, $p<0.0001$, adjusted $R^2 = 0.31$, **Figure 12**), as well as when comparing the number of parasitoid adults and the number of parasitoid pupae ($F_{1, 136} = 77.38$, $p <0.001$, adjusted $R^2 = 0.36$, **Figure 13**).

The transition success from parasitoid larvae to pupae varied among clutches from 0.5 to 1.0 with a mean of 0.98 ± 0.05 (SEM). After arcsine transformation, the

transition success from larvae to pupae showed a strong negative relationship with larvae clutch size ($F_{1, 131} = 5.67$, $p = 0.02$, adjusted $R^2 = 0.03$, **Figure 14**). The transition success from parasitoid pupae to adults varied from 0 to 1.0 with a mean of 0.82 ± 0.24 (SEM). A significant negative relationship was also found when comparing the number of parasitoid pupae to the adult transition success ($F_{1, 135} = 11.72$, $p = 0.0008$, adjusted $R^2 = 0.07$, **Figure 15**).

DISCUSSION

The mode for bodyguard lifespan was a single day, meaning that the majority of bodyguards died relatively quickly, within a day or so after parasitoid larval emergence (**Figure 8**). However, the fact that most bodyguards survived for at least 24 hours and did not die immediately after parasitoid emergence, is of importance as it is a novel behavior recorded for the *P. rapae-C. glomerata* interaction. On average, bodyguard caterpillars survived for 3 days after parasitoid emergence, despite not feeding during this time. No bodyguards survived more than 8 days except one caterpillar, which survived for a remarkable 12 days after parasitoid larval emergence. It is important to note that due to this research being conducted in the laboratory (a natural enemy-free space), the bodyguard lifespans we observed are likely upper estimates for what might occur in more natural situations where environmental stressors (i.e., desiccation) and natural enemies are likely to reduce them.

According to the usurpation hypothesis, the longer the bodyguard remains alive, the longer they can defend parasitoid pupae from predators. My initial hypothesis was that bodyguard survival would decrease with parasitoid clutch size. This prediction was supported by our rearing data. Although the variance was high, we detected a strong

negative relationship between days of bodyguard survival and both the number of parasitoid larvae and the number of parasitoid pupae (**Figure 9** and **Figure 10**, respectively) per host. This indicates that bodyguards survived for fewer days when they had high parasitoid loads, and conversely smaller clutches of parasitoids tended to have greater bodyguard survival. When relating bodyguard lifespan to all 3 measures of parasitoid load, it was found that a parasitoid load of roughly 20 parasitoids per host is the optimal clutch size and the point of diminishing returns as at a parasitoid load of 20 parasitoids, the bodyguard is able to survive a maximum of 8 days but clutch sizes larger than 20 see a decrease in days of bodyguard survival. The optimal clutch size of 20 found in this study is similar to the Lack clutch size of 23 reported previously for a British strain of *Cotesia glomerata* on *Pieris rapae*, which normally parasitizes *Pieris brassicae* (Le Masurier 1991). Adult parasitoid load does not have a strong influence on how long the bodyguard remains alive (**Figure 11**); instead larval and pupal clutch size are the better determinants of bodyguard survival. This is not surprising, since host feeding occurs in the larval stage of *C. glomerata*, which is what is most likely to impact post-emergence host survival. Overall, if bodyguards are important for parasitoid fitness, selection should favor parasitoids that lay smaller clutches per caterpillar host to ensure greater survival. A possible explanation for the significant negative relationship observed between days of bodyguard survival and parasitoid larvae and pupae loads is that the threshold at which the caterpillar is able to survive and support the largest clutch size is surpassed as a larger portion of the caterpillar is eaten the larger the parasitoid clutch is. If the parasitoid larvae eat too much of the caterpillar, resulting in a high degree of catastrophic damage to the caterpillar, then the bodyguard is more likely to die quicker.

As expected, a strong positive relationship is seen when comparing the number of parasitoid adults to the number of parasitoid larvae and the number of parasitoid pupae (**Figure 12** and **Figure 13**, respectively). These trends are observed as the number of individuals from the previous life stage sets an upper bound on the number of successful individuals in the next stage. We compared both early life stages to the number of parasitoid adults because that is the ultimate measure of realized offspring fitness. On average, the higher the number of parasitoid larvae and pupae, the higher the number of adults will be. However, there were fewer instances under which parasitoid larvae and pupae clutch sizes exceeded 40. On multiple instances where the number of larvae exceeded 40, an average of only about a fourth or less of those larvae successfully developed into adults (**Figure 13**). In these situations, sibling competition might lead to decreased development success, and the female parasitoid should be under selection to reduce clutch sizes.

