DISSERTATION

WINNERS AND LOSERS IN TOXIC RELATIONSHIPS AFFECTING PARASITOID WASPS

Submitted by

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ABSTRACT

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Parasitoids are insects that develop on or inside another insect host, ultimately killing the host to complete their own development. Nearly every terrestrial plant-herbivore system has a suite of associated parasitoids. Success of parasitoids and their ability to regulate herbivore populations depends on interactions between multiple trophic levels. Of particular interest is the role of toxic chemicals that mediate interactions among species of plant-herbivore-parasitoid (tri-trophic) systems. My dissertation explores interactions involving toxins that affect parasitoids via multiple trophic levels.

Plants produce a number of toxins to defend against herbivores that can also have consequences for parasitoids. In Chapter 2, I review the current published research on plant defense toxins and parasitoid interactions. The effects on parasitoids vary based on mechanism of interactions, study system, and life history of parasitoids. I first discuss the evidence of plant defense impacts on host immunity and the direct impacts of plant chemistry on parasitoid fitness. Then I explore the well-studied glucosinolate defenses found in the plant families Brassicaceae and Capparaceae as a case study on plant toxin and parasitoid interactions. I also review the current evidence for the commonly presented hypothesis that plant defense effects are reduced at higher trophic levels. Finally, I examine the recent advances in research on reciprocal influence of parasitoids on plant toxin expression.

Despite the growing number of studies exploring the effects of plant toxins on parasitoids, the effects of variability in the expression of plant toxins on parasitoids has received little attention. Variability in plant toxins negatively affects herbivores but the influence of toxin variability on the ability of parasitoids to suppress herbivore populations is unclear. In Chapter 3, I studied the effects of variability in the plant defensive toxin, xanthotoxin, on development of a polyembryonic parasitoid of a generalist caterpillar. Parasitized caterpillars were fed artificial diets containing either different constant concentrations of xanthotoxin or multiple diets containing varying levels of xanthotoxin but with the same mean as the constant concentration treatment. Parasitoids performed worse on diets containing constant high levels of xanthotoxin. However, parasitoids were unaffected when herbivores fed on diets varying between high and low levels of xanthotoxin, compared to constant diets with the same mean. Herbivore suppression is therefore greatest when experiencing varying plant defense diets which strengthens bottom-up impacts and maintains equal top-down pressures.

Parasitoids may also influence expression of plant defense toxins. Many solitary parasitoids reduce herbivore feeding by killing the host before it completes development. However, gregarious parasitoids often cause the host to feed more, removing the plant's advantage of attracting these parasitoids. Since toxins are costly to produce, plants whose herbivores are consistently attacked by gregarious parasitoids which increase herbivore damage are expected to increase toxin production. In Chapter 4, I compared the induction response of glucosinolate defenses in *Brassica rapa* plants to feeding by caterpillars parasitized by either solitary or gregarious parasitoids. Plants produced increased concentrations of defensive toxins when fed upon by caterpillars parasitized by gregarious parasitoids than when unparasitized or parasitized by solitary parasitoids. By using caterpillars at the same earlier developmental stages,

which feed the similar amounts, I show that plants respond to parasitoid identity rather than feeding amount. This research demonstrates the unique response plants can have to herbivores attacked by parasitoids with different life histories.

Toxins in tri-trophic systems are not only used by plants to defend against colonizing insects, but also by parasitoids in competition. A single herbivore species is often attacked by more than one parasitoid species (multiparasitism). When multiparasitism occurs, the larval parasitoids of the different species must compete for control of the host, usually to the death. In one such system, the solitary parasitoid *Cotesia rubecula* generally outcompetes the gregarious *C. glomerata* when they share a host.

In the final chapter (Chapter 5), I explore the role of oviposition fluids as a source of toxins in larval competition between *C. rubecula* and *C. glomerata*. Oviposition fluids are injected along with eggs into a host by adult *C. rubecula*. These fluids are responsible for many physiological changes in the host, but their role in competition has received little attention. I injected caterpillars parasitized by *C. glomerata* with individual oviposition fluids from *C. rubecula* without a *C. rubecula* egg or larva. Many *C. glomerata* individuals were deformed in caterpillars injected by *C. rubecula* oviposition fluids. I demonstrate a physiological suppression mechanism that a *C. rubecula* larva uses to outcompete heterospecific larvae inside the host. Furthermore, I provide evidence for *C. rubecula* larvae using multiple mechanisms to suppress competitor development at several life stages. This study demonstrates a secondary competitive function beyond the physiological host changes for *C. rubecula* oviposition fluids and highlights the importance of competition in driving characteristics of parasitoids.

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DEDICATION

To my family, who encouraged me to follow my passion and always believed in my success.

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CHAPTER 1: BACKGROUND AND STUDY SYSTEMS

1.1 INTRODUCTION

It has long been recognized that understanding of plant-herbivore systems requires consideration for the role that the third trophic level plays - natural enemies, such as parasitoids (Price et al. 1980). Parasitoids are ubiquitous in terrestrial systems, with nearly every herbivorous insect having its own suite of parasitoids (Price 2002). Many studies have focused on the direct interactions of herbivores and their parasitoids, but less attention has been given to interactions between parasitoids and other trophic levels (i.e., plants and other parasitoids). Success of parasitoids can be influenced by other trophic levels through chemical interactions, often involving toxic substances. For example, plant defense toxins against herbivores can have important consequences for parasitoids (Ode 2006). Interactions between plant defenses and parasitoids can affect the impact that parasitoids have on herbivore populations and the role of parasitoids in minimizing herbivore damage to plants (Ode 2006, Ode et al. 2016). Toxins injected by ovipositing parasitoids can cause physiological changes in the host that benefit their developing offspring, which may affect other parasitoid species attacking the same host (Magdaraog et al. 2016).

My dissertation focuses on toxic compounds in tri-trophic (plant-herbivore-parasitoid) systems affecting parasitoid success. First, I review the literature discussing plant defense chemistry impacts on parasitoids and the mechanisms driving some of these interactions. Next, I explore the role of variable plant chemical defenses in herbivore diets over time on suppression of herbivores by parasitoids. Third, I investigate how different parasitoids influence expression of plant defenses. These chapters explore outcomes and mechanisms of interactions between

plant defense toxins and parasitoids. In the final chapter, I present my research on the use of oviposition fluids as toxins against competing parasitoid larvae sharing a host. These chapters also explore the influence of life history differences on parasitoid success. Parasitoid life history is discussed in the context of plant defense toxin impacts on parasitoids, influencing reciprocal expression of plant defenses, and as a factor in competitive ability of larval parasitoids.

1.2 PARASITOID BIOLOGY AND LIFE HISTORY

Parasitoids are insects which live on or inside of another insect host, ultimately killing the host to complete their own development (Godfray 1994). The intimate relationship with the host resource makes parasitoids well-suited study organisms for examining the forces driving interspecific interactions. Parasitoids require host insects to complete their development and the host provides the only resources for development of larval parasitoids. Thus, changes in host availability or quality will have strong impacts on fitness of their parasitoids.

Parasitoids can substantially change physiology of their host, including suppressing host immune response and changing salivary and oral secretions (Fleming 1992, Moreau and Asgari 2015, Tan et al. 2019). Many parasitoids achieve this by injecting oviposition fluids including venoms and virus-like particles along with their eggs during oviposition (Fleming 1992, Asgari and Rivers 2011, Moreau and Asgari 2015). Oral secretions are often altered by parasitism and these changes can be detected by plants on which the herbivore feeds (Poelman et al. 2011). Some parasitoids also influence feeding of the host, leading to greater consumption by parasitized hosts (Harvey 2005).

A number of life history strategies are employed by parasitoids. Endoparasitoids develop while feeding from the inside of the host while ectoparasitoids develop while feeding externally. Most ectoparasitoids are idiobiont, meaning they prevent the host from developing after

oviposition, often via paralysis. Many endoparasitoids are koinobionts, allowing their hosts to continue developing as the immature parasitoid develops. Gregarious parasitoids are those in which multiple offspring develop in a single host. In solitary parasitoids, only one offspring can develop per host. On occasion, more than one solitary parasitoid egg may be laid in a host, but only one will survive to pupation (Godfray 1994, Harvey 2005). Koinobiont endoparasitoids are the focus of the dissertation research with particular attention paid to differences between solitary and gregarious parasitoids.

Gregarious and solitary parasitoids differ in their influence on the host as well as in their competitive ability. Gregarious parasitoids typically cause their herbivore hosts to take longer to develop and feed more than unparasitized herbivores or herbivores parasitized by solitary parasitoids (Strand 1989, Coleman et al. 1999, Harvey 2005). Since gregarious parasitoids tolerate many larvae developing together inside a single host, they often lack characteristics to compete with other immature parasitoids (Laing and Corrigan 1987, Magdaraog et al. 2012, Harvey et al. 2013). Solitary parasitoids typically reduce herbivore feeding by emerging from younger hosts than gregarious parasitoids, which permit their host to develop through later stages and avoid the later herbivore instars where the most feeding occurs (van Loon et al. 2000, Harvey 2005). Inside the host, they are typically superior competitors compared to gregarious species. Many solitary parasitoids possess enlarged mandibles and increased mobility, allowing them to physically combat competing immature parasitoids (Magdaraog et al. 2012, Harvey et al. 2013).

1.3 STUDY SYSTEMS

1.3.1 *Trichoplusia ni* and *Copidosoma floridanum*

Trichoplusia ni (cabbage looper) is a generalist caterpillar which feeds on several hundred species of plants (Sutherland and Green 1984). It is thus capable of feeding on diets with a wide range of plant defense compounds, including the furanocoumarin xanthotoxin found in parsnip plants (Lee and Berenbaum 1989, Lampert et al. 2011). Cabbage loopers can be easily reared on an artificial diet. The diet can be used to test differences in plant defensive chemical concentrations with constant nutritional content.

Copidosoma floridanum is a polyembryonic egg-larval parasitoid wasp of cabbage loopers. Adult females lay one or two eggs inside a cabbage looper egg. Because haploid eggs develop as males and diploid are female, the sex of offspring is determined at oviposition (Godfray 1994). Because this species is polyembryonic, oviposition of a single parasitoid egg results in the production of single-sex male or female broods and oviposition of two eggs (one of each sex) will form mixed-sex broods (Ode and Strand 1995). Eggs of *C. floridanum* clonally divide and multiply as the host egg hatches and develops through several instars. During this time, the wasp egg(s) hatch and develop rapidly inside the host. Development of parasitized cabbage loopers is a couple days longer than unparasitized loopers and they grow approximately 50% larger (Strand 1989). The parasitoid larvae will eventually consume nearly all of the host organs and tissues in the final, 5^{th} instar of the host, leaving only the hardened cuticle of the exoskeleton and major tracheae unconsumed thereby forming a mummified host when they pupate. A brood of *C. floridanum* from a single mummy can produce approximately 1500-2000 individuals from just a single parasitoid egg (Ode and Strand 1995).

1.3.2 The *Pieris rapae – Cotesia* system

The imported cabbageworm, *Pieris rapae*, is a specialist caterpillar of plants in the family Brassicaceae. Plants in this family produce defensive glucosinolates, which break down into

toxic compounds such as isothiocyanates when they come in contact with the plant enzyme myrosinase as a result of herbivory (Hopkins et al. 2009). Cabbageworms possess a nitrile specifier protein which allows them to divert production of glucosinolates to less toxic nitriles which are then excreted (Wittstock et al. 2004). Adult butterflies lay eggs directly on plants and the larvae develop through five instars before pupating. In North America, the primary natural enemies of cabbageworms are two *Cotesia* parasitoids (Herlihy et al. 2012).

Cotesia glomerata is a gregarious endoparasitoid which typically lays between 20 and 30 eggs per cabbageworm host (Van Driesche 1988). Female *C. glomerata* typically parasitize 1st or 2nd instar caterpillars and offspring develop until the final, 5th instar of the host when they finally emerge as larvae and pupate next to the host (Gols et al. 2019). Immature wasps feed primarily on fat body and hemolymph while the host continues to feed and develop. Larval *C. glomerata* possess an anal vesicle (for excretion) and very small mandibles, thus lacking possess any physical characteristics that improve competitive ability or mobility (Laing and Corrigan 1987). Caterpillars parasitized by *C. glomerata* often feed more than unparasitized caterpillars, and this effect is exacerbated as the brood size of *C. glomerata* increases (Smallegange et al. 2008). Larval parasitoids spin yellow silk cocoons immediately upon emerging from their host in which they will pupate. The host dies of starvation soon after the parasitoids emerge.

Cotesia rubecula is a solitary endoparasitoid of the imported cabbageworm. Similar to *C. glomerata*, it prefers to oviposit in 1st and 2nd instar caterpillars (Vyas et al. 2019) and larvae feed on host fat body and hemolymph while the host continues to develop. Larval *C. rubecula* possess enlarged mandibles during the 1st instar (called a “hunting morph”) which allow them to physically kill competing parasitoids inside the host. They also possess a caudal appendage which may assist in movement through the host (Laing and Corrigan 1987). *Cotesia rubecula*

emerges from the penultimate, 4th instar of the host and therefore greatly reduces feeding by the parasitized caterpillar compared to unparasitized caterpillars (van Loon et al. 2000). Larvae spin white cocoons for pupation immediately after emerging and the host dies of starvation shortly after.

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CHAPTER 2: INTERACTIONS BETWEEN PLANT CHEMICAL DEFENSES AND PARASITOID FITNESS

2.1 INTRODUCTION

A great deal of progress has been made in understanding the interactions among insects and plants in tri-trophic (plant-herbivore-natural enemy) systems. Many studies have focused on the effects of bottom-up (plant traits) and top-down forces (natural enemies) on limiting herbivore populations (Vidal and Murphy 2018). However, much less attention has been given to the bottom-up effects of plant defensive chemistry on natural enemies of herbivores. Plant defense undoubtedly affects herbivore fitness, which can lead to impacts on their natural enemies as well (Ode 2006). Parasitoids, which share an intimate relationship with their herbivore hosts, can be sensitive to the changes in herbivore host quality mediated by plant chemistry.

A plethora of studies have examined the positive impacts of plant chemistry on parasitoid fitness through herbivore induced plant volatiles, which provide important cues for successful host location (Geervliet et al. 1996, Fatouros et al. 2005, Hare 2011, Poelman et al. 2013, Frati et al. 2017, Turlings and Erb 2018, Takabayashi and Shiojiri 2019). Attraction to these volatiles increases foraging efficiency and thus improves reproduction potential for the parasitoid. Far fewer studies focus on the potential negative impacts of plant chemical defenses on parasitoids. Here I will explore the current literature on the impacts of plant defense chemistry on parasitoid fitness, both positive and negative. A number of research advances have been made since previous reviews on the subject (see Ode 2006, Gols and Harvey 2009). I will consider (1) differences in mechanisms of plant defense interactions, (2) factors involved in determining the

impacts of defense chemistry on parasitoids, and (3) how these impacts influence or are influenced by implications for plant fitness.

2.2 PLANT DEFENSE THEORY

An underlying theme of plant defense allocation theories is that production of defenses must provide a net fitness benefit for the plant, which is often not explicitly measured (Erb 2018). A number of theories have attempted to explain resource allocation for defenses and distribution of defenses within different plant tissues (Stamp 2003). In a few systems, studies have explicitly demonstrated the direct fitness costs of producing higher amounts of defensive chemicals (Stowe 1998, Baldwin 1998, Marak et al. 2003, Lankau and Kliebenstein 2009, Stowe and Marquis 2011). Studies on artificially selected lines of high and low defense plants have demonstrated the impact of producing higher concentrations of defenses in the absence of herbivory on traits including reduced seed production (Lankau and Kliebenstein 2009) and reproductive dry weight (Marak et al. 2003). The costs of producing chemical defenses are outweighed by the benefits in the presence of herbivores, where high defenses are favorable for fitness (Baldwin 1998, Lankau and Kliebenstein 2009). Many defenses are therefore inducible, allowing costs to be allocated towards defense only when the perceived risk of herbivory is high (Karban and Baldwin 1997, Baldwin 1998). However, the relative costs and benefits associated with these defenses may need to consider impacts of chemical defenses on parasitoids as well.

2.3 PLANT DEFENSE IMPACTS ON HERBIVORE IMMUNITY

Plant defenses play an important role in mediating immune responses of herbivores (Kaplan et al. 2016). Herbivores' primary immune defense against parasitoids is encapsulation, where parasitoids are surrounded by hemocytes which melanize and kill parasitoids by asphyxiation (Godfray 1994). Research has suggested that herbivore immunity (i.e.,

encapsulation ability) is lowered on diets with higher defensive chemicals (Bukovinszky et al. 2009, Smilanich et al. 2009, Lampert 2012, Richards et al. 2012, Kaplan et al. 2016, Hansen et al. 2017, Slinn et al. 2018). Buckeye caterpillars perform better with higher iridoid glycosides in their diet, which they sequester as an anti-predator defense (Richards et al. 2012). However, feeding on diets high in iridoid glycosides also reduced encapsulation ability (Smilanich et al. 2009, Richards et al. 2012). In another specialist herbivore, *Pieris rapae*, encapsulation responses to the parasitoid *Cotesia glomerata* were reduced when feeding on wild cabbage varieties with higher chemical defense levels compared to feeding on cultivated varieties. Increased defenses of cabbage induced as a result of prior caterpillar feeding also reduced encapsulation response compared to uninduced plants (Bukovinszky et al. 2009). Plant defense interactions with herbivore immunity may even affect competition between parasitoids. While *C. glomerata* is normally an inferior larval competitor, induction of increased plant defenses reduces encapsulation for *C. glomerata* individuals and improves the chances of *C. glomerata* outcompeting superior parasitoids inside the host (Poelman et al. 2011).