Parasitoid pupal transition success had a slight negative relationship with the parasitoid load, since almost all of the larvae successfully develop into pupae (~98%) (**Figure 14**), but at very high clutch sizes, a small proportion of the larvae fail to pupate. By contrast, clutch size had a stronger influence on the pupa-adult transition, wherein adult parasitoids failed to emerge from a large proportion of pupae in largest clutch sizes (**Figure 15**). Presumably, in large clutches, intraspecific competition reduces resource availability to larvae, forcing them to pupate at smaller sizes that can be below the threshold for successful adult development. Additionally, in nature, the transition success from life stage to life stage may be even lower due to attack by hyperparasitoids, although the bodyguarding behaviors may serve as an effective deterrent.

To our surprise, we observed a few cases in which very small clutch sizes also had low adult emergence. Theoretically, females choosing to lay smaller clutches should see a high transition success rate and high survival of each life stage as there is low sibling competition and more resources available for each individual. Speculations as to why we may have observed these cases are that the host immune response was able to overcome the parasitism and a minimum threshold clutch size needs to be oviposited to overcome the host immune response or the parasitoid female may have been toward the end of her life so the remaining oviposited eggs were of lower quality. The remaining older eggs could have lower yolk provisioning, having enough to perhaps develop to the pupal stage but not quite enough yolk to undergo full metamorphosis. Further research is needed to determine the factors influencing the low life stage transition success of small clutches.

To aid in assessing the relevant push and pull factors involved in parasitoid clutch size decisions, I developed a heuristic model (**Figure 16**). In this model, when a female parasitoid is constrained by natural enemies in which the host is more likely to be eaten by generalist predators, a low clutch size is selected for as the female needs to spread her eggs on more hosts to minimize enemy mortality. At large clutch sizes, parasitoids are mainly limited by intraspecific larval competition. High clutch sizes can also be more attractive as a resource to natural enemies and hyperparasitoids. Any hyperparasitoids that forage in a density-dependent manner would be more likely to be attracted to and remain in patches with higher pupal clutch size. Both extremes on the clutch size spectrum have important constraints so there is likely an intermediate clutch size that is a balance of both constraints, an optimal clutch size, that can result in high larvae to adult

survival. However, because ecological factors like enemy pressure tend to exhibit strong spatial and temporal variation, the 'optimal clutch size' can be a moving target, which is perhaps why we observed such large variation in clutch size in *C. glomerata*.

This model can apply generally to parasitoids without bodyguard hosts but what is unique about parasitoids with bodyguards, like *Cotesia glomerata,* is that the parasitoid reduces clutch size to access bodyguarding services. In situations with low enemy pressure, I would predict larger clutch sizes and shorter bodyguarding lifespans, with the converse occurring in high enemy pressure environments. Natural selection will favor the largest clutches that are successful and there are several advantages of host manipulation in this system. Parasitoids are at their most vulnerable and exposed during the time they egress from the host as larvae to their pupation, so the bodyguard may only need to survive for the duration of this process, a possible reason for why we saw a mode of a single day of bodyguard survival; if the bodyguard is not able to survive, then it won't be effective. *C. glomerata* pupate within a few hours of emergence and, on average, the bodyguards survived three days (at least under laboratory conditions), which is plenty of time. The adults emerge after approximately a week after pupation so the bodyguard does not typically survive until adult parasitoid emergence, but the bodyguard does protect the most vulnerable (particularly to secondary hyperparasitoids) and exposed larval and early pupal stages of the parasitoid. New pupae may be more vulnerable than old pupae because the time can allow for the silk to harden, which could be a possible reason for why we saw an average bodyguard survival of three days. More data on what the mortality factors are for this system can provide answers as to what the real reason(s) behind the bodyguarding phenotype.

The results of this study add an additional trade-off to those we already know are present in parasitoid reproductive strategy decisions. Local *C. glomerata* populations might have evolved a bodyguarding phenotype because it has been documented to sustain up to 70% hyperparasitism in some regions. This suggests that natural enemies are a stronger selecting force as the parasitoids trade a slightly smaller clutch size for more protection from hyperparasitoids. Thus, determining who the natural enemies are (for the primary parasitoid) and how they respond to parasitoid clutch size in the field is the next step in developing our understanding of this novel phenotype.

SIGNIFICANCE

Caterpillar pests can cause severe damage to agricultural crops, resulting in economic losses. This research is significant in agriculture especially because parasitoids are a common biocontrol method for caterpillar pests on organic farms. The longer the bodyguard caterpillar remains alive and can fend off natural enemies and hyperparasitoids, the higher the chances of parasitoid survival and the higher the parasitoid abundance will be, allowing for more successful biocontrol of caterpillar pests. Understanding the interactions between bodyguards and parasitoids will allow for further research into the effectiveness of parasitoids as pest control agents. Even though *Cotesia glomerata* does not kill the *Pieris rapae* caterpillar until its fifth and last instar when the caterpillar has already consumed crops to nearly complete development, the parasitoid can decrease the size of or prevent future generations of the cabbage white butterfly from proliferating, preventing future damage to cruciferous crops. If natural selection is assumed to maximize fitness, then we should see *Cotesia glomerata* clutch sizes averaging 20 parasitoids.

Hopefully, this natural method of reducing caterpillar pest populations will encourage the emergence of more effective organic farming and reduce the use of chemicals, such as pesticides and herbicides, used on other farms that have harmful effects on the environment and human health.

CHAPTER 2 FIGURES

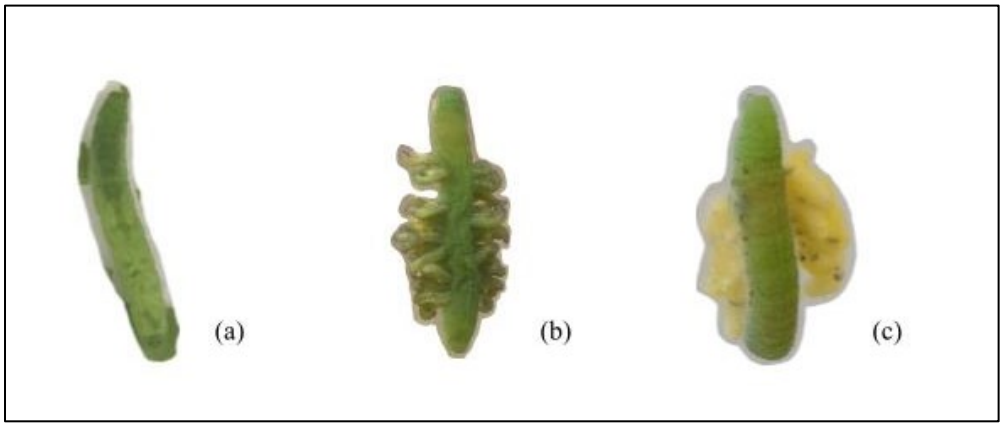

Figure 7. Development of parasitoid *C. glomerata* inside of bodyguard host *P. rapae.* (a) Parasitoid larvae developing inside of the caterpillar host. (b) Parasitoid larvae puncturing through the caterpillar's cuticle. (c) Parasitoid pupae on either side of the caterpillar host.

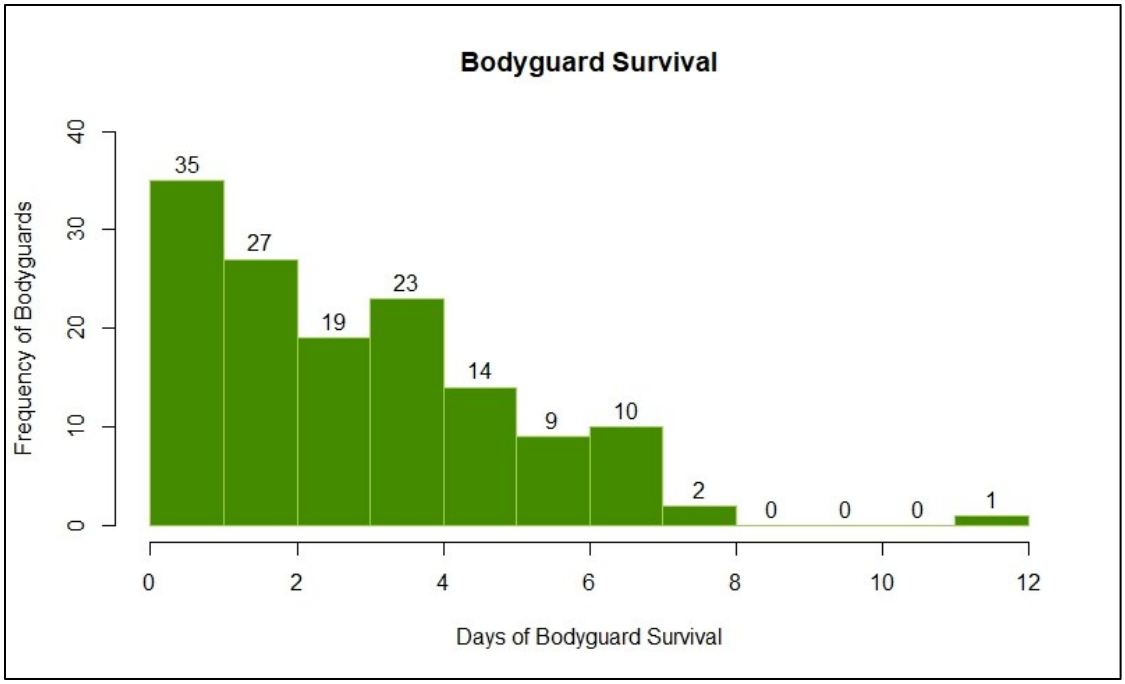

Figure 8. The lifespan of parasitized *P. rapae* was highly variable, ranging from 0-12 days (mean = 3 ± 2.1 (SEM) days). Exact bodyguard frequencies per day of survival are labeled on top of each bar.