A great deal of field research on the relationship between plant chemical defenses and herbivore immunity to parasitism has been gathered from long-term studies involving tropical plants in the genus *Piper. Piper* is a mostly neotropical genus of shrubs with a wide range of inter- and intra-specific variation in defensive chemistry. It has become a model system for examining the role of phytochemical diversity in community ecology of tri-trophic systems (Dyer and Palmer 2004). Immunity of *Eois* caterpillars, which feed on *Piper,* is strongly influenced by chemical diversity of host plants (Hansen et al. 2017, Slinn et al. 2018). Caterpillars feeding on plants which are better defended tend to have lower immunity and thus greater proportions of parasitism in the field (Hansen et al. 2017). Long-term data sets on plant-

herbivore-parasitoid interactions on *Piper* revealed higher parasitoid diversity on plants with higher chemical defenses (Glassmire et al. 2016). This suggests that chemical diversity may therefore shape community structure of parasitoid populations via changes in herbivore immunity (Glassmire et al. 2016, Hansen et al. 2017). However, these measurements of field associations may be clouded by increased diversity of plant volatiles leading to higher attraction of parasitoids (Glassmire et al. 2016).

Despite the evidence for the negative impacts of plant chemistry on herbivore immunity, there are also numerous examples of herbivores using alternative food sources to improve their chances of surviving when parasitoid pressure is high. This has been well studied in *Drosophila* larvae, where consumption of higher ethanol diets increases mortality for parasitoids inside the fly larvae and adult parasitoids lay less eggs on flies feeding on high ethanol (Milan et al. 2012, Kacsoh et al. 2013). Larvae perform more poorly on high ethanol diets, but this is favored under higher parasitoid pressure (Kacsoh et al. 2013). This use of sub-optimal food sources to improve defense against parasites is denoted as "self-medication" (Abbott 2014). Self-medication is documented in a few herbivore-parasitoid systems (Singer et al. 2004, 2009, Smilanich et al. 2011, Abbott 2014, Poyet et al. 2017), but whether these impacts to parasitoid fitness are based on direct contact with toxins, changes in host quality, or changes in host immunity is less clear. Given the lower fitness and lower immune response for many herbivores on higher defense plants, evidence of self-medication indicates that the direct effects of plant chemistry may be more important for herbivore defense in some species than innate immunity (Abbott 2014).

2.4 DIRECT IMPACTS OF PLANT CHEMISTRY ON PARASITOID FITNESS

Parasitoids may directly encounter plant defense compounds in the hemolymph or tissues of the host as they are feeding. This has led to development of the "nasty host hypothesis" stating

that herbivores will experience less parasitism on higher defense plants as their tissues are more toxic for their parasitoids (Gauld et al. 1992). Often studies do not measure presence of plant toxins in host tissues or hemolymph, but direct effects can be explored with sequestering herbivores. Many herbivores sequester compounds from their host plant as a defense against natural enemies, forcing parasitoids to experience these toxins directly (Le Guigo et al. 2011, Beran et al. 2014, van Geem et al. 2014, Stenoien et al. 2019). Interestingly, evidence suggests that sequestration serves as a strong defense against predators, but can prove weak or even hindering as a defense against parasitoids, despite the presence of toxins in the host tissues (Le Guigo et al. 2011, Smilanich et al. 2011, Reudler et al. 2011). Cabbage aphids sequester glucosinolate defenses from their host plants and grow larger on better defended wild plants. Despite higher defenses, aphids on wild plants experience higher parasitism rates and parasitoids were larger, indicating that sequestration did not help aphids avoid parasitism (Le Guigo et al. 2011). On *Arabidopsis*, aphid predators were negatively impacted by glucosinolate concentrations while parasitoid performance was positively associated with glucosinolate concentration (Kos et al. 2012a). Catalpa sphinx caterpillars, *Ceratomia catalpae,* sequester large amounts of iridoid glycosides with concentrations reaching 50% of the dry weight of hemolymph (Bowers 2003). Larvae of the parasitoid, *Cotesia congretata*, which attacks catalpa sphinx, suffer no decrease in numbers of offspring and achieve very high survival despite high glycoside concentrations in host caterpillars (Lampert et al. 2010). Willow feeding leaf beetles, *Chrysomela lapponica*, sequester defenses from their host plant but actually experienced higher parasitism by tachinid flies when feeding on plants with higher defense chemistry (Zvereva and Rank 2003). These examples contradict the nasty host hypothesis and these sequestering hosts

may primarily provide refuge from predators which would consume the host resource rather than parasitoids (Lampert et al. 2010).

Parasitoids can also experience direct contact with defense compounds in other herbivores which do not intentionally sequester plant compounds (Barbosa et al. 1986, 1991, Ode et al. 2004, McGovern et al. 2006, Lampert et al. 2008, 2011b). Contrary to the trends found in sequestering hosts, direct effects of plant defenses on parasitoids in other herbivores tend to be negative. One of the earliest examples demonstrated that herbivore diets containing as little as 0.3 percent α-Tomatine, an alkaloid in tomatoes, could negatively affect parasitoid eclosion, development time, adult weight, and lifespan and showed that the compound could be detected in adult parasitoid tissues (Campbell and Duffey 1979). This work was followed by many examples of nicotine in the hemolymph of the tobacco hornworm, *Manduca sexta*, impacting both generalist and specialist parasitoids (Barbosa et al. 1986, 1991). While most impacts to the parasitoid followed impacts on host quality, parasitoids experienced increased mortality from nicotine in the diet, and in-turn the host hemolymph, despite most of the compound being excreted by the host (Barbosa et al. 1991). Furthermore, nicotine even affects hyperparasitoids at the fourth trophic level, which performed better on diets lowest in nicotine (Harvey et al. 2007a).

Even in specialist herbivores which are generally less impacted by certain plant defenses, compounds may not be fully metabolized, leading to detrimental effects on their parasitoids. Parsnip webworm, *Depressaria pastinacella*, is a specialist herbivore capable of feeding on parsnip plants containing high concentrations of toxic furanocoumarins. These compounds are highly toxic to generalist herbivores and their parasitoids, but have little effect on webworms, which can efficiently metabolize them (McGovern et al. 2006, Lampert et al. 2011b). However, furanocoumarins were found in webworm hemolymph, and high concentrations reduced brood

size in its specialist parasitoid, despite a lack of effect on the webworms (Lampert et al. 2008, 2011b). *Drosophila suzukii* flies tend to lay more eggs on diets containing the alkaloid atropine when in the presence of parasitoids. These compounds have little impact on the flies, but parasitoids developing in flies feeding on atropine suffered higher mortality and were much smaller than from flies on atropine free diets. Though atropine in flies was not measured, mortality of parasitoids developing in the fly pupal stage when encapsulation does not occur indicates a direct mortality effect of atropine on parasitoids (Poyet et al. 2017). Unlike in self-medication, the host is not negatively impacted in these scenarios but the parasitoid still is.

2.5 GLUCOSINOLATE DEFENSES AND PARASITOID INTERACTIONS

Thus far, much of the fitness effects on parasitoids demonstrated in this review have focused on carefully manipulated artificial diets containing plant chemical defenses. However, many recent developments in research on the interactions between plant defenses and parasitoids comes from studies using the chemical variation of plant individuals, populations, and species. A number of studies have explored the impacts of different species (Harvey et al. 2003, Sznajder and Harvey 2003, Gols et al. 2008c, 2009, Le Guigo et al. 2011) and different cultivars or populations (Fuentes-Contreras and Niemeyer 1998, Harvey et al. 2007b, Gols et al. 2008b, Kos et al. 2011, Li et al. 2016, Harvey and Gols 2018) of plants which often have great differences in defensive chemistry. Arguably the most work in this area has involved glucosinolate defenses of Brassicaceae plants.

Glucosinolates are amino-acid derived compounds that are catalyzed into highly toxic compounds, such as isothiocyanates, when they come in contact with the separately stored myrosinase enzyme during herbivory (Wittstock et al. 2003, Hopkins et al. 2009). More than 120 glucosinolates have been identified and most are classified broadly into aliphatic (primarily

derived from methionine), indole (derived from tryptophan), or aromatic (typically derived from phenylalanine) (Fahey et al. 2001). Both generalist and specialist herbivores alike can experience negative impacts from many of the glucosinolates and their hydrolysis products (Li et al. 2000, Agrawal and Kurashige 2003, Wittstock et al. 2003, Müller et al. 2010, Kos et al. 2012b). It is not surprising that these compounds may impact parasitoids as well (Gols et al. 2008b, Kos et al. 2012b, Gols and Harvey 2009).

Glucosinolates often have greater impacts on generalist herbivores and parasitoids than on specialists. Wild populations of *Brassica oleracea* with higher concentrations of indole glucosinolates negatively impact pupal mass of the specialist *Pieris rapae* and its parasitoid, *Cotesia rubecula*. However, chemical differences in the same plant populations also drastically affected mortality of the generalist, *Mamestra brassicae*, and its parasitoid, *Microplitis mediator* (Gols et al. 2008b). Similarly, glucosinolates more heavily impacted a generalist parasitoid of the diamondback moth, *Plutella xylostella*, than a specialist parasitoid. While the specialist *Diadegma semiclausum* experienced negative effects on body size and development on highly defended wild *Brassica* that largely followed similar effects on the host, the generalist parasitoid *D. fenestrale* also experienced increased mortality on highly defended wild plants (Gols et al. 2008a). It is possible that the effects of glucosinolates on parasitoids depend on parasitoid specialization rather than that of the host. For example, the parasitoid *Hyposoter ebeninus* performed better on higher glucosinolate plants regardless whether it was reared on specialist *P. rapae* or the generalist *Spodoptera exigua* (Kos et al. 2012b).

Many studies of glucosinolate impacts on parasitoids have focused on a few specialist caterpillars and their parasitoids. Plant chemistry effects on parasitoids in these specialist systems seem most often related to changes in insect host quality, as the changes in parasitoid

fitness mirror impacts on the host (Harvey et al. 2007b, 2011, Gols et al. 2008b, 2009, Harvey and Gols 2018). For instance, both *Pi. rapae* and *Pi. brassicae* caterpillars feeding on different populations of *B. oleracea* plants both suffered increased development time and decreased adult mass as did the pupal parasitoid *Pteromalus puparum* in both hosts (Harvey et al. 2007b, 2011). In another study, performance of both *Pi. brassicae* and its parasitoid *C. glomerata* were worse when caterpillars fed on plants with higher glucosinolates induced by jasmonic acid (a plant defense hormone) application (Qiu et al. 2009). Two species of parasitoids and their specialist host diamondback moth all performed best on cultivated cabbage populations over highly defended wild populations (Harvey and Gols 2018). Glucosinolates appear to have a minimal effect on direct mortality of parasitoids of specialist herbivores, whereas direct mortality may increase for generalist parasitoids when hosts feed on higher glucosinolate concentrations (Gols et al. 2008a).

Plant defenses should be considered a key component in mediating interactions between species utilizing the same plant even across time scales. Some studies involving brassicaceous plants explore interactions of plant chemistry beyond the typical single unit plant-herbivore-parasitoid model. Parasitoids can experience impacts from defense induction due to herbivores feeding at different locations or times. Root feeding flies increase concentrations of the aliphatic glucosinolate sinigrin in *Brassica nigra* plants which leads to longer development time in *Pi. rapae* caterpillars as well as reduced body size in both its parasitoid *C. glomerata,* and a hyperparasitoid (Soler et al. 2005). Egg deposition by *Pi. brassicae* butterflies (even without hatching and feeding) induces defenses in *B. nigra* which can reduce performance of subsequent herbivores and their parasitoids (Pashalidou et al. 2015). Induction effects can even influence competition between parasitoids in different hosts. Plants that were fed on by *C. rubecula*

parasitized caterpillars induced responses that led to higher mortality from encapsulation for *C. glomerata* in caterpillars that fed later, but *C. rubecula* development was unaffected. Contrarily, if the plant was induced by *C. glomerata* parasitized caterpillars, *C. glomerata* in subsequently feeding caterpillars performed better (Poelman et al. 2011). Though glucosinolate concentrations were not measured, they are known to affect encapsulation success (Bukovinszky et al. 2009) and likely play a role in this interaction.

2.6 EVIDENCE FOR DECREASED IMPACTS OF PLANT CHEMISTRY FOR PARASITOIDS RELATIVE TO HOSTS

Some publications on interactions between plant defenses and parasitoids have suggested that the effects of plant chemistry may be diluted at higher trophic levels (Gols et al. 2008c, Gols and Harvey 2009). Excretion or detoxification of plant defenses by the host herbivore could reduce the effects for parasitoids. Indeed, reduced chemical exposure for parasitoids has been demonstrated for some plant compounds including furanocoumarins (McGovern et al. 2006) and iridoid glycosides (Lampert et al. 2011a, van Nouhuys et al. 2012). Performance of *Pi. brassicae* caterpillars are affected by feeding on *Brassica nigra* plants versus *Sinapsis arvensis* which differ in defensive chemistry. However, its parasitoid *C. glomerata* developed equally well on the two plants (Gols et al. 2008c). Similarly, parasitoids of diamondback moth experienced smaller variations in mass and development time when the host fed on different plant populations than experienced by unparasitized caterpillars (Harvey and Gols 2018). The diluted effects of chemistry on parasitoids can translate to reductions at the fourth trophic level as well (Harvey et al. 2007a, van Nouhuys et al. 2012). The hyperparasitoid *Lysibia nana* performs equally well on *C. glomerata* and *C. melitaearum* despite the latter sequestering iridoid glycosides from its host (van Nouhuys et al. 2012).

The decreased effects of plant chemistry with increasing trophic levels may offer some explanation for the better performance of many parasitoids with increasing plant defense. Since many parasitoids do experience less chemistry than their herbivore host (McGovern et al. 2006, Lampert et al. 2011a, van Nouhuys et al. 2012), parasitoids can avoid the direct impacts of defenses while benefitting from plant chemistry impacts on host immunity. Thus far, immunocompromising has been discussed in regards to reduced encapsulation, yet the effects may extend beyond this, whereby fitness of parasitoids is increased on hosts experiencing higher plant defenses (Harvey et al. 2007a, Reudler et al. 2011). For example, the parasitoid *C. marginiventris* grows faster on hosts which feed on higher amounts of iridoid glycosides despite unparasitized hosts having slower development (Reudler et al. 2011). Moreover, the hyperparasitoid *L. nana* experienced higher fitness when attacking the hornworm parasitoid *C. congregata* developing in caterpillars fed low nicotine diets compared to nicotine free diets (Harvey et al. 2007a). In one of the more extreme examples, *Microplitis mediator* parasitoids of the generalist caterpillar *Mamestra brassicae* experienced higher survival on higher defense plants on which unparasitized caterpillars had the highest mortality. This is likely due in part to compromised immune response of the host but also indicates that the effects of plant chemistry may accumulate as the host develops and are avoided by the parasitoid which emerges earlier in development. *Microplitis mediator* emerges from the penultimate instar of the host and may avoid the negative impacts of plant defense that the host experiences if effects are cumulative through development, leading to mortality effects primarily in the last instar (Harvey and Gols 2011). These diluted effects may also explain the lack of support in many systems for the "nasty host hypothesis" (Gauld et al. 1992). Better defended plants may serve as a refuge for parasitoids, where predators that would consume the host fare worse (particularly in sequestering

hosts), but parasitoids experience lesser effects and reduced encapsulation, leading to greater survival (Smilanich et al. 2009, 2011, Lampert et al. 2010).

However, some parasitoids do experience increased negative effects from plant chemistry compared to their hosts, particularly among generalist herbivores. The polyembryonic parasitoid of cabbage loopers, *Copidosoma floridanum*, experiences reduced brood size on both furanocoumarin (Lampert et al. 2011b) and iridoid glycoside defenses (Lampert and Bowers 2013) in addition to mirroring the negative effects of these chemicals on the host. Parasitized woolly bear caterpillars can successfully self-medicate by feeding on nutritionally suboptimal, but better defended plants which increases mortality for developing parasitoids (Singer and Stireman III 2003, Singer et al. 2004, 2009). A common theme among these generalists, is a parallel decrease in host quality and increased mortality for both host and parasitoid indicating that these effects may be in part due to decreases in host quality (Lampert et al. 2011b, Lampert and Bowers 2013). Cases where parasitoids are more strongly influenced by plant defenses than their host appear less common in specialist herbivores, though examples do exist, particularly for gregarious parasitoids (where multiple parasitoids develop per host) which can experience reduced brood size from direct mortality effects (Barbosa et al. 1986, Lampert et al. 2008). Generalizations about the effects of plant chemical defense on parasitoids requires caution and these effects will depend on many conditions, including but not limited to life history and diet breadth of both the parasitoid and its host.

2.7 PARASITOID CONTRIBUTIONS TO PLANT FITNESS

A key component of the consideration of plant responses to parasitoids depends on the mutualistic benefit they do (or don't) provide for the plant. Parasitoids are described as an indirect defense for plants, because attraction of parasitoids to herbivore induced plant volatiles

can lead to reduced in herbivore damage to the plant (Turlings and Erb 2018) which has led to the use of parasitoids as biological control (Peterson et al. 2016). However, the idea of parasitoids as an indirect defense has been controversial and explicit evidence for the assumption that parasitoids benefit plant fitness is surprisingly somewhat scarce (van der Meijden and Klinkhamer 2000, Hare 2011). Idiobiont parasitoids, which immediately prevent the host from continuing development through paralysis or death, certainly provide benefits by preventing any further feeding by parasitized herbivores (Buteler et al. 2008, Hare 2011). For example, plants with wheat stem sawfly that were parasitized by an idiobiont parasitoid produced much heavier seeds compared to plants with unparasitized sawflies, even though these larvae were not parasitized until near the end of development (Buteler et al. 2008). Presence of idiobiont chalcid parasitoids of the seed weevil *Ceutorhynchus* sp. halved the amount of weevil damaged fruits of *Hormathophylla spinosa* plants and increased overall seed production by as much as 30% (Gómez and Zamora 1994, Munguía-Rosas et al. 2016). In the mutualistic yucca and yucca moth system, moths pollinate the plants but the larvae also feed directly on developing seeds. Idiobiont parasitoids of yucca moth larvae were able to reduce the costs of pollination from seed feeding and contribute to mortality of non-pollinating moth species, leading to increased seed production (Crabb and Pellmyr 2006).