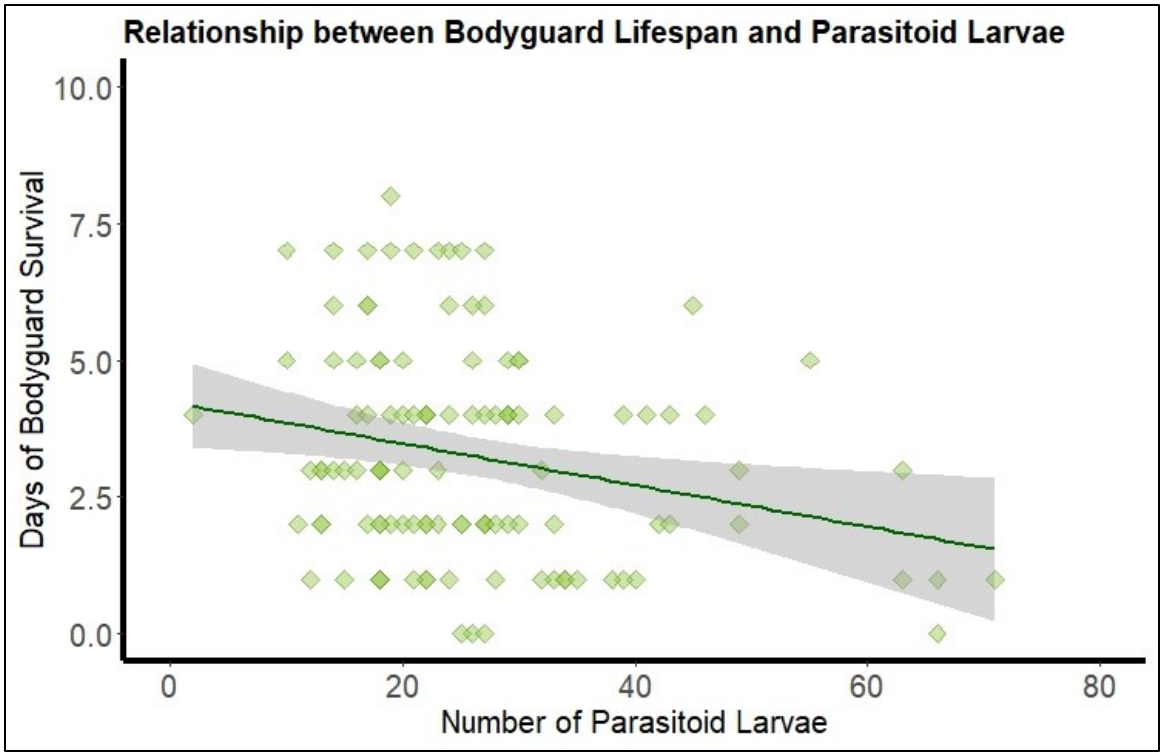

Figure 9. Bodyguards survived for less time when they had high parasitoid larvae loads (mean = 25.56 ± 12.2 (SEM) parasitoid larvae, maximum = 71 parasitoid larvae).

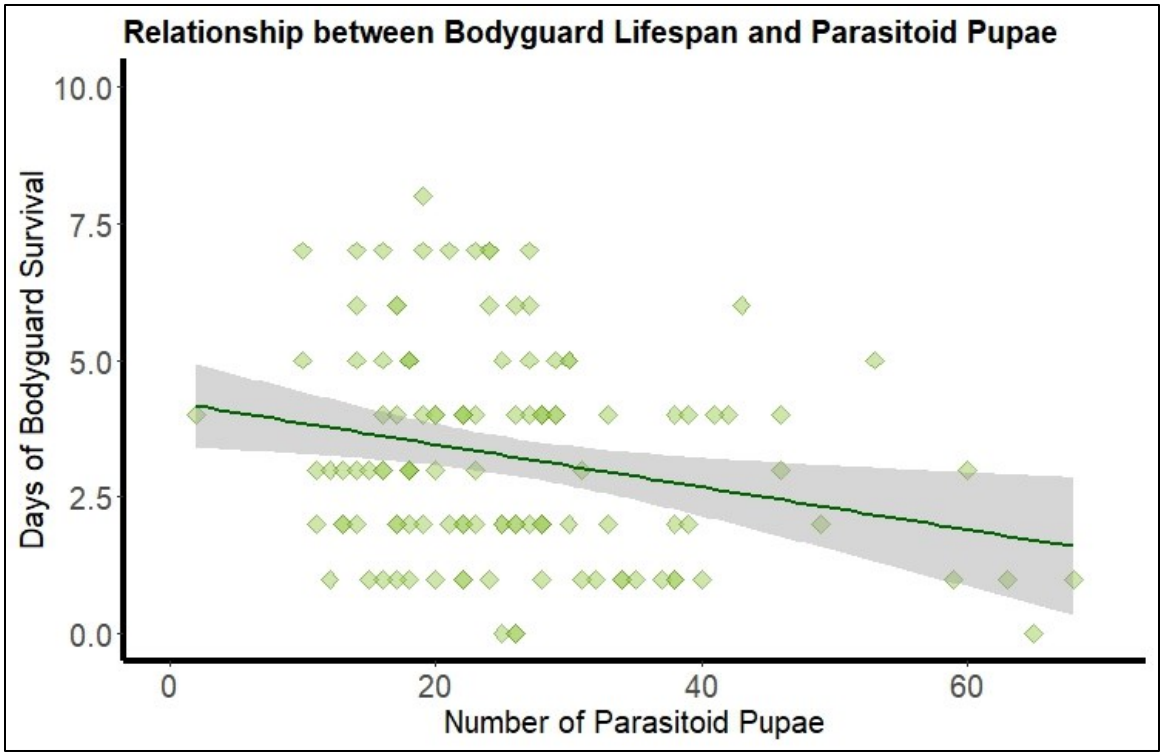

Figure 10. Bodyguards survived for less time when they had high parasitoid pupae loads (mean = 25.28 ± 11.8 (SEM) parasitoid pupae, maximum = 68 parasitoid pupae).

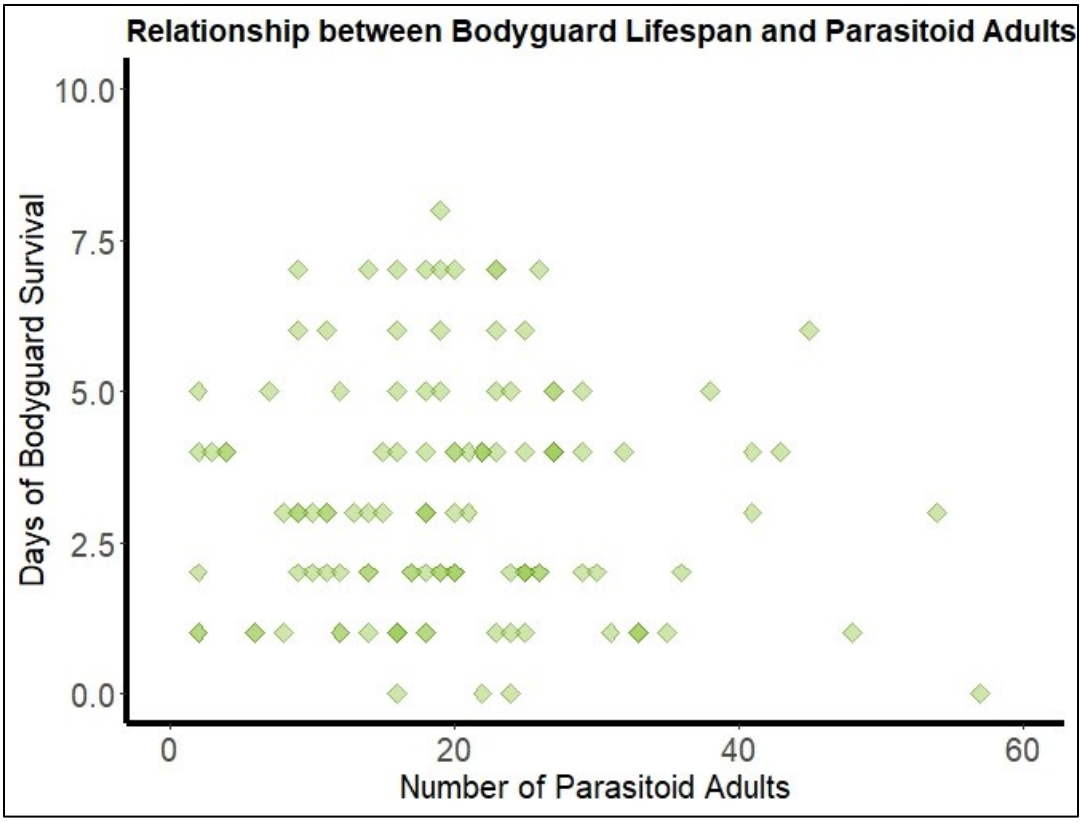

Figure 11. No clear correlation was observed between days of bodyguard survival and the number of parasitoid adults. Bodyguards survived the most amount of time when there was an emergence of roughly 20 parasitoid adults (mean = 19.76 ± 10.4 (SEM) parasitoid adults, maximum = 57 parasitoid adults).