Many direct measures of parasitoid impacts on plant fitness, however, have been performed with koinobiont parasitoids, which allow the host to continue developing after parasitism. Relationships between koinobionts and plant fitness are less clear, as the herbivore damage continues long after arrival of the parasitoids (Hare 2011). Some koinobionts do benefit to plant fitness, especially those parasitizing herbivores that feed directly on reproductive tissues. *Sinapsis arvensis* plants had similar seed production to undamaged plants when fed on by

parasitized *Pieris brassicae*, which feeds on flowers and seed pods, compared to feeding by unparasitized caterpillars. Plants benefited more when the caterpillar was parasitized by solitary *C. rubecula* over gregarious *C. glomerata* parasitoids. The solitary parasitoid (one offspring develops per host) killed the host much earlier and reduced feeding more compared to the gregarious parasitoid (multiple offspring develop per host) (Gols et al. 2015). Typically the earlier the host is killed, the greater the benefit to the plant. Many significant reductions in plant damage from koinobiont-parasitized hosts are due to solitary parasitoids as they often kill their host much earlier (van der Meijden and Klinkhamer 2000, Harvey 2005). *Arabidopsis* plants fed on by unparasitized *Pieris rapae* produced seeds only on regrown shoots while plants with caterpillars parasitized by the solitary *C. rubecula* produced comparable numbers of seeds to undamaged plants (van Loon et al. 2000). Maize plants produced 30% more seeds when fed on by caterpillars parasitized by solitary *Cotesia marginiventris* or *Campoletis sonorensis* than when fed on by unparasitized caterpillars (Hoballah and Turlings 2001)

Controversy over the benefit of parasitoids to plant fitness stems from lack of explicit measurements of fitness and from evidence that some koinobiont parasitoids increase potential damage. Gregarious parasitoids in particular can increase feeding of the herbivore host compared to unparasitized herbivores (Strand 1989, Harvey 2005, Smallegange et al. 2008, Ode et al. 2016). Caterpillars parasitized by the gregarious *C. glomerata* consume equal or more plant tissue than unparasitized caterpillars leading to lower seed production (Coleman et al. 1999, Smallegange et al. 2008). Cabbage loopers, *Trichoplusia ni*, consume approximately 50% more plant tissue when parasitized by the polyembryonic gregarious parasitoid, *Copidosoma floridanum* (Strand 1989). Gregarious parasitoids which decrease plant fitness may provide selective pressures for plants to increase their defense responses to herbivores parasitized by

these parasitoids compared to solitary parasitoids, but this also depends on the long term impacts of gregarious parasitoids on herbivore populations (Ode et al. 2004, 2016, Ode 2006).

2.7.1 Influence of parasitoids on plant defense expression

Recently, a few studies have begun exploring the reciprocal influence of parasitoids on expression of plant defense. Evidence from distributions of *Copidosoma sosares*, a parasitoid of parsnip webworm, indicates that plant chemistry expression can be influenced by parasitoid presence. Concentrations of furanocoumarins in plants were much higher in parsnip populations where the parasitoid is largely absent compared to populations where parasitoids are common. Additionally, some of these furanocoumarin concentrations were correlated with parasitoid, but not webworm fitness, suggesting that reduced furanocoumarin levels may be a result of selection by parasitoid presence (Ode et al. 2004). In another example, volatile production and genetic expression of plant defense in *B. oleracea* was influenced by feeding from caterpillars parasitized by different parasitoids. This also differentially affected subsequent colonization by diamondback moths (Poelman et al. 2011). Cabbage loopers parasitized by *C. floridanum* induced 1.5 times greater amounts of indole glucosinolates in wild cabbage plants compared to unparasitized caterpillars, likely due to *C. floridanum* parasitized caterpillars feeding 50% more (Ode et al. 2016). Differential defense responses of plants to parasitized caterpillars may be mediated by changes in salivary enzyme activity of parasitized caterpillars, which has been explicitly demonstrated in *Helicovera* sp. feeding on tomato and tobacco plants (Tan et al. 2019). The extent to which parasitoids influence defense expression across systems is in need of further investigation.

2.8 FUTURE DIRECTIONS

There is now widespread of evidence that plant defense chemistry affects interactions with not only herbivores, but also their parasitoids. Given the prevalence of these impacts (both positive and negative), further research is needed to determine the selective force of parasitoids on evolution and expression of plant defensive chemistry. Only a few studies thus far have provided evidence that parasitoids can influence defense expression. Much of the focus on plant chemistry and parasitoid interactions remains unidirectional in the tri-trophic context, focusing on bottom-up impacts on parasitoid fitness. Many top-down studies focus only on interactions of parasitoids on herbivores with less attention for the impact of parasitoids on expression of plant traits. This is a ripe area of research which could illuminate important factors involved in coevolution of plant-herbivore-parasitoid systems.

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CHAPTER 3: MEAN LEVEL BUT NOT VARIABILITY OF PLANT DEFENSE IN HERBIVORE DIET IMPACTS FITNESS OF ITS POLYEMBRYONIC PARASITOID

3.1 SYNOPSIS

Herbivore populations can be regulated by bottom-up factors such as plant defenses and top-down factors such as natural enemies. In many cases, natural enemy populations are also adversely affected by plant defenses, leading to a tension where plant defense can suppress herbivore populations directly but potentially also release herbivores from top-down control. Over their lifespans, individual herbivores and their natural enemies may experience substantial variation in plant defense. Recent studies have demonstrated that herbivore fitness declines substantially when fed diets with elevated variation in plant anti-herbivore toxins compared to herbivores fed a diet with a constant level of defense even though the mean toxin levels encountered were the same. However, the impacts of defense variability on natural enemies and top-down control of herbivores is unknown. We conducted feeding trials that manipulated the mean and variation of a plant defense toxin experienced by the caterpillar *Trichoplusia ni* and its parasitoid *Copidosoma floridanum*. Increasing the mean defense concentration experienced by *T. ni* and *C. floridanum* decreased the emergence success and fitness of *C. floridanum*. However, the level of variation around the mean had little impact on *C. floridanum* emergence success or fitness. This pattern contrasted with the impact of defense variability on unparasitized *T. ni*, where both increases in the defense mean and variation consistently decreased *T. ni* growth, fecundity, and fitness. Thus, increased defense variability suppressed herbivore fitness with no perceptible cost to top down control. While increased defense mean concentrations also suppressed herbivore fitness, it did so at a substantial cost to top-down control. Our study

highlights the importance of defense variability through plant diversity as a means of regulating herbivore populations.

3.2 INTRODUCTION

Herbivores experience both bottom-up pressures from defense traits of their host plants as well as top-down pressure from their predators, parasitoids, and pathogens (Hunter and Price 1992). The combination of bottom-up and top-down impacts should be considered when examining the factors regulating herbivore populations. Yet, often only one set of bi-trophic interactions are examined. Plant defensive chemicals are important bottom-up factors in suppressing herbivore fitness (Stamp 2003). Furthermore, these chemicals can have consequences for parasitoids developing in these herbivores through decreases in host quality (Lampert and Bowers 2010, Harvey et al. 2011), increased herbivore immunity (Kaplan et al. 2016), or direct exposure of developing parasitoids to plant defense chemicals (Barbosa et al. 1986, McGovern et al. 2006, Lampert et al. 2008). In other cases, performance of parasitoids may increase if plant anti-herbivore toxins cause a decrease in the immune response of the herbivore (Bukovinszky et al. 2009, Smilanich et al. 2009, Lampert 2012, Quintero et al. 2014). When examining the impacts of plant defense traits on herbivores, the effect of plant chemicals on top-down controls such as parasitoids should be considered as well.

Herbivores encounter variability in defenses both spatially and temporarily throughout development (Hakes and Cronin 2011, Quintero et al. 2014). Herbivores can experience variation moving across different parts of the same plant or between individual plants (Wetzel et al. 2016, Pearse et al. 2018), through ontogenetic variation in plant defense production (Quintero et al. 2014), or through induction (Karban et al. 1997). Furthermore, some studies have suggested that the ability of herbivores to metabolize plant defense toxins may change

ontogenetically (Quintero and Bowers 2018, Boege et al. 2019). However, few studies have examined the effects of variability on performance; instead, most studies focus on the effects of mean trait values. Nevertheless, some recent studies have provided evidence that variability, in and of itself, can impact herbivores (Grettenberger and Tooker 2016, Wetzel et al. 2016, Pearse et al. 2018). Variability in the diet may impact some herbivores through effects of non-linear averaging (Wetzel et al. 2016). Feeding on diets of varying defense can also lead to physiological mismatch of gut acclimation (Wetzel and Thaler 2016, Pearse et al. 2018).

By extension, the effects of plant defense variability on herbivores may also result in negative consequences for their natural enemies. However, the effects of plant defense variability on parasitoids has not been explicitly measured. Given the widespread occurrence of spatial and temporal variation in plant defenses, and the documented effects on herbivores (Wetzel et al. 2016, Pearse et al. 2018), variation in plant defenses likely affects higher trophic levels as well and it is unclear how this might affect top-down control of herbivore populations. One observation is that if herbivores are less mobile than their predators, variable plant defenses may increase top-down predation of herbivores because predators readily move between high quality patches with abundant herbivores and low-quality patches with fewer herbivores (Riolo et al. 2015). Top-down effects tend to be stronger than bottom-up effects on herbivorous insects (Vidal and Murphy 2018) so consideration of tri-trophic interactions is crucial in understanding the effects of variable plant traits on herbivore success (Ode 2006, Gols 2014).

In this study, we explored the impacts of toxin variation in the diet of the cabbage looper *Trichoplusia ni* (Lepidoptera: Noctuidae) on development of its parasitoid *Copidosoma floridanum* (Hymenoptera: Encyrtidae). Cabbage loopers can be reared on artificial diets in which precise concentrations of plant toxins can be incorporated, making them well-suited for

studies of the impacts of defenses on *T. ni* caterpillars (Pearse et al. 2018) and *C. floridanum* (Lampert and Bowers 2010, 2013, Lampert et al. 2011). Using an artificial diet, we established the effect of xanthotoxin, a plant defense produced by many plants in the Apiaceae, across a range of mean concentration values by manipulating the concentration of xanthotoxin in the diet without changing nutritional content. We then fed individual parasitized caterpillars varying concentrations of xanthotoxin in their diet to explicitly test the effects of variation in toxin concentration and compare them to the effects of mean concentrations. This design allowed us to measure the emergent effects of toxin variability by removing other variables that might obscure these effects.

We hypothesized that the negative effects of toxin variability in the diet of the herbivore (see Pearse et al. 2018) will decrease host quality resulting in negative impacts on the parasitoid. Alternatively, the effects of toxin variability may dissipate with increasing trophic levels and variability in host diet may not be strongly experienced by parasitoids and may maintain similar top-down control of herbivores. Because natural enemies experience plant defenses indirectly via their herbivore prey/host, they may not be exposed to the same degree of variation in a defensive trait as would be the herbivore. Such effects may be very important in the regulation of herbivore populations, as negative effects of a plant resistance trait on natural enemies can effectively negate the benefit of that trait as a plant defense (Ode 2006, Peterson et al. 2016). We also compared the effects of mean and variability of xanthotoxin on the potential for top-down control of herbivores by examining the number of parasitoids (emerged from parasitized hosts) per caterpillar egg (laid by unparasitized moths) in the generation following the study. While diet variability can reduce herbivore fitness (Wetzel et al. 2016, Pearse et al. 2018), it remains

unknown whether this will also limit the potential for top down control through negative effects on higher trophic levels.

3.3 METHODS

3.3.1 Insect colonies

The cabbage looper is a polyphagous caterpillar, feeding on plants across several different families, including several members of the Apiaceae (Sutherland and Green 1984) that contain xanthotoxin. *Copidosoma floridanum* is a polyembryonic egg-larval parasitoid of several noctuid moths (Noyes 1988). Polyembryonic parasitoids lay either one egg (male or female) or two eggs (one male and one female) inside the egg stage of its host. Once the host egg hatches, embryos produced by these parasitoid eggs clonally multiply forming as many as a few thousand individuals per host before finally pupating within the mummified cuticular remains of the host's final instar (Strand 1989, Ode et al. 2018).

Laboratory colonies of *T. ni* and *C. floridanum* were maintained in separate Sanyo MIR-554 environmental chambers set at 25° C and 16L:8D photoperiod (30% RH) and reared for several generations on artificial diet before use in experiments. Adult *T. ni* were kept in 3.78 L plastic containers and provided with cotton soaked in 10% honey water as a food source. The top of each container was covered with a paper sheet on which *T. ni* adults laid eggs; these egg sheets were changed daily to provide *C. floridanum* females young host eggs for oviposition. Some egg sheets were exposed to adult female *C. floridanum* and then placed in petri dishes and kept at 25° C until hatching. Unparasitized egg sheets were surface sterilized with a 10% bleach solution and then air-dried. After hatching, first instar *T. ni* larvae (both parasitized and unparasitized) were transferred to 37 mL plastic cups (Solo® soufflé cups [Dart Container Corp., Mason, MI, USA]; two larvae per cup) containing ~15 ml artificial diet (modified from Shorey

and Hale 1965; Table 1), and reared until pupation. Moth pupae were collected and placed in the adult rearing containers where they were allowed to emerge. Mummies formed from parasitized caterpillars were kept in 15 ml glass test tubes plugged with a cotton stopper and allowed to emerge. Because *C. floridanum* adults generally only live a few days, broods were usually used within only six hours of emergence.

3.3.2 Experimental design

Newly emerged (< 6 hours old) adult *C. floridanum* females were allowed to oviposit on egg sheets containing less than 12-hour old *T. ni* eggs. *Copidosoma floridanum* lays mostly mixed sex broods when mated (Ode and Strand 1995). To ensure that some all-male broods were produced, some of the eggs were parasitized with unmated females; unfertilized eggs develop into males due to the haplodiploid development of wasps. Paper towels with parasitized eggs were then placed in petri dishes at 25° C until hatching.

Upon hatching, parasitized first instar larvae were placed in individual 37 mL SOLO® soufflé cups containing ~15 ml of artificial diet. Artificial diets used in the experiments only differed from the diet used for colony maintenance (see above) in the addition of xanthotoxin with concentrations of 0.5, 1.0, 1.5, or 2.0 mg of xanthotoxin per gram of diet. To establish the overall impact of xanthotoxin concentration on parasitoid fitness, individual parasitized first instar caterpillars were placed on artificial diet containing 0, 0.5, 1.0, 1.5, or 2.0 mg xanthotoxin/g diet (n = 54, 55, 55, 52, 52 respectively). Caterpillars were weighed on the sixth day of development to measure growth differences across the diet treatments. Larvae were reared on the same diet until mummies were formed. The values from this experiment were used to construct curves of the mean effect of xanthotoxin concentration on *C. floridanum* fitness measures to use as a baseline comparison for the fitness effects of variable diets.

To test the effects of xanthotoxin variability, larvae were switched every three days between two diets which had a combined average of 1.0 mg xanthotoxin/g diet. Diet pairs represented either high variability (0 and 2.0 mg xanthotoxin/g diet), low variability (0.5 and 1.5 mg xanthotoxin/g diet, or no variability (both 1.0 mg xanthotoxin/g diet) (n = 80 per treatment). Treatments varied in the magnitude of variation, but not the mean, of xanthotoxin experienced by the caterpillars. Half of the larvae started on the higher concentration of xanthotoxin first, while the other half were placed on the lower diet first to account for any initial diet effects. On the sixth day, prior to switching diets, caterpillars were weighed to measure developmental growth rate differences between treatments. Larvae that died after day six were dissected to confirm parasitism status. The small number of larvae that were not parasitized (3/240 in variability, 17/268 in mean experiment) were removed from any further analyses.

After mummy formation, mummies were placed in a glass test tube fitted with a cotton stopper and kept at 25° C until adult eclosion. Adult wasps were allowed to emerge for 24 hours, after which the emerged adults and mummified host remains were frozen at -20˚C. In each brood of adult *C. floridanum*, the first 200 wasps were sexed and the remaining adults were counted. Mummies were dissected to determine the number of unemerged larvae, pupae, and pharate adults and combined with the number of emerged adults to determine total brood size. Brood size, the total combined number of emerged and unemerged *C. floridanum* individuals from a single host, was used as a direct measure of parasitoid fitness.

3.3.3 Statistical analysis

Statistical analyses were performed using R version 3.6.2. Survival (coded as yes or no for each brood) was analyzed using logistic regression with xanthotoxin concentration (or treatment for the variability experiment) as a predictor. Generalized linear models and AIC

model selection criteria were used to fit the baseline curve shape for all other fitness measures. Analyses were performed with xanthotoxin concentration as a single predictor variable for baseline fitness curves using the linear model (lm) function in R. Variability treatment effects on day six mass, pupation time, and brood size (response variables) were compared with one-way ANOVA, with treatment as the predictor, using the lm function analyzed with type III sum of squares. Emergence was analyzed using a logistic regression with proportions as the response variable. Brood types were combined in all analysis since there was no effect of brood type on any response variables tested. Results are given with mean and standard error (mean ± SE).

We also wanted to project the effects of variable diets on population dynamics of the parasitoid and host using an estimate of subsequent generation parasitoid-host ratio (i.e. adult wasps per host egg). Lifetime egg production data of *T. ni* (from Pearse et al. 2018) was combined with the brood size and survivorship data presented here on *C. floridanum*. We projected the effects of variable toxins on herbivore population dynamics using the combined impacts of variable defense on herbivores and parasitoids. Parasitized hosts failing to yield any adult parasitoids were considered as having an emerged brood size of zero. These data were combined with the number of emerged adult parasitoids to calculate average emerged brood size. The average brood size in each mean and variability treatment was divided by the corresponding lifetime egg production of *T. ni* to calculate adult wasps per host egg. This calculation uses both the subsequent generation of adult wasps (number emerged from a parasitized caterpillar) and the eggs laid by an unparasitized individual of the same generation (from Pearse et al. 2018). This accounts for loss of fitness in the next generation through brood size or egg production due to xanthotoxin effects. Standard error was propagated based on the individual errors from the respective data sets. Since the 1.0 mg/g xanthotoxin treatment in the mean experiment and the no

variability treatment in this study are equivalent other than switching and handling of caterpillars, which was not performed for the mean experiment, these two values were used to normalize the two experiments. The emerged wasp averages in the variability treatment were multiplied by the difference between no variability and the 1.0 mg/g treatments (a factor of 1.78).