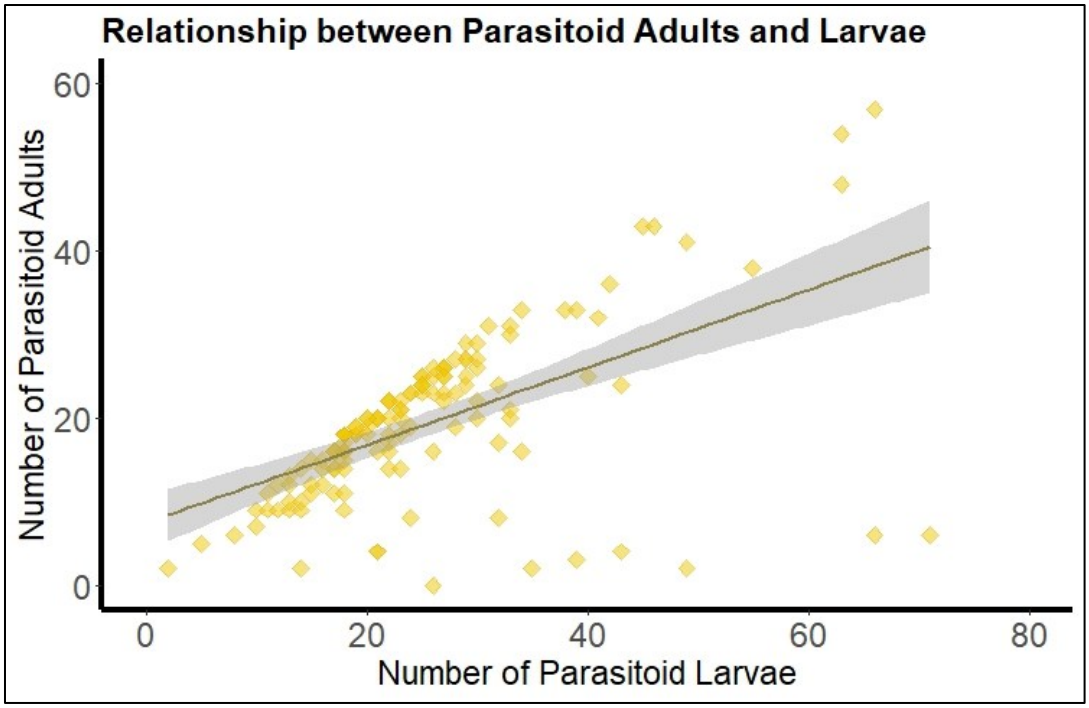

Figure 12. The number of parasitoid adults is positively correlated with parasitoid larvae.

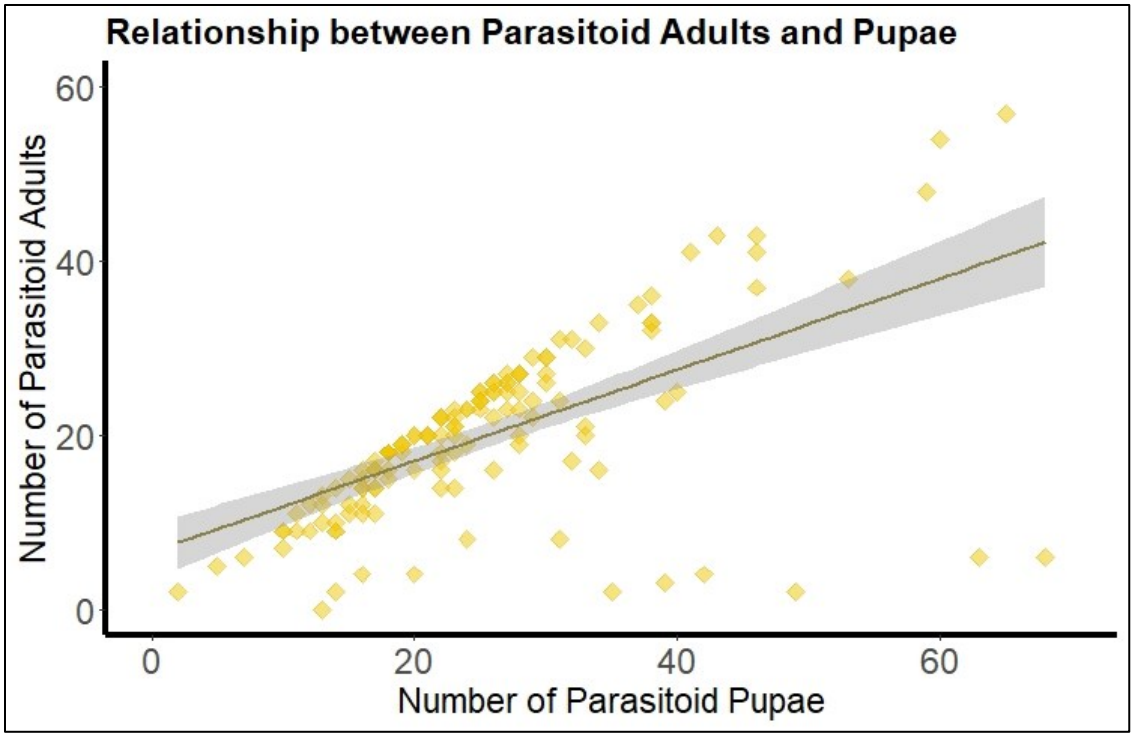

Figure 13. The number of parasitoid adults is positively correlated with the number of parasitoid pupae.

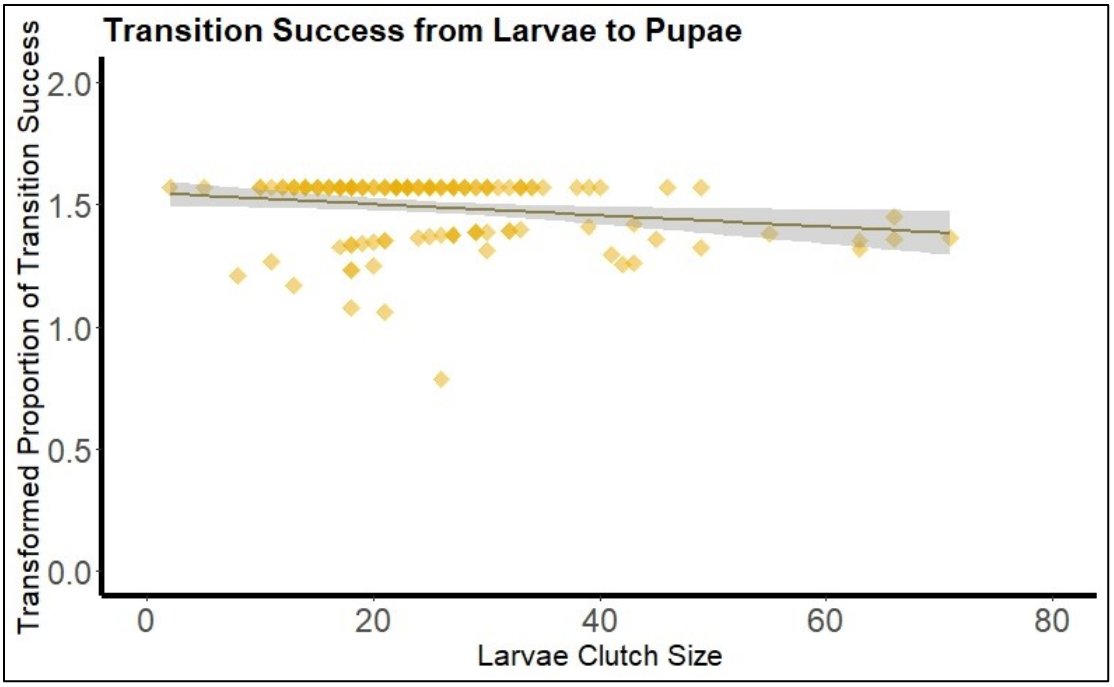

Figure 14. Proportion of transition success of larvae to pupae with an arcsine transformation has a slight negative relationship with larvae clutch size. Proportion transition success from larvae to pupae had a mean of 0.98 ± 0.05 (SEM).