3.4 RESULTS

3.4.1 Constant diet experiment

The likelihood that any *C. floridanum* successfully emerged significantly declined when their hosts fed on diets with higher mean concentrations of xanthotoxin (Figure 3.1A). Only 8.7% of the parasitized hosts produced any adult wasps when fed diets containing the highest concentrations of xanthotoxin (2.0 mg/g). Most mortality was caused by death of the host prior to mummy formation with the host being 13.5 times more likely to die on 2.0 mg/g diet than the no xanthotoxin diet ($\chi^2 = 38.00$, df = 1, $p < 0.0001$). Only 13.1% of total brood mortality in all mean treatments combined occurred after mummy formation. Stresses of parasitism caused high mortality of hosts even in diets containing no xanthotoxin where only 60.9% of hosts survived to form mummies.

Xanthotoxin had sublethal effects on parasitoid fitness with increasing concentration as well. Day six mass of host caterpillars decreased logarithmically with increasing xanthotoxin levels (Figure 3.2A). Average host mass ranged from 9.02 ± 0.79mg on diets with no xanthotoxin to only 1.39 ± 0.25mg with 2 mg/g xanthotoxin in the diet. Xanthotoxin concentration also increased the amount of time to pupation (Figure 3.3A). Pupation time ranged from 16.9 ± 0.22 days on average on 0 mg xanthotoxin diet to 22.8 ± 0.61 days on 2.0 mg xanthotoxin diet and larger larvae tended to pupate faster (Figure 3.4). For brood size, levels of xanthotoxin in the host diet had no impact on brood size until concentrations were greater than

1.0 mg/g, leading to two distinct clustered groups (Figure 3.5). Brood size on 1.0 mg/g or lower xanthotoxin diets averaged 1366.4 ± 59.5 individuals per host while the average of the higher xanthotoxin diets was about 40% less with only 797.7 ± 82.7 individuals per host. Emergence was similar, with broods having similar emergence at 1.0 mg/g or lower concentration diets and far lower emergence on the two higher xanthotoxin diets (Figure 3.6). This is likely because brood size was strongly correlated with emergence (logistic regression: $\chi^2 = 24.197$, df = 1, $p < 0.0001$). Smaller broods tended to have poor emergence, since these broods often leave too much host tissue unconsumed preventing the adult wasps from successfully emerging (Ode and Strand 1995).

3.4.2 Variable diet experiment

Conversely, variability had little to no impact on parasitoid fitness. The magnitude of xanthotoxin variability had no impact on *C. floridanum* brood survivorship (Figure 3.1B). Survivorship in all variability treatments was consistent with survivorship on the 1.0 mg/g xanthotoxin constant diet treatment. Again, most mortality occurred before parasitoid pupation, with 84.1% of total mortality caused by host death. In addition, there was no effect of variability on host growth (Figure 3.2B) with a combined treatment average day six mass of 3.1 ± 0.15mg which was very similar to the average on the 1.0 mg xanthotoxin treatment in the mean experiment. Starting diet did not influence day six mass for any treatment in the variability experiment ($F = 1.68$, df = 4, $p = 0.1571$). Larger hosts again had lower pupation time but variability of xanthotoxin in the diet did not impact parasitoid pupation time (Figure 3.3B). Average pupation time with all variability treatments combined was 20.81 ± 0.26 days. Despite the effect of high constant xanthotoxin on brood size, this was not affected by variability either (Figure 3.5B). Average brood size across all variability treatments combined was nearly 1100

individuals per host. Emergence proportions were similar with emergence being equivalent across all variability treatments (Figure 3.6B).

3.4.3 Calculated effects on next generation

By combining these results and those from Pearse et al. (2018) on the egg production of *T. ni,* we calculated the parasitoid pressure on the next generation of herbivores in variable systems as the number of adult wasps per host egg. Due to the larger brood sizes and higher emergence resulting from caterpillars in the mean experiment, the results of the variability experiment were normalized to the mean experiment results (see methods). More variable diets had many more parasitoids per host than non-variable diets. Low variability and high variability diets had nearly twice the parasitoid pressure of non-variable diets (Figure 3.7). The lowest parasitoids per host egg were in the 2.0 mg/g treatment where host egg production was low, but high mortality and low brood size of parasitoids resulted in very low overall parasitoid densities.

3.5 DISCUSSION

Our results demonstrate the reduction in consequences of plant defense variability at higher trophic levels. The polyembryonic parasitoid *C. floridanum* experiences little effects from variation in xanthotoxin levels in the host diet. This was despite the clear negative effects of high levels of xanthotoxin on parasitoid fitness including slower development, decreased brood size, poor emergence, and low overall survivorship of hosts and broods. However, when parasitoids were reared in hosts feeding on diets with varying levels of xanthotoxin, parasitoids performed similarly regardless of the difference in magnitude of variation.

It is possible that the parasitoids do not actually experience the variability in the host. Parasitoids feed primarily on the hemolymph and at least some portion of xanthotoxin in the caterpillar's diet enters the hemolymph (Lampert et al. 2011). Yet if the amount (or rate) of

unmetabolized xanthotoxin entering the hemolymph during herbivore feeding is constant, parasitoids may not experience the same variability as the host. Regardless of whether parasitoids experience variability, the host was expected to still be negatively affected by variability as previously shown (Pearse et al. 2018). While host day six mass follows an extremely similar trend to previous results for constant xanthotoxin levels, no negative emergent effect of variability was observed for day six mass. Caterpillar mass was instead closer to the mean value of variability treatments. This suggests parasitism changes the effect on the host as well, possibly by regulating the feeding rates of the host to mitigate the effects of a changing diet. Therefore, caterpillars may not experience the variability when parasitized, leading to the lack of variable diet effect compared to that observed for unparasitized caterpillars (Pearse et al. 2018). Alternatively, it is possible that the weight increase from *C. floridanum* broods inside the host outweighed any effects of variability, but we find this unlikely as the parasitoids are still only embryos at such an early stage in development (Strand 1989).

A number of publications have shown negative effects of plant defenses on parasitoids (Ode 2006), to which we can contribute with the clear negative impacts of high xanthotoxin concentrations on fitness and survival of *C. floridanum*. Yet we present novel evidence for reduced impact of defense on a parasitoid compared to the effects on its host herbivore (as shown by Pearse et al. 2018). Here, we show that the effects of variation in plant defenses are reduced with increasing trophic level. This could have enormous consequences for the role of variation in tri-trophic interactions and population dynamics. Herbivores may experience increased bottom-up control from the emergent effects of variable defenses while maintaining the top-down effects of parasitoids which are relatively unaffected by variable defense. Thus, population growth of

herbivore populations would be severely limited in environments where individual herbivores experience variation in plant defenses.

Our results combined with those from Pearse et al. 2018 show that parasitoid individuals per host egg are much higher in the variable environments than those on the constant mean (Figure 3.7). While high constant defense environments may minimize herbivore performance, these environments still have intense negative consequences for parasitoid performance, including very high mortality of the host and also the parasitoid (which would have killed the host eventually anyways). In low defense environments, parasitoids are more successful, but the high performance of herbivores outweighs the increases in parasitoid performance and resulting parasitoid to host ratios are still lower than on variable systems. Since parasitoids are relatively unaffected by xanthotoxin up to 1.0 mg/g in the hosts diet, the detrimental effects of intermediate xanthotoxin on herbivore success leads to the highest parasitoid pressure among constant defense environments. However, increasing defenses even slightly could lead to harsh negative consequences for parasitoids. Overall, herbivore suppression would be maximized in highly variable environments which have negative emergent effects on herbivore fitness but do not heavily impact parasitoid fitness.

The decreased diversity of most agricultural systems results in enormous pressure from insect pests (Altieri and Nicholls 2004). Here, we demonstrate that defense variability, by not influencing parasitoids and negatively influencing herbivores, provides greater relative top-down impact from parasitoids. Variability can constrain herbivores directly through physiological mismatch (Pearse et al. 2018, Wetzel and Thaler 2016), and indirectly through interactions (or lack of) with other trophic levels. Riolo et al. modeled the effects of heterogeneity in quality of plant patches for herbivores, showing that even small variations in diversity of patches created

dynamic changes in herbivore and natural enemy communities (Riolo et al. 2015). More diverse patches increased parasitoid recruitment and led to lower host densities and higher parasitoid to herbivore ratios. Our results provide further support for increased herbivore suppression with increasing plant diversity.

We also suggest considering the scale of this variability for increasing herbivore suppression. Here, we focused on variation for individual herbivores, which will experience variability spatially and temporally during development through defense induction and moving to different tissues of the same plant or between plants. Thus, systems which incorporate diversity within plants or between neighboring individual plants, are likely to maximize the bottom-up and top-down effects of herbivore suppression. We have demonstrated that variability in plant defenses has little impact on parasitoid performance and therefore, increasing variability in defenses will suppress herbivore performance directly through time dependent effects (Pearse et al. 2018) while maintaining the potential for top-down herbivore suppression.

Table 3.1: Diet Recipe. Ingredient amounts are for each diet and are listed used in order of addition. All ingredients were mixed with a Waring industrial blender. Xanthotoxin was added for experimental diets but not colony rearing.

Ingredient	**Amount**	**Source**
Soy Flour	12.5 g	Bio-Serv
Wheatgerm	37.5 g	Bio-Serv
Casein	25 g	Bio-Serv
Torula Yeast	31.25 g	Bio-Serv
Alfalfa meal	25 g	Bio-Serv
Ascorbic Acid	3 g	Bio-Serv
Sorbic Acid	1.5 g	Bio-Serv
Methyl Paraben	2.5 g	Sigma-Aldrich
Tetracycline	62.5 mg	Sigma-Aldrich
Xanthotoxin	0, 0.5, 1, 1.5, or 2 g	Sigma-Aldrich
Pinto Beans + Water (cooked)	62.5 g + 250 mL	
7.4% Formalin	10 mL	Mollinckrodt (Formaldehyde)
Raw Linseed Oil	6.75 mL	Sonyside
Agar (heated with water)	11.25 g + 500 mL	MoorAgar
Vitamin Mix	5 g	Bio-Serv

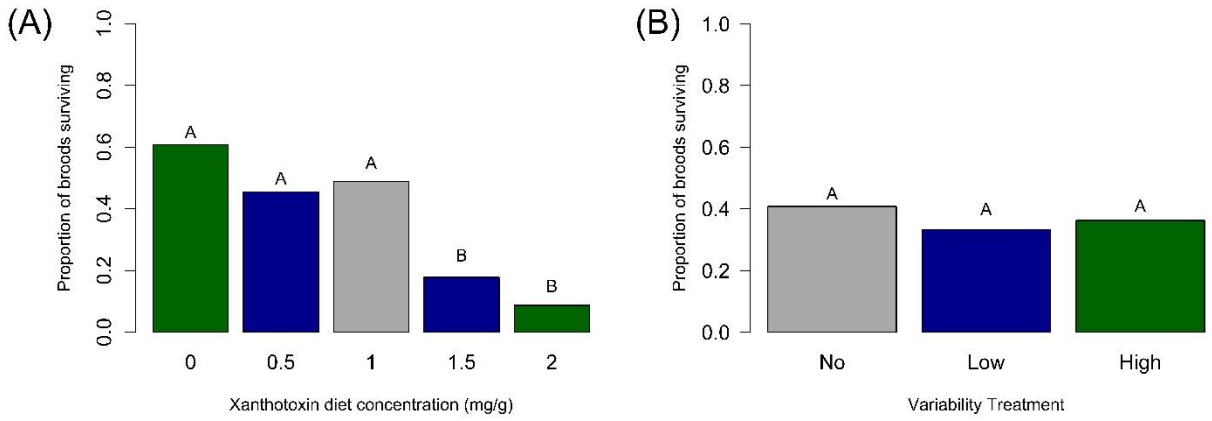

Figure 3.1. *C. floridanum* brood survivorship on constant xanthotoxin diets (A) and variable diets with a mean of 1.0 mg xanthotoxin/g of diet (B). Bars are calculated from exact proportions of broods surviving out of the total. Pairwise comparisons were performed as post-hoc analysis of odds ratios following logistic regression (A: LR $\chi^2 = 38.71$, df = 1, $p < 0.0001$; B: LR $\chi^2 = 1.416$, df = 2, $p = 0.4926$). Bars with different letters above are significantly different groups.

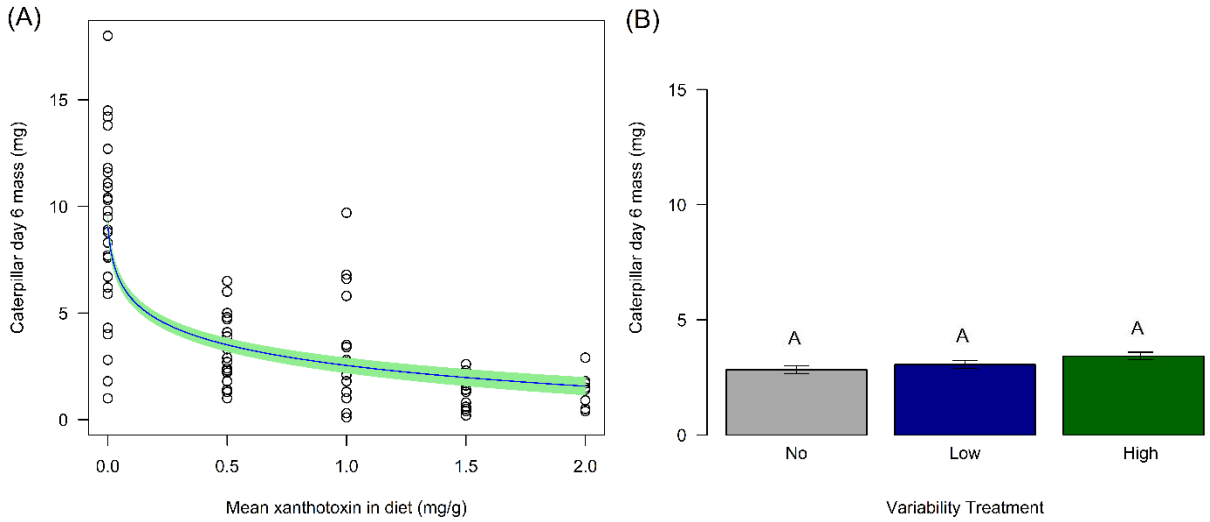

Figure 3.2. Host mass on the sixth day of development on constant xanthotoxin diets (A) and variable diets (B). Regression line is a logarithmic function of xanthotoxin concentration with a small added value (0.01) to account for zero values and mass as the response variable ($F_{1,82}$ = 104.01, $p < 0.0001$). There were no significant differences between variable diet treatments (one-way ANOVA; $F_{2,189}$ = 1.24, $p = 0.2918$). Error bars displayed are standard error.

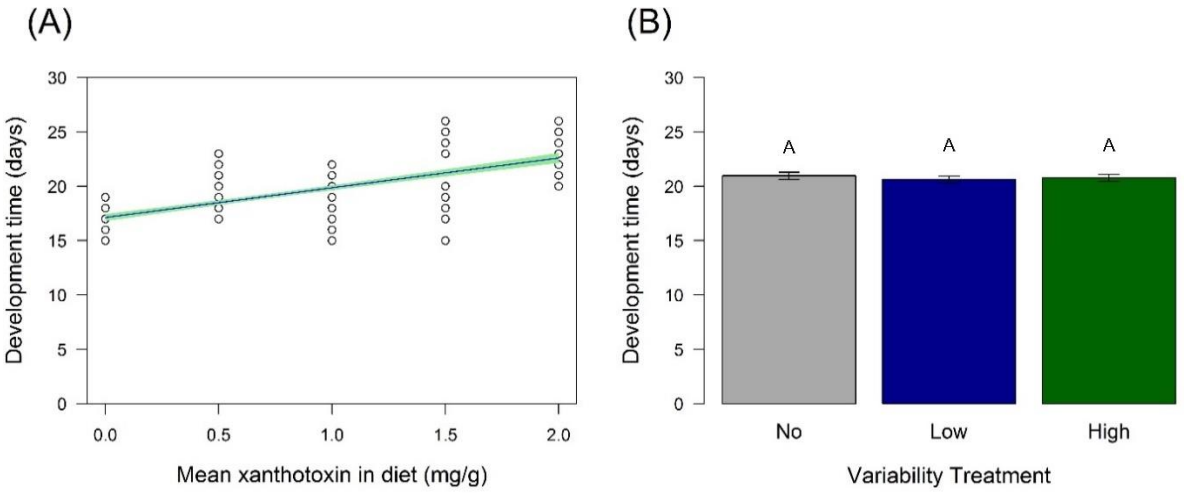

Figure 3.3. Parasitoid development time on constant xanthotoxin diets (A) and variable diets (B). Line fit is based on a linear regression of xanthotoxin concentration versus development time. ($F_{1,39} = 55.44$, $p < 0.0001$). There were no significant differences between treatments in the of variable diets (one-way ANOVA, $F_{2,96} = 0.14$, $p = 0.873$). Error bars displayed represent standard error.