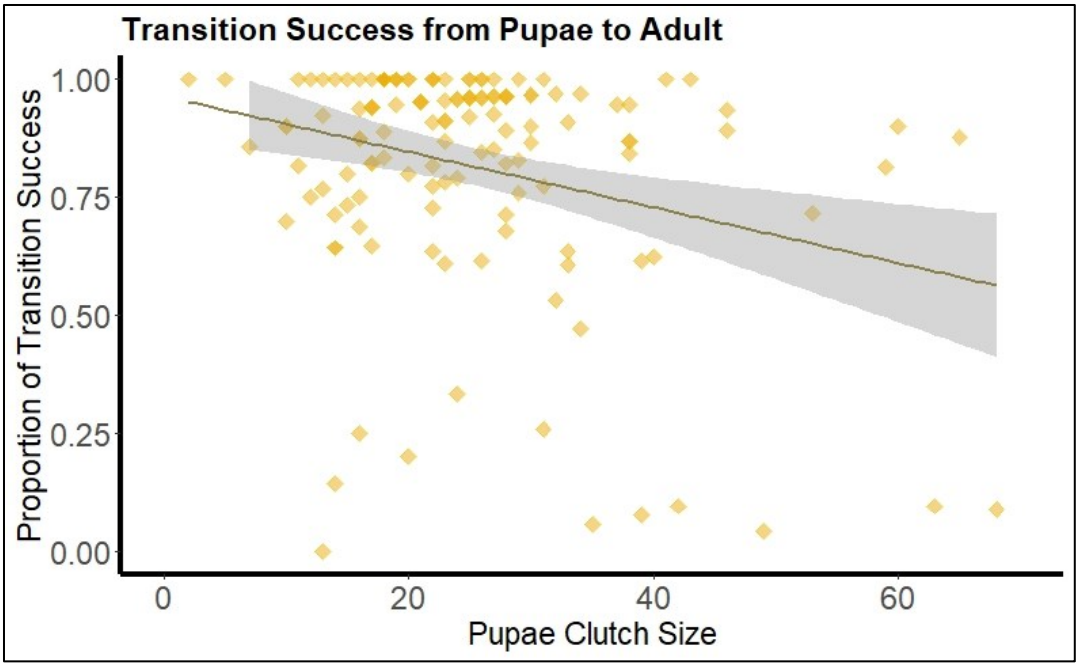

Figure 15. The proportion of transition success from pupae to adults had a significant negative relationship with pupae clutch size with mean = 0.82 ± 0.24 (SEM).

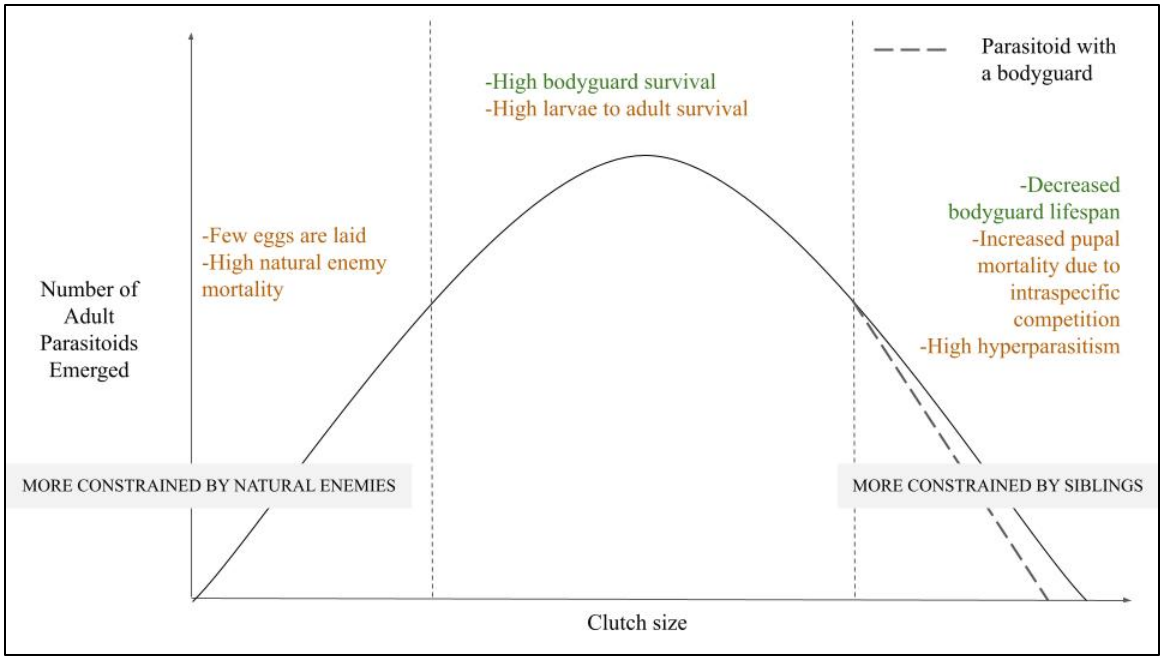

Figure 16. Heuristic model demonstrating the trade-offs and constraints on parasitoid clutch size.

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