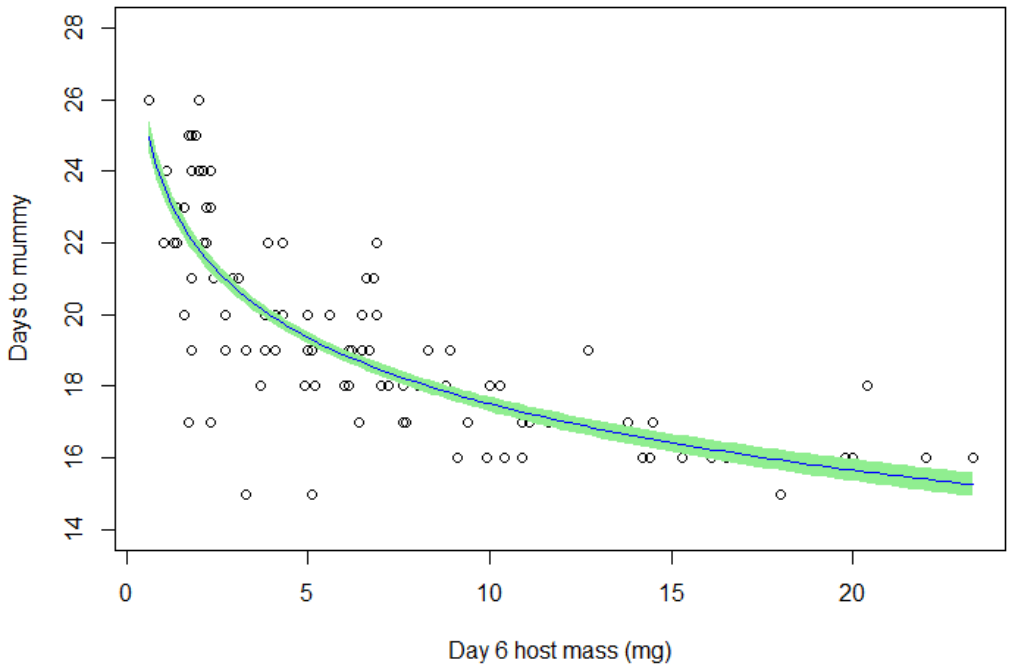

Figure 3.4. Relationship between host day six mass and parasitoid development time. Fit regression line represents development time as a logarithmic function of caterpillar mass at day six ($F_{1,38} = 78.26$, $p < 0.0001$).

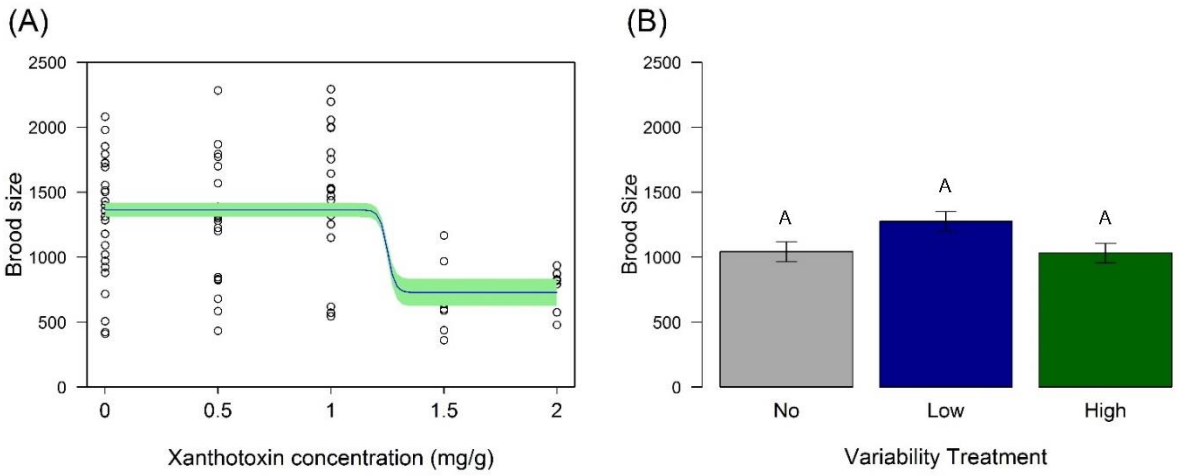

Figure 3.5. Parasitoid brood size on constant xanthotoxin diets (A) and variable diets (B). A logistic curve was fit in the mean experiment with brood size as a function of xanthotoxin concentration ($F_{1,85} = 28.82$, $p < 0.0001$). No significant differences were found between variable diet treatments (one-way ANOVA, $F_{2,79} = 2.49$, $p = 0.0896$).

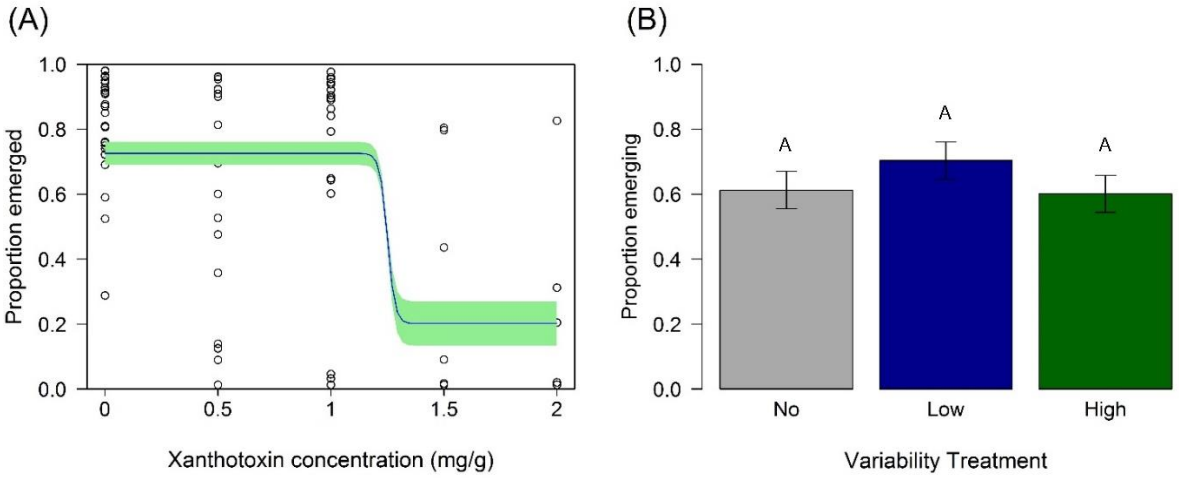

Figure 3.6. Emergence success on constant xanthotoxin diets (A) and variable diets (B). Proportions were analyzed using logistic regression of proportion with treatment as a predictor ($\chi^2 = 13.07$, df = 1, p = 0.0003). Pairwise comparisons were performed as post-hoc analysis of odds ratios following logistic regression ($\chi^2 = 1.71$, df = 2, p = 0.4251). No significant differences were found between variable diet treatments.

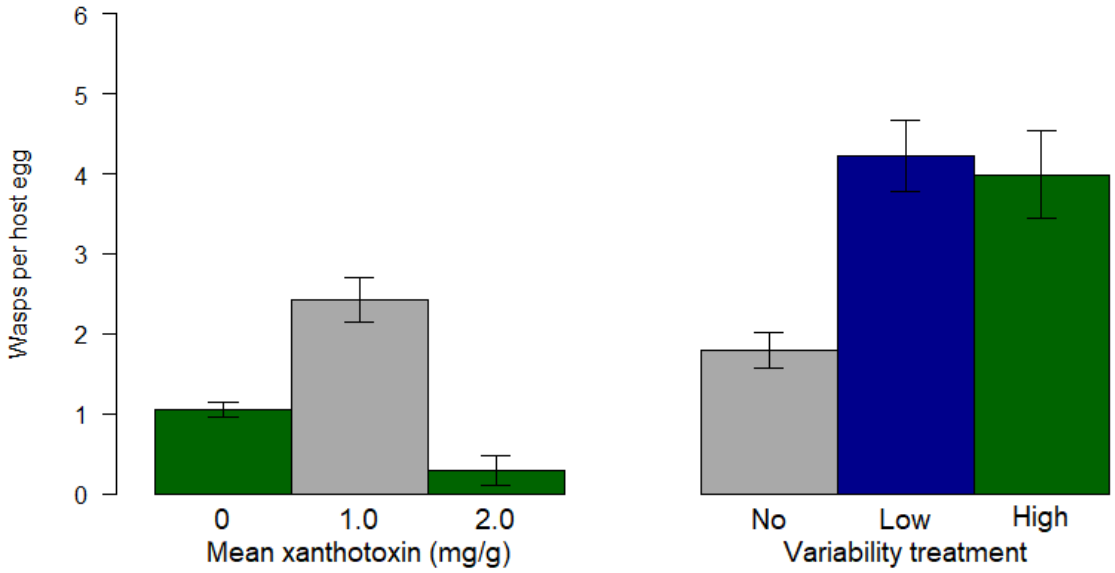

Figure 3.7. Parasitoids per host egg in second generation after feeding on variable diets. Values were calculated by combining brood data and lifetime fecundity data from Pearse et al. 2018. Left bars are calculated from constant xanthotoxin diets and right bars are based on the variability treatments. Error bars were propagated from the errors of the respective data sets.

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CHAPTER 4: PLANTS INDUCE DEFENSE CHEMICALS BASED ON IDENTITY OF PARASTIOID ATTACKING AN HERBIVORE

4.1 SYNOPSIS

Plants are capable of inducing increased levels of defensive chemicals in response to herbivore damage. These defenses can have consequences for not only the herbivore, but also their parasitoids. Many parasitoids are thought to increase plant fitness, by decreasing herbivore feeding in the near term or reducing herbivore populations in the long term, making defenses which negatively impact parasitoids disadvantageous for the plant. This is particularly true of solitary parasitoids (where only one individual completes development per host) that cause their hosts to feed less than unparasitized herbivores. However, gregarious parasitoids (where more than one individual develops per host) can decrease plant fitness if parasitized herbivores feed more than unparasitized herbivores. We explored the relationship between plant defense induction and caterpillars parasitized by solitary and gregarious parasitoids. We wanted to determine: 1) whether plants induced higher chemical defense against a gregarious parasitoid that increases herbivore feeding and 2) whether differential responses to being fed on by herbivores parasitized by solitary or gregarious parasitoids were based on feeding amount or identity of parasitized caterpillars. We tested induction of glucosinolate defenses in *Brassica rapa* using *Pieris rapae* caterpillars at equivalent size and development stage parasitized by either a solitary (*Cotesia rubecula*) or gregarious (*C. glomerata*) parasitoid. We demonstrate that *C. glomerata*-parasitized *P. rapae* elicit an increase in glucosinolate production by plants independent of the amount of feeding damage. Feeding by caterpillars of different instars parasitized by *C. glomerata* induced similar levels of glucosinolate production. These results

provide insight into the influence of parasitoids on plant defense expression and indicate the need for considering parasitoids in understanding plant defense and herbivore interactions.

4.2 INTRODUCTION

Feeding damage by herbivory often induces increased production of defensive chemicals in plants (Baldwin 1998, Textor and Gershenzon 2009, Stowe and Marquis 2011) with more damage corresponding to greater induction response (Karban and Baldwin 1997). While this leads to negative consequences for herbivores (Agrawal 1999, van Dam et al. 2000, Textor and Gershenzon 2009), it may also negatively affect natural enemies such as parasitoids (Ode 2006). Parasitoids develop on or inside an herbivore host and provide indirect defense for plants by ultimately killing the herbivore (Bustos-Segura et al. 2019). These relationships between plant defenses and parasitoids present a trade-off for plants in balancing defense against herbivores while minimizing loss of indirect defense from parasitoids. In theory, parasitoids may influence expression of plant defenses by altering the amount of tissue consumed by their herbivorous host. Yet, to date, only a few studies have explored the impact of herbivore parasitism on expression of plant defenses (Poelman et al. 2011, Ode et al. 2016).

Many parasitoids provide benefits to plant fitness (van Loon et al. 2000, Ode 2006, Smallegange et al. 2008, Bustos-Segura et al. 2019), but sometimes do not (Coleman et al. 1999, Ode et al. 2004, Smallegange et al. 2008). Solitary parasitoids, in which one larva develops per host, often emerge from earlier stages of the host and greatly reduce the amount of feeding by the host (Harvey et al. 1999, van Loon et al. 2000, Harvey 2005). However, gregarious parasitoids, in which many larvae develop per host, can cause hosts to feed as much or more than unparasitized hosts (Strand 1989, Coleman et al. 1999, Harvey 2005). As such, these parasitoids may not provide a benefit to the plant, and the negative impacts on plant fitness increase with

parasitoid brood size (Coleman et al. 1999, Smallegange et al. 2008). The immediate benefits of indirect defense can therefore be lost with gregarious parasitoids. Differences in the amount of herbivore feeding caused by gregarious versus solitary parasitoids could in itself influence plant responses, causing greater induction in response to gregarious parasitoids. For instance, feeding damage by cabbage loopers parasitized by the gregarious parasitoid *Copidosoma floridanum* induced higher amounts of indole glucosinolates compared to unparasitized hosts. In turn, increased concentrations of indole glucosinolates were correlated with decreased fitness of the parasitoid (Ode et al. 2016).

While parasitoid identity may affect the amount of plant tissue consumed by a parasitized herbivore and therefore the level of induced defense responses by plants, whether parasitoid identity irrespective of the amount of damage to the plant can influence induction of plant defenses is unknown. Yet precedence for this idea exists in a handful of studies that have shown that parasitoid identity determines plant volatile production after herbivory, through parasitoid-specific changes in oral secretions (Poelman et al. 2011, 2012, Cusumano et al. 2019). While most feeding damage by herbivores, parasitized or unparasitized, occurs later in development (Harvey 2005), initial plant induction responses begin much earlier. If plants are able to differentially respond to herbivores parasitized by different parasitoids, they likely do so earlier in development (Karban et al. 1999).

In this study, we explored the induction response of glucosinolate defenses in *Brassica rapa* plants to feeding by unparasitized *Pieris rapae* caterpillars or caterpillars parasitized by either the gregarious *Cotesia glomerata* or the solitary *C. rubecula* (Hymenoptera: Braconidae). Like many plants in the Brassicaceae, *B. rapa* produces inducible glucosinolate defenses (Abdel-Farid et al. 2010) which are broken down into toxic isothiocyanates upon contact with the plant

enzyme myrosinase as a result of herbivore damage (Hopkins et al. 2009). *Pieris rapae* (Lepidoptera: Pieridae) is a specialist herbivore of glucosinolate-containing plants (Wittstock et al. 2004). *Cotesia glomerata* is a gregarious parasitoid wasp which attacks early instar pierid caterpillars and emerges from the final instar of the host. In many cases, *C. glomerata* can negatively impact fitness of the host plant by causing the host caterpillar to grow larger, particularly when large numbers of eggs have been laid (Coleman et al. 1999, Smallegange et al. 2008). The solitary *C. rubecula* is a specialist parasitoid wasp of *P. rapae* which attacks early instars and emerges from the penultimate, 4th instar of the host, before most feeding occurs (Harvey et al. 1999, van Loon et al. 2000).

We tested whether *B. rapa* plants can differentially induce glucosinolate defenses in response to parasitized and unparasitized *P. rapae* and whether any differences were due to the amount of herbivore feeding or identity of the parasitoid. Specifically, we explored whether caterpillars parasitized by the gregarious *C. glomerata* elicited different plant induction responses than the solitary *C. rubecula* parasitized caterpillars or unparasitized caterpillars based on parasitoid specific cues (such as oral secretions) rather than differences in feeding amount. We predicted *B. rapa* plants would induce higher levels of glucosinolates when fed on by *C. glomerata* parasitized caterpillars. By using caterpillars of equal size across parasitism treatments (which also fed the same amount), we show that parasitoid identity, more than amount of caterpillar feeding, is largely responsible for observed defense metabolite induction effects. We demonstrate that plants can respond differently with defense metabolite production to caterpillars parasitoid by different parasitoids.

4.3 METHODS

4.3.1 Insects and plants

All insects used in the experiments were from laboratory-reared colonies at Colorado State University. Colonies of *P. rapae* and *C. glomerata* were started from adults and parasitized caterpillars, respectively, collected from crucifer plots at the Agricultural Research and Development Education Center in Wellington, CO in 2015. Colonies of *C. rubecula* were started from pupae collected on crucifers near the University of Minnesota campus in St. Paul, MN in 2015. All laboratory colonies were periodically supplemented with field collected individuals throughout the study.

Caterpillars were reared on collard green plants (*Brassica oleracea,* "Georgia Southern") in a greenhouse with a 16L:8D photoperiod (18.3-26.7° C, 40% RH) prior to use in experiments. Parasitoid colonies were maintained in Sanyo MIR-554 environmental chambers set at 15° C (16L:8D photoperiod, 30% RH) until used for parasitism at room temperature. For parasitized treatments of both wasp species, caterpillars were parasitized individually as 2nd instars and then returned to collard green plants until reaching the appropriate age for the experiment (4th or 5th instar). Parasitism was confirmed by observing oviposition on caterpillars in each replicate. Each caterpillar was removed immediately after oviposition to avoid superparasitism. Parasitism status of caterpillars was confirmed either by presence of visible larvae through the host cuticle (in late parasitoid instars), by emergence of parasitoids, or by dissection. If parasitoids were not found via dissection in parasitized treatments, these replicates were assumed to not be parasitized and were removed from analyses.

Experimental plants were grown from *Brassica rapa dichotoma* seed (accession PI 649170, origin India) supplied by the USDA-GRIN seed database. Plants were grown in four-

inch pots and supplied with 5g of Osmocote fertilizer (14-14-14) at the time of planting. Plants were 4-6 weeks old with several fully expanded leaves when used for the experiment. Only *B. rapa* was used for experiments examining glucosinolate induction.

4.3.2 Experimental design

4.3.2.1 Induction of glucosinolates in *B. rapa*:

All caterpillars were weighed before being placed on plants in the experiment. Caterpillars were used as 4^{th} or 5^{th} instars and were categorized by size and parasitism for five total caterpillar treatments: 4^{th} instar (n=34) and 5^{th} instar (n = 26) *C. glomerata*-parasitized *P. rapae*, 4^{th} instar *C. rubecula*-parasitized *P. rapae* (n = 30), and 4^{th} instar (n = 21) and 5^{th} instar (n = 15) unparasitized *P. rapae*. As *C. rubecula* kill and emerge from the host 4^{th} instar, there was no 5^{th} instar *C. rubecula*-parasitized *P. rapae* treatment. Initial pre-feeding leaf samples were taken from each plant immediately before placing caterpillars on the plants to establish a glucosinolate baseline for determining induction effects. Caterpillars were placed on individual plants (one caterpillar per plant) in 34x34x61cm mesh cages under a 16L:8D photoperiod in a greenhouse (18-27° C, 40% RH) and allowed to feed for three days. Care was taken to place caterpillars on a leaf of approximately the same age as the pre-feeding sample leaf.

After the three-day feeding period, caterpillars were removed, and post-feeding leaf samples were taken from damaged leaves that were similar age to the initially sampled leaf. Any damaged leaves were removed and photographed. Leaf margins of damaged leaves were redrawn in pictures and the area of removed tissue was calculated using ImageJ software (U.S. National Institutes of Health) to quantify feeding damage. All caterpillars were weighed again after removal from the plant except those from which parasitoids emerged or butterfly pupae were formed (n = 16 caterpillars emerged parasitoids or formed pupae) as these pupal stages generally

result in loss of mass compared to caterpillars creating inaccurate estimates of weight gain. Parasitism of caterpillars was confirmed visually (as large parasitoids can be viewed through the host cuticle), by emergence of parasitoids, or by dissection. Leaf samples were taken from control plants (n = 15) and caged in the same manner as treatment plants but did not experience herbivory in the three days between samples. These plants were used to determine if the sampling method caused any induction responses.

4.3.2.2 Comparison of parasitoid survival on collards and *B. rapa*

In order to explore the effects of plant chemistry on parasitoid survival, we compared brood sizes of *C. glomerata* and survival of *C. rubecula* larvae in caterpillars feeding on the experimental plants, *B. rapa* with parasitoid larvae from caterpillars feeding on cultivated *B. oleracea* (collard greens). These collards contain very low levels of glucosinolates (R. Paul unpublished data). Collards were used as a low chemistry alternative food plant. Caterpillars were parasitized and reared until 4th instar as mentioned previously. Upon reaching 4th instar, caterpillars were randomly split into two groups and one group was placed on a *B. rapa* plant and the other on a collard. Caterpillars were allowed to feed under greenhouse conditions for three days as previously described and then all caterpillars were removed and dissected. Brood size for *C. glomerata* was calculated as the number of intact (living) *C. glomerata* larvae in each host. Caterpillars parasitized by *C. glomerata* in which no parasitoids were found were removed from analysis as it is unclear at what point in development parasitoids were killed. Survival of *C. rubecula* was assessed on presence or absence of the single larva. Another set of caterpillars were parasitized by *C. glomerata* as 2nd instars as described and then immediately dissected to determine clutch size (number of eggs laid) before any caterpillar immune responses could occur. The primary immune response of caterpillars against parasitoids is encapsulation, where

parasitoids are surrounded and suffocated by melanized hemocytes (Godfray 1994). Presence of encapsulation was recorded in all caterpillars in this experiment. Encapsulation is not visible after three days (R. Paul and D. Vyas pers. obs.), so any encapsulation observed in the dissections occurred after the start of experimental feeding.

4.3.3 Glucosinolate analysis

Leaf samples were collected from each plant by removing an entire fully expanded leaf (to help avoid induction response from sampling) and tearing off the end of the removed leaf. The torn piece was then rolled into a centrifuge tube to prevent further damage, flash frozen in liquid nitrogen, and then stored at -80° C to prevent further damage until processing for glucosinolate analysis. Frozen leaf samples were first dried in a lyophilizer for 48 hours. Freeze-dried leaf tissue ($\bar{x}$ = 44.1 mg ± 0.1 SE) was then weighed and ground to a fine powder by adding a small metal ball to each tube and using a Qiagen Tissulyser set at 30 times per second for two minutes. To each tube, 1mL of 70% methanol was added along with 50µL of a 5mM sinigrin internal standard solution to calculate sample differences in extraction efficiency. Absence of natural sinigrin in *B. rapa* was confirmed in preliminary glucosinolate analysis of leaf tissue from several plants. Samples were vortexed and then sonicated for six minutes in a room temperature water bath. Following sonication, the samples were centrifuged for 10 minutes at 13,200 rpm and the supernatant was added to an anion exchange column. This extraction process was then repeated a second time (minus the standard addition) to maximize collection of glucosinolates and this supernatant was also added to the column.

Columns were constructed from 1000mL pipette tips stuffed with glass wool. To each column, 0.5mL of DEAE-Sephadex slurry (A25 chloride form, Millipore Sigma; 5g sephadex in 75 mL of water) was added (as the anion exchanger) and allowed to drain before adding the

supernatants. Once both sets of supernatants were added, the columns were rinsed twice with 1mL of methanol and twice with 1mL of pure water. During this process, glucosinolates remain bound to the Sephadex, allowing other plant compounds that are not bound to be washed out. Next, the bottom of each column was capped and 50µL of prepared sulfatase was added and then the top was covered with parafilm and left overnight. Enzyme activity removes sulfate groups from the glucosinolates, releasing them from the anion exchanger. The following day, columns were washed with 2mL of HPLC water and flow-through collected. These samples were dried on a vacufuge at 45° C and then reconstituted in 100µL of water to concentrate samples for analyses by UHPLC-PDA-MS.

4.3.4 UHPLC-PDA-MS/MS analysis and quantification

Chemical analysis of glucosinolates was performed using a Shimadzu LC-30AD Liquid Chromatograph with SIL-30AC autosampler and SPD-M30A photodiode array detector (PDA). A Shimadzu LCMS-8040 mass spectrometer was used to confirm identities of glucosinolates. Separation was achieved using a Phenomenex NX-C18 column (100mm, 2.0mm I.D., 3µm pore size) and a pure water-acetonitrile gradient (Table 1) with a flow rate of 0.4mL/min. Quantities were measured by PDA using UV absorbance at 229nm. Compound identities were deduced from established literature and confirmed by retention time and coupled MS/MS analysis using the H^+-desulfoglucosinolate ion for each compound with 35eV ionization energy. Individual glucosinolate quantities were calculated with UV absorbance chromatogram peak areas. Pre- and post-feeding samples were standardized using quantities of the internal sinigrin standard and absolute values were calculated by standardizing to an external sinigrin standard. Individual glucosinolate amounts were calculated by multiplying quantities by the established response factors for individual compounds (Grosser and van Dam 2017).

A total of six glucosinolates were found in *B. rapa* plants: gluconapin (GNP), glucocochlearin (GCC), glucobrassicanapin (GBN), glucobrassicin (GBR), 4-methoxyglucobrassicin (MET), and neoglucobrassicin (NEO). Half of these were classified as aliphatic glucosinolates, derived from the amino-acid methionine: GNP, GCC, GBN. The other half were indole glucosinolates, derived from tryptophan: GBR, MET, NEO. GNP was the most abundant compound with nearly an order of magnitude greater initial concentration than the other glucosinolates (Table 3.2; contrast: $t = 6.703$, $df = 501$, $p < 0.0001$). GBN was second most abundant with higher initial concentrations than GCC and MET but all other glucosinolates had similar initial concentrations (Table 3.2).

4.3.5 Statistical analysis

Analyses were performed using R Studio (version 1.2.5001) running R version 3.6.2. Model assumptions were checked in all analyses using residual plots following transformations. The effects of parasitism treatment and individual glucosinolate concentrations on caterpillar leaf consumption, growth, and final size were analyzed using separate mixed models for each log-transformed response variable. Comparisons between groups were performed with post-hoc pairwise analyses and p-values were adjusted for multiple comparisons using Tukey HSD with the emmeans package. All analyses for glucosinolate effects as the response variable were split by individual glucosinolates and concentrations were square root transformed prior to analysis. Initial analysis consisted of a mixed model with treatment, leaf area consumed, and time (pre or post) as interactive fixed effects, with a random effect of individual plant, to account for covariation between sequential measurements of the same plant. P-values of time by treatment interaction effects for these models were used to pre-screen for differences and justify pursuing further pairwise comparisons. Paired t-tests were used to analyze differences in pre-feeding and

post-feeding concentrations of glucosinolates within treatments. Significance for paired t-tests was assessed using a Bonferroni adjustment for a family of six tests and corresponding $\alpha = 0.0083$.

To determine the impacts of plant type on *C. glomerata* brood size in the secondary experiment (collards versus *B. rapa*, representing glucosinolate differences), treatments for *C. glomerata* were compared using ANOVA. Individual pairwise comparisons of brood sizes between the two plants (collards and *B. rapa*) and freshly parasitized caterpillars were analyzed using students t-test for each pair analysis. Caterpillars that did not have any parasitoids were excluded from analysis, since it could not be determined at what point the brood was killed and whether that was due to treatment plants or natural host immunity. A logistic regression was fit for examining encapsulation in this experiment with treatment as a predictor. Broods with no intact parasitoids were included only if encapsulation was observed. Encapsulation is only visible for three days (R. Paul and D. Vyas, pers. obs.), and therefore must have occurred during the three-day experiment. All means are presented as mean with standard error (mean ± SE) and significance of effects was assessed using $\alpha = 0.05$. The effect of plant type on *C. rubecula* survival was analyzed by fitting a logistic regression model with survival and plant species as a predictor. Encapsulation was not analyzed for *C. rubecula* as it was never observed in the study.

4.4 RESULTS

4.4.1 Induction of glucosinolates in *B. rapa*

Plants experiencing herbivory by *C. glomerata* parasitized caterpillars showed the strongest induction response. Gluconapin (GNP) was induced by feeding from 4^{th} and 5^{th} instar *C. glomerata*-parasitized caterpillars regardless of caterpillar size (4^{th} instars: $t = 3.75$, $df = 20$, $p = 0.0012$; 5^{th} instars: $t = 3.29$, $df = 16$, $p = 0.0046$) but was not induced by other caterpillar

treatments (Figure 4.1). GNP concentrations were approximately 1.7 and 2.0 times greater after feeding by 4th and 5th instar *C. glomerata*-parasitized caterpillars respectively. Response of glucobrassicanapin (GBN) was similar to GNP where strong induction response was caused by *C. glomerata* parasitized caterpillar feeding for both instars (4th: $t = 4.03$, $df = 20$, $p = 0.0007$; 5th: $t = 3.17$, $df = 16$, $p = 0.0059$; Figure 4.2), resulting in concentrations 2.32 and 2.16 times initial levels for each instar respectively.

GNP and GBN were the only glucosinolates which had any within-treatment differences between pre and post values (Figure 4.3). Plants exhibited high variability in chemical responses to caterpillars but induction of GNP and GBN by *C. glomerata* parasitized caterpillars was more consistent than other glucosinolate responses or caterpillar effects. Control plants, which were sampled but did not experience herbivory, did not differ in glucosinolate concentrations over the three day sample period (total glucosinolates: $t = 0.78$, $df = 75$, $p = 0.8632$).

Induction of GNP and GBN was not affected by differences in weight or feeding amount of caterpillars of the same host-parasitoid combination. Final weight of 4th instar caterpillars after the three days of feeding was comparable across all parasitoid groups (Figure 4.4), but GNP and GBN were only induced by *C. glomerata*-parasitized caterpillars. Similarly, *C. glomerata*-parasitized 5th instars and unparasitized 5th instars achieved approximately the same size (211.3 ± 6.4 and 223.7 ± 7.2 mg, respectively), but only *C. glomerata*-parasitized caterpillars induced significant glucosinolate responses. Larger caterpillars ate significantly more leaf tissue, measured by leaf area (Figure 4.5), with 5th instars of *C. glomerata*-parasitized and unparasitized larvae eating approximately five times more than 4th instars (Figure 4.6). Parasitism did not affect the amount of feeding over the three-day period during the experiment since there was no difference in feeding amounts within the same instar groups. However, *C. rubecula* parasitized

caterpillars gained less mass in the feeding period than either 4th instar unparasitized ($t = 3.20$, $df = 107$, $p = 0.0152$) or 4th instar *C. glomerata* caterpillars ($t = 3.94$, $df = 107$, $p = 0.0013$; Figure 4.7) despite feeding on similar amounts of leaf tissue (Figure 4.6). Overall, leaf damage was positively associated with the amount of mass each caterpillar gained (Figure 4.8). No differences in induction effects were found between 4th and 5th instars within unparasitized or *C. glomerata*-parasitized caterpillars that would indicate size effects on induction.

4.4.2 Comparison of parasitoid survival on collards and *B. rapa*

Brood size of *C. glomerata* was reduced in parasitized caterpillars feeding on *B. rapa* compared to those feeding on collards ($t = 2.50$, $df = 5$, $p = 0.0166$). Brood size from caterpillars feeding on *B. rapa* was on average 16.77 ± 1.71 individuals per host, while brood size from caterpillars feeding on collard greens was 22.92 ± 1.76 individuals per host. Clutch size from freshly parasitized caterpillars was on average 25.50 ± 1.72 individuals per host which was not different from brood size of the 4th instar caterpillars after feeding on collards ($t = 1.03$, $df = 43$, $p = 0.3085$). Active encapsulation of *C. glomerata* larvae was higher in caterpillars feeding on *B. rapa* than collards ($z = 1.85$, $p = 0.0644$). On *B. rapa,* six out of 24 caterpillars showed signs of encapsulation, but encapsulation was only observed in one out of 25 caterpillars that fed on collard greens. Survival of *C. rubecula* was not affected by plant type (LR $\chi^2 = 0.56$, $df = 1$, $p = 0.4552$) with 10/12 *C. rubecula* present in caterpillars on collards and 14/15 present in caterpillars on *B. rapa.* Encapsulation was not observed in any *C. rubecula*-parasitized caterpillars.

4.5 DISCUSSION

Our results demonstrate that *B. rapa* plants can induce chemical defenses based on the species of parasitoid attacking *P. rapae*. Induction of two aliphatic glucosinolates, GNP and

GBN, occurred after feeding by *C. glomerata* parasitized caterpillars only. Feeding from caterpillars parasitized by *C. glomerata* induced GNP and GBN even when they consumed similar amounts of plant tissue to unparasitized and *C. rubecula* parasitized caterpillars as 4th instars. These results support the expectation that plants would increase induction against gregarious parasitoids, which can cause increased damage to the plant even compared to unparasitized hosts. To our knowledge, only one other study has shown differential induction responses of plant defensive chemistry by parasitoids, but only compared parasitized and unparasitized caterpillars (Ode et al. 2016). Our results are the first to show that parasitoid identity (rather than presence of parasitism) results in differential induction of plant defense chemicals.

By carefully controlling the amount of time for feeding and starting size of the caterpillars, we were able to distinguish between plant responses based on caterpillar feeding amount versus parasitoid status. Differences in feeding amount during the experiment only resulted from different instars, as feeding damage was similar among all species at the same instar. Since *C. rubecula* emerges from the host during the penultimate, 4th instar, while *C. glomerata* emerges from the final, 5th instar, the amount of leaf tissue consumed varies greatly between hosts parasitized by these two wasps. Even in just the three days of the experiment, feeding by 5th instar caterpillars was approximately 4 to 5 times or more than that of the 4th instars. This demonstrates the enormous feeding difference between hosts of *C. rubecula* and *C. glomerata*-parasitized or unparasitized caterpillars shown in previous studies (Coleman et al. 1999, Harvey et al. 1999, van Loon et al. 2000, Smallegange et al. 2008). Stronger induction effects from *C. glomerata*-parasitized caterpillars, even at the same instar, indicates that plants respond to differences in species as well as the presence of parasitoids in the host, which has

been observed for volatile production as well (Fatouros et al. 2005, Poelman et al. 2011, 2012, Cusumano et al. 2019).

Induction of glucosinolates in response to feeding by *C. glomerata* parasitized caterpillars may benefit the plant by reducing fitness of the gregarious parasitoid. Parasitoid brood sizes of *C. glomerata*-parasitized caterpillars feeding on cultivated collards, which contain lower levels of defensive glucosinolates was significantly higher than those feeding on *B. rapa*. Several hosts feeding on *B. rapa* contained less than 10 individual parasitoids and 25% of caterpillars had active encapsulation around parasitoid larvae. This late encapsulation of *C. glomerata* larvae (rather than eggs) only occurred once in caterpillars feeding on collards in the experiment and is not observed in field populations of *P. rapae* on cultivated cabbage cultivars (R. Paul and D. Vyas pers. obs.). It is unclear whether the death of *C. glomerata* individuals within the host is a result of reductions in host quality or direct contact with defensive chemistry. While there may be a number of differences between collards and *B. rapa,* we think the evidence indicates the likelihood of glucosinolates playing a role in this effect on *C. glomerata.* Impacts on caterpillar host quality, such as slower development, reduced body size or poorer nutrition, are unlikely to cause direct death of parasitoids in the three-day feeding time frame. Instead, it is more likely that glucosinolates, or their breakdown products, are encountered directly by *C. glomerata* which would explain the rapid death and supports the induction effect. Direct impacts of plant defense compounds on mortality of parasitoids have been demonstrated in previous studies (Barbosa et al. 1991, Lampert et al. 2008). *Pieris rapae* does not sequester glucosinolates, instead diverting their metabolism to nitriles over their toxic isothiocyanate derivatives (Wittstock et al. 2004). However, impacts of aliphatic glucosinolates on *P. rapae* in other studies indicate that this metabolism may not be efficient enough to prevent all toxicity from glucosinolates (Kos et al.

2012b) leading to exposure to toxic compounds (such as isothiocyanates) for the developing parasitoids. The observed encapsulation response against *C. glomerata* larvae could instead be due to increased immune function of the host, though this is unlikely given the previous literature demonstrating that higher glucosinolates lead to decreased immune response in *P. rapae* (Bukovinszky et al. 2009).

Recent literature has explored the effects of plant defense chemistry on parasitoids, revealing both positive (Bukovinszky et al. 2009, Harvey and Gols 2011, Kos et al. 2012a) and negative effects (Lampert et al. 2008, Qiu et al. 2009, Lampert and Bowers 2013, Ode et al. 2016). Induction of glucosinolates by *C. glomerata*-parasitized caterpillars may benefit the plant through reduction in brood size (and thus reduced feeding [Smallegange et al. 2008]) for current *C. glomerata* parasitism. Gregarious parasitoids, including *C. glomerata*, can incur negative effects on plant fitness through increased feeding by the host (Coleman et al. 1999, Smallegange et al. 2008). However, small broods of *C. glomerata* can still benefit the plant through reduction in the duration (and therefore feeding) of the final instar, as small broods will still kill the host (Smallegange et al. 2008). In a similar study using *Trichoplusia ni* and its gregarious polyembryonic parasitoid *Copidosoma floridanum*, plants induced higher levels of glucosinolates with feeding from parasitized caterpillars (versus unparasitized) and higher glucosinolates were negatively correlated with parasitoid fitness including brood size. This plant response is not surprising given that *C. floridanum* parasitized caterpillars grow 50% larger and consume more plant tissue than unparasitized hosts (Ode et al. 2016). In the case of *C. glomerata,* negative impacts to plant fitness mostly occur with large brood sizes (Smallegange et al. 2008). Specific induction of glucosinolates by *B. rapa* may reduce the impacts of large parasitoid broods on

damage to the plant in the short term. However, gregarious parasitoids may still benefit plants overall through reduction in herbivore populations.

In conclusion, we found evidence for specific plant responses to feeding by different herbivore-parasitoid combinations that was not dependent on herbivore size and damage. We find further support for specific defense metabolite production to counter feeding by herbivores containing gregarious parasitoids which can be detrimental to the plant. Though we did not measure plant fitness impacts, we provide some support for decreased gregarious parasitoid survival on plants with higher glucosinolates, indicating that these defenses may reduce potential feeding increases from gregarious parasitism. Our results suggest that parasitoids can influence reciprocal selection and expression of plant defense traits. Further research should examine these selective forces and the extent to which plant response varies in the presence of different types of natural enemies with varying life histories.

Table 4.1 Water-acetonitrile gradient used as mobile phase for separation of glucosinolates with UHPLC-PDA-MS/MS

Run time (min)	Acetonitrile in mobile phase
0	1%
0-3	1%
5	10%
7-10	50%
10.1-12	1%

Table 4.2 Initial glucosinolate concentrations (in mg/g of leaf tissue) found in leaves of *B. rapa* (n = 102) used for induction experiments. Means are given with standard error. Different letters following glucosinolate means belong to significantly different groups based on post-hoc pairwise comparisons with Tukey adjustment ($p < 0.05$) based on one-way ANOVA ($F_{5,501} = 315.12$, $p < 0.0001$).

Glucosinolate	Mean ± SEM (mg/g)	Min	Max
GBN	0.5791 ± 0.0732 b	0	14.305
GBR	0.2695 ± 0.0223 ab	0	2.6585
GCC	0.0750 ± 0.0080 a	0	0.7301
GNP	9.0157 ± 0.5428 c	0	64.2342
MET	0.0914 ± 0.0055 a	0	0.7958
NGB	0.4296 ± 0.0420 ab	0	6.1246

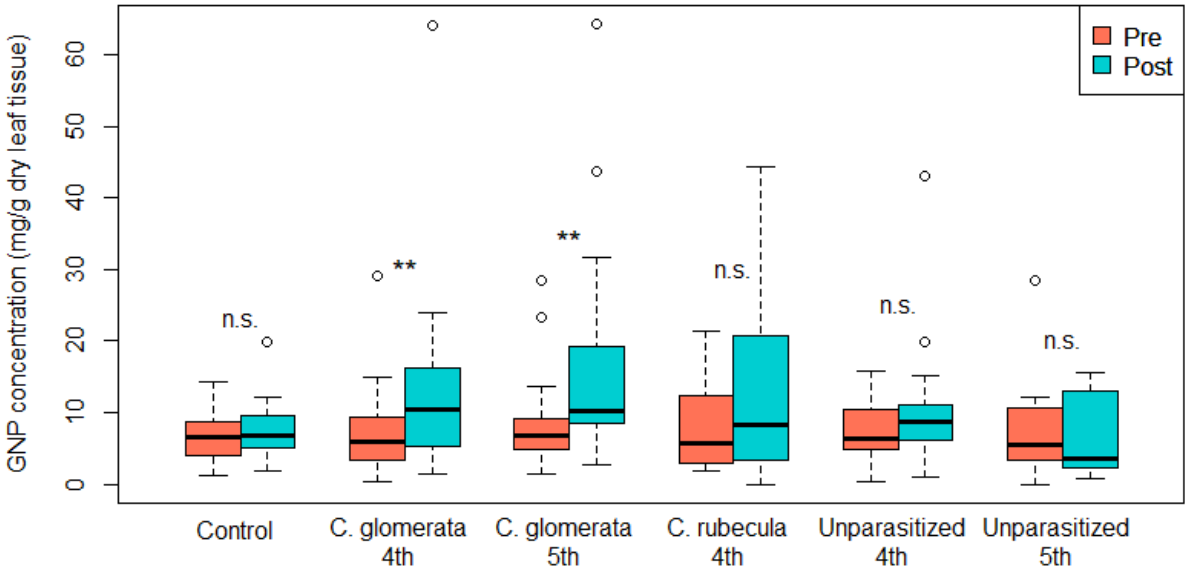

Figure 4.1 Gluconapin (GNP) induction by treatment. Boxes represent median with first and third quartiles with whiskers extending to minimum and maximum values within 1.5 times inter-quartile range. Individual points above boxes are more than 1.5 times inter-quartile range. Significant differences between pre and post feeding concentrations are indicated above the boxes by asterisks based on Bonferroni adjusted $\alpha = 0.0083$ (paired t-test, ** $p < 0.005$).

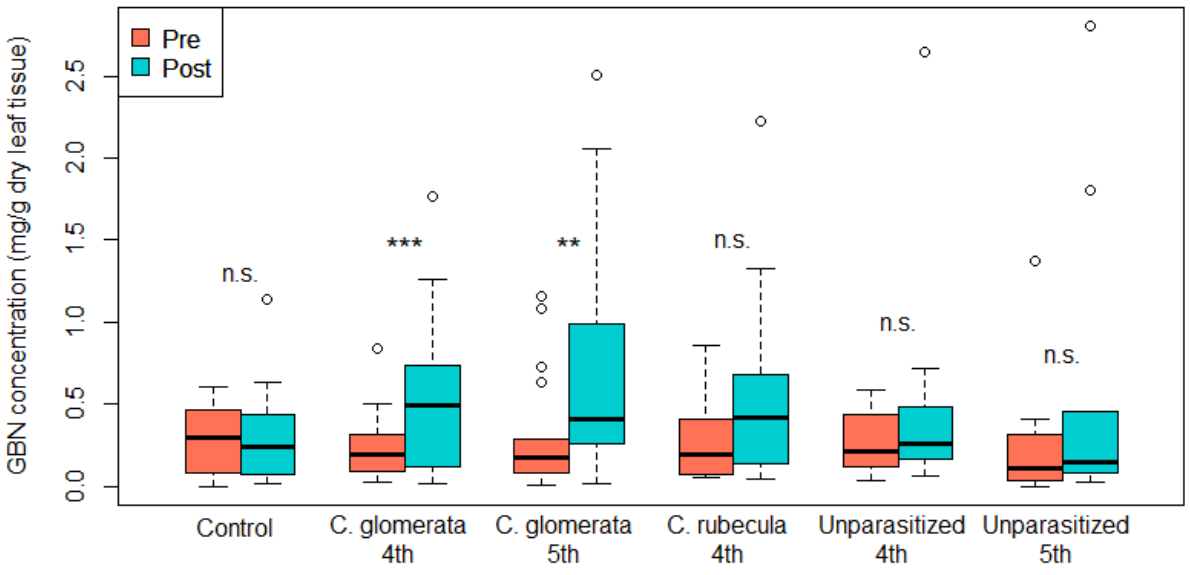

Figure 4.2 Glucobrassicanapin (GBN) induction by treatment. Boxes represent median with first and third quartiles and whiskers extending to minimum and maximum values within 1.5 times inter-quartile range. Individual points above boxes are more than 1.5 times inter-quartile range. Significant differences between pre and post feeding concentrations are indicated above the boxes by asterisks based on a Bonferroni adjusted $\alpha = 0.0083$ (paired t-test, ** $p < 0.005$, *** $p < 0.001$).

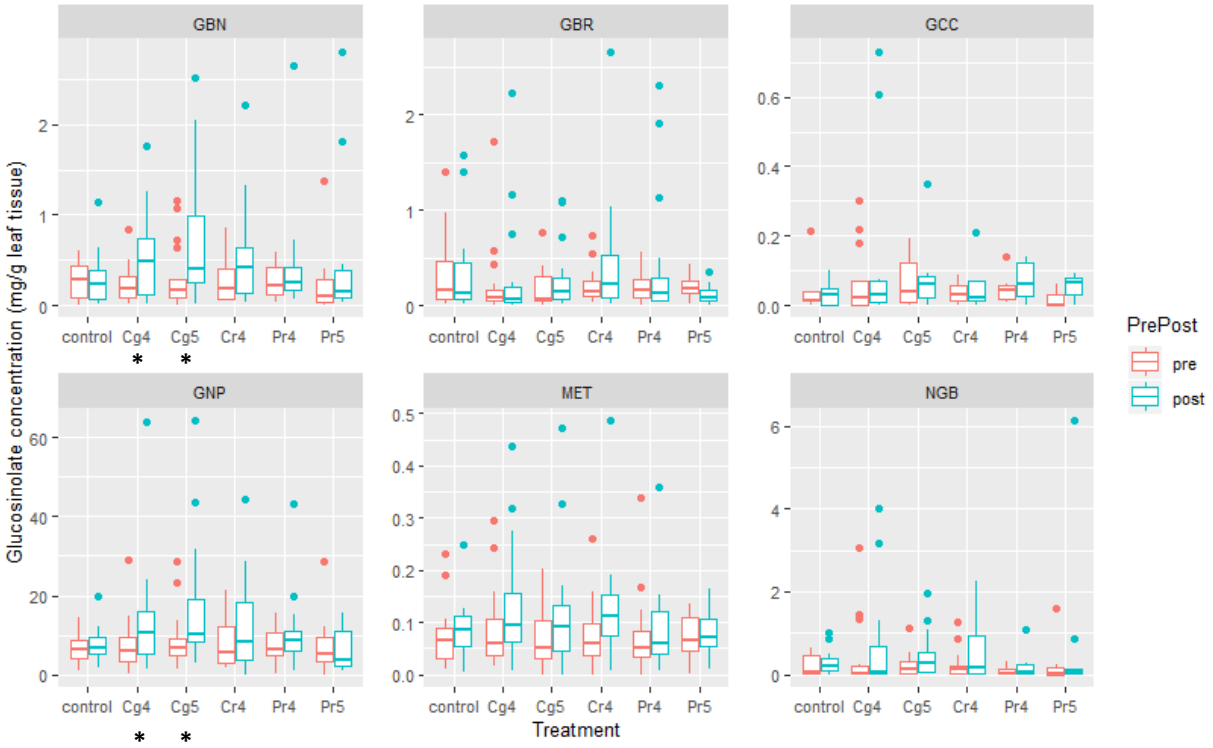

Figure 4.3 Amount of individual glucosinolates found in leaf tissue before and after caterpillar feeding. Note differences in scales between individual glucosinolates. Each plot represents a different glucosinolate: GBN = glucobrassicanapin, GBR = glucobrassicin, GCC = glucococchlearin, GNP = gluconapin, MET = 4-methoxyglucobrassicin, NGB = neoglucobrassicin. Treatments are labeled by parasitoid species and instar: Cg – *C. glomerata*, Cr – *C. rubecula*, Pr – unparasitized *P. rapae*, 4 – 4th instar, 5 – 5th instar. Control treatments were plants that did not experience herbivory but were sampled the same way. Boxes display the median with first and third quartiles. Whiskers extend to minimum and maximum values within 1.5 times inter-quartile range. Individual points above or below boxes are beyond 1.5 times inter-quartile range. Significant differences between pre-feeding and post-feeding concentrations are marked by asterisks below the x-axis based on paired t-tests with Bonferroni adjusted α = 0.0083.

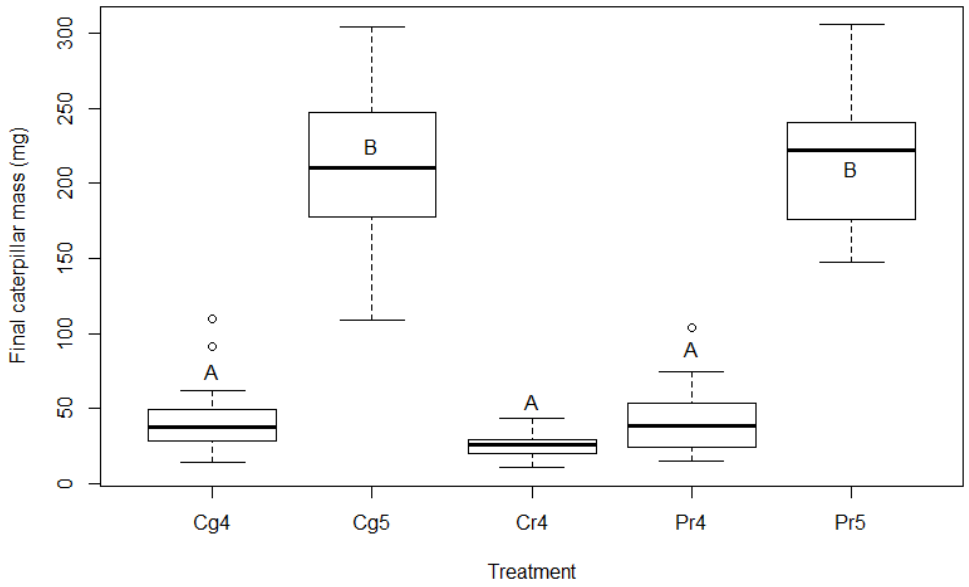

Figure 4.4 Final caterpillar size by treatment. Treatments are labeled with parasitoid species and instar number: Cg – *C. glomerata* parasitized, Cr – *C. rubecula* parasitized, Pr – unparasitized *P. rapae*, 4 – 4th instar, 5 – 5th instar. Plot whiskers extend to minimum and maximum values within 1.5 times inter-quartile range. Individual points above boxes are more than 1.5 times inter-quartile range. Letters represent significantly different groups ($p < 0.05$) from ANOVA post-hoc pairwise comparisons with Tukey adjustment ($F_{4,113} = 140.47$, $p < 0.0001$).

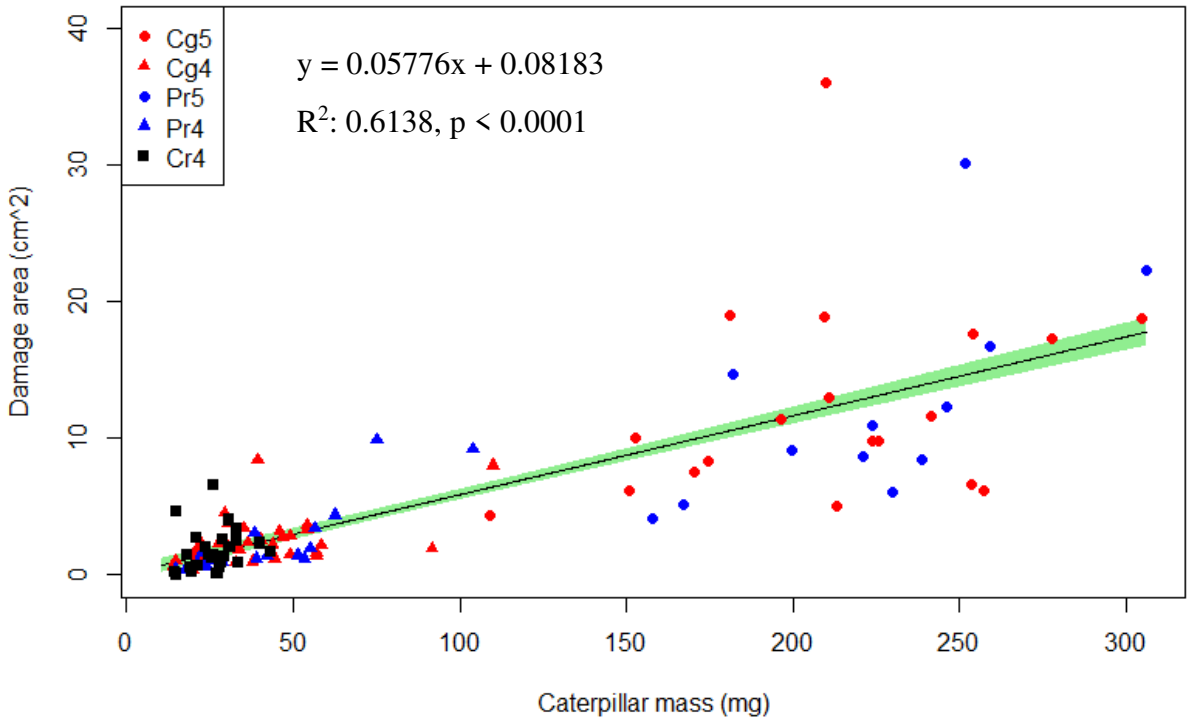

Figure 4.5 Relationship between caterpillar final size and damage area of leaves. Colors represent different parasitoid-host combinations and shapes correspond to different instars. Treatments in legend are labeled with parasitoid species and instar number: Cg – *C. glomerata* parasitized, Cr – *C. rubecula* parasitized, Pr – unparasitized *P. rapae*, 4 – 4th instar, 5 – 5th instar. Response data were log transformed for analysis of significance (linear regression: t = 12.56, df = 104, p < 0.0001).

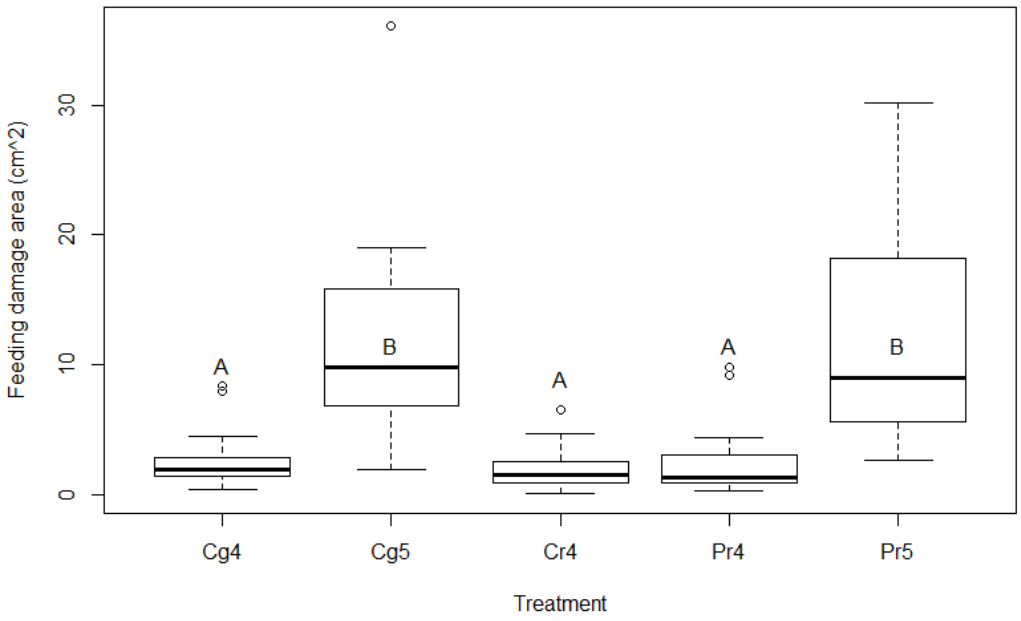

Figure 4.6 Caterpillar leaf consumption by treatment. Treatments are labeled with parasitoid species and instar number: Cg – C. glomerata parasitized, Cr – C. rubecula parasitized, Pr – unparasitized *P. rapae*, 4 – 4th instar, 5 – 5th instar. Plot whiskers extend to minimum and maximum values within 1.5 times inter-quartile range. Individual points above boxes are more than 1.5 times inter-quartile range. Letters represent significantly different groups ($p < 0.05$) determined by ANOVA post-hoc pairwise comparisons with Tukey adjustment (ANOVA: $F_{4,99} = 19.949$, $p < 0.0001$).

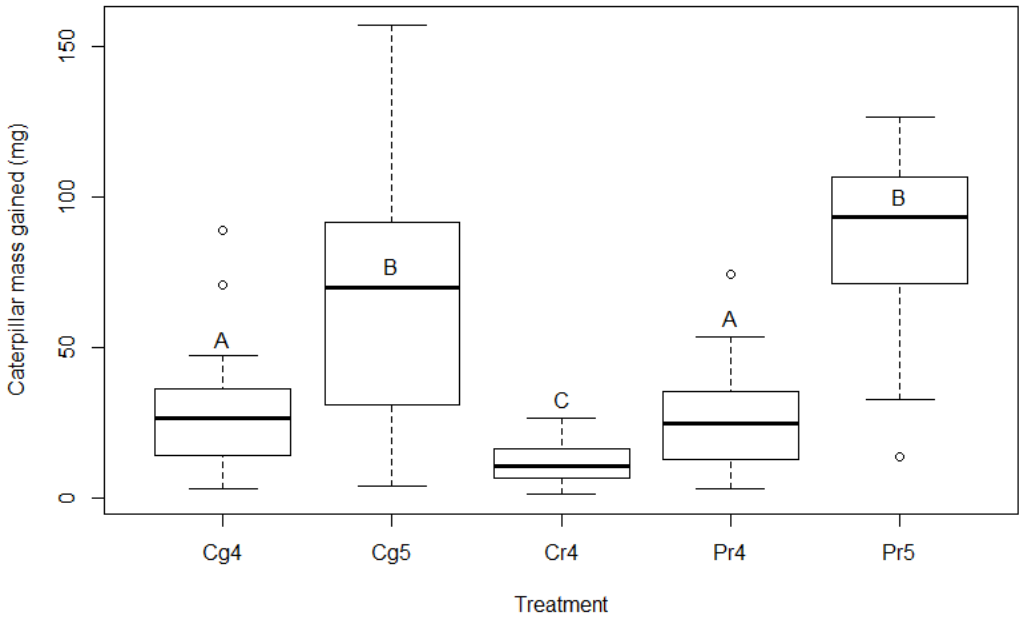

Figure 4.7 Caterpillar growth (mass gained) by treatment. Treatments are labeled with parasitoid species and instar number: Cg – C. glomerata parasitized, Cr – C. rubecula parasitized, Pr – unparasitized *P. rapae*, 4 – 4th instar, 5 – 5th instar. Plot whiskers extend to minimum and maximum values within 1.5 times inter-quartile range. Individual points above or below boxes are beyond than 1.5 times inter-quartile range. Different letters represent significant differences ($p < 0.05$) between treatments based on post-hoc pairwise comparisons with Tukey adjustment. (ANOVA: $F_{4,107} = 27.67$, $p < 0.0001$).

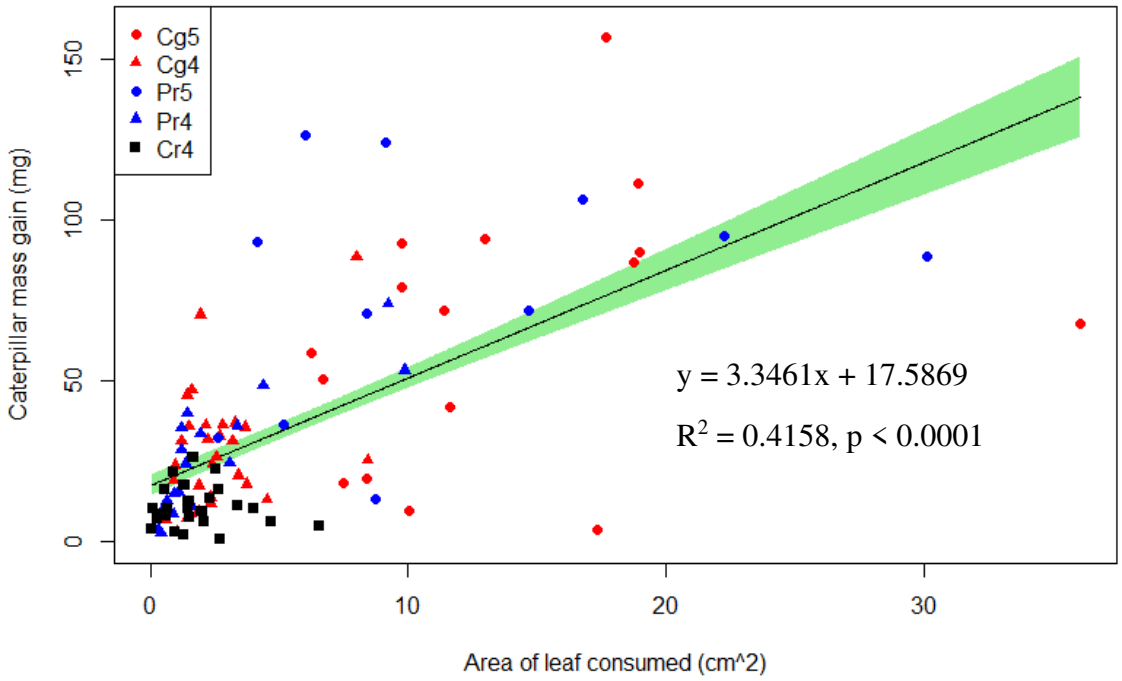

Figure 4.8 Plot of tissue consumed versus caterpillar growth. Colors represent different host-parasitoid combinations. Treatments in legend are labeled with parasitoid species and instar number: Cg – *C. glomerata* parasitized, Cr – *C. rubec*ula parasitized, Pr – unparasitized *P. rapae*, 4 – 4th instar, 5 – 5th instar. Response data were log transformed for analysis of significance (linear regression: t = 12.96, df = 104, p < 0.0001).

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CHAPTER 5: OVIPOSITION FLUIDS FROM ADULT FEMALE WASPS MEDIATE INTERSPECIFIC COMPETITION BETWEEN LARVAL PARASITOIDS

5.1 SYNOPSIS

Interspecific competition is often highly asymmetric, resulting in one species consistently outcompeting another. These interactions have important implications for community dynamics. However, the mechanisms of competition are often poorly understood. Parasitoids wasps, which develop inside another insect host, often engage in lethal interference competition as larvae when the host is attacked by multiple parasitoids. Solitary parasitoids (one larva per host) typically outcompete gregarious parasitoids (many larvae per host) which is often attributed to physical combat. Solitary larvae possess enlarged mandibles that they can use to kill competitors, a feature most gregarious species lack. Other potential mechanisms of interference competition have been observed, including the use of chemical (or physiological) suppression, but few explicit tests on the mechanisms behind these have been performed.

Parasitoids manipulate the environment of their host through oviposition fluids (calyx fluid and venom) injected with eggs into the host by the adult female. These fluids are primarily responsible for suppression of host immunity but may serve other roles that have not been determined. The role of these fluids in mediating larval competition is largely unexplored.

We examined the role of oviposition fluids of the solitary parasitoid *Cotesia rubecula* on mediating competition with the gregarious parasitoid *C. glomerata*. When sharing a host, *C. rubecula* wins practically all competitive interactions between these species, supposedly through physical combat. We injected venom and calyx fluid from *C. rubecula* into hosts parasitized by *C. glomerata* to examine the role of these oviposition fluids in interspecific competition. Here we

demonstrate that both venom and calyx fluid from *C. rubecula* can inhibit development of competing *C. glomerata*. *Cotesia glomerata* exposed to these fluids often failed to develop or were misshapen and died prematurely. We show that *C. rubecula* uses multiple mechanisms to outcompete *C. glomerata* at several stages of development using primarily physiological means. Our results show a previously unknown secondary competitive function for oviposition fluids of *C. rubecula.* These findings highlight the importance of larval interference competition in influencing characteristics of parasitoids.

5.2 INTRODUCTION

Competition is widely recognized as an important ecological phenomenon influencing outcomes of species interactions and community dynamics. Many examples of competition are highly asymmetric, where one species consistently outcompetes the other (DeBach 1966, Lawton and Hassell 1981, Murdoch et al. 1996, Reitz and Trumble 2002). In some cases, new sympatric association between species can result in asymmetric competition and displacement of the weaker competitor (DeBach 1966, Reitz and Trumble 2002, Gao and Reitz 2017). Yet in many cases, the mechanisms driving these competitive outcomes are not well understood.

A prime example of asymmetrical competition is between insect parasitoids, which develop as larvae in or on another insect host (Godfray 1994). Often, hosts are simultaneously parasitized by multiple parasitoids (multiparasitism) and larval parasitoids are forced to compete for control of the host; death is often the cost of losing (Godfray 1994, Harvey et al. 2013). Parasitoid larvae thus employ a number of strategies, including interference competition which can involve direct chemical suppression or physical killing of competitors (Harvey et al. 2013). Yet there are few studies which explicitly explore the mechanisms involved in larval competition between parasitoids inside the host.

Solitary parasitoids, which develop only one offspring per host, are typically superior larval competitors to gregarious parasitoids, which have multiple larvae per host (Pexton and Mayhew 2004, Harvey et al. 2013). Larvae of solitary parasitoids often possess enlarged mandibles which can be used to physically kill competing parasitoids inside the host and have increased mobility compared to gregarious species (Laing and Corrigan 1987, Pexton and Mayhew 2004, Magdaraog et al. 2012, Wang et al. 2019). Competitive superiority of solitary parasitoids is typically attributed to these traits (Pexton and Mayhew 2004, Magdaraog et al. 2012, Harvey et al. 2013). However, it is unlikely that a solitary larva could find and physically kill every competitor inside the host, especially when there are often 50 or more gregarious individuals (Harvey et al. 2013).

The use of chemicals to hinder development (physiological suppression) may allow parasitoid larvae to combat competitors more effectively because they do not need to physically find competitors (Harvey et al. 2013). While physical combat certainly plays a role in competition for some species (Vinson and Iwantsch 1980a, Laing and Corrigan 1987, Wang et al. 2008), evidence of physiological suppression has been noted for many different parasitoids (Vinson and Iwantsch 1980a, Godfray 1994, Harvey et al. 2013), even in solitary species possessing enlarged mandibles (Laing and Corrigan 1987, Wang et al. 2019). Despite the numerous examples of physiological suppression in intrinsic competition (larval competition inside the host) (Vinson and Iwantsch 1980a, Wang et al. 2008, Harvey et al. 2013, Liang and Liu 2017, Yang et al. 2018, Wang et al. 2019), the mechanisms behind this are not well understood. Parasitoid larvae can release toxic substances that kill intrinsic competitors (Uka et al. 2006), or may influence the ability of competitors to survive immune responses by the host (Poelman et al. 2014). Parasitoids often manipulate the environment of their host to suit their

own needs, such as adjusting nutrient or oxygen content inside the host, which may make conditions intolerable for conspecifics (Fisher 1961, Wang and Messing 2003, Cusumano et al. 2012). However, little attention has been given to the competitive role of oviposition fluids that are injected with the parasitoid egg by the adult female.

Oviposition fluids injected with parasitoid eggs help developing larvae combat unique challenges in developing within a host, such as overcoming host immune responses and nutritional deficiencies. Insect immune systems are capable of identifying parasitoid eggs and surrounding them with hemocytes which can melanize and kill the parasitoids in a process known as encapsulation (Godfray 1994, Strand and Pech 1995). As a result, many wasp parasitoids have venoms and/or virus-like particles which are injected along with the eggs to manipulate the host conditions to favor the parasitoid (Vinson and Iwantsch 1980b, Strand and Pech 1995). Parasitoid venoms are often composed of proteins which help suppress host immune response, such as inhibiting melanization during encapsulation (Kitano 1982, Asgari et al. 2003b). Members of the families Braconidae and Ichneumonidae possess polydnaviruses which are stored in a region of the ovaries called the calyx (Godfray 1994). Polydnaviruses (PDVs) are responsible for many developmental and hormonal effects in the host, especially related to immune suppression, and may require the venom for full effect (Fleming 1992, Gundersen-Rindal et al. 2013). The effects of venoms and PDVs act similarly to pathogens, even causing host castration in some cases (Tagashira and Tanaka 1998, Digilio et al. 2000, Bai et al. 2009). These oviposition fluids are thus prime candidates for investigating physiological suppression during intrinsic competition between parasitoids.

To our knowledge, only one study to date has explicitly identified a role for oviposition fluids in competition. In three competing species of parasitoids attacking *Mythimna separata*

caterpillars, injections of virus-like particles from the solitary *Meteorus pulchricornis* prevented development of two gregarious parasitoid species, *Cotesia kariyai* and *C. rufricus*. Eggs of these gregarious parasitoids generally did not hatch and larvae that developed often appeared malformed and died as early instars (Magdaraog et al. 2016). In other systems, the role of oviposition fluids in intrinsic competition between parasitoids has yet to be explored.

Competitive interactions between the gregarious *Cotesia glomerata* and solitary *C. rubecula*, (two originally European parasitoids attacking *Pieris rapae* in North America) have led to displacement of the gregarious species in much of North America (Herlihy et al. 2012). Under conditions of multiparasitism, *C. rubecula* is a superior competitor, winning intrinsic interactions nearly every time unless *C. glomerata* parasitizes the host several days before *C. rubecula* (Laing and Corrigan 1987). The superiority of *C. rubecula* is credited to its enlarged mandibles in the first instar "hunting morph" (Laing and Corrigan 1987). Niche separation of hosts in Europe where *C. glomerata* primarily attacks *P. brassicae* (Geervliet et al. 2000) and avoidance of multiparasitism by *C. glomerata* in parts of North America allow these species to coexist (Vyas et al. 2019). This further supports the importance of intrinsic competition as a driver of community patterns for parasitoids.

We investigated the mechanisms by which the solitary parasitoid, *C. rubecula* outcompetes *C. glomerata* in the host. It is unlikely that *C. rubecula* is able to find and physically kill all competing *C. glomerata*, which can lay 30 or more eggs per host (Van Driesche 1988). We were interested in whether *C. rubecula* uses physiological suppression to outcompete gregarious *C. glomerata*, and whether calyx and/or venom from the parent plays a role in competition. Oviposition fluids from *C. rubecula* have important functions in suppressing host immunity (Asgari and Schmidt 1994, Asgari et al. 2003b, 2003a), but their role in

competition remains unknown. We compared developmental success of *C. glomerata* in caterpillars injected with calyx fluid (containing PDVs) and/or venom of *C. rubecula* via microinjections. Here we demonstrate that calyx and/or venom alone are capable of causing significant reductions in *C. glomerata* survival, revealing an additional physiological mechanism for the competitive success of *C. rubecula* beyond physical combat. Our results indicate that *C. rubecula* employs mechanisms from both the adult and larva to mediate intrinsic competition with *C. glomerata*.

5.3 METHODS

5.3.1 Insects

Parasitoids and caterpillars used in experiments were maintained in laboratory reared colonies at Colorado State University in Fort Collins, CO. *Pieris rapae* colonies were originally collected as adults on canola plants near the CSU Agricultural Research, Development, and Education Center (ARDEC, GPS: 40.652703, -104.994627) in 2015. *Cotesia glomerata* were reared from parasitized caterpillars collected at ARDEC in 2015. Colonies of *C. rubecula* were started from pupae collected near the University of Minnesota campus in St. Paul, MN in 2015. All insects were reared for several generations in the laboratory before experimentation. Caterpillars were reared on *Brassica oleracea* (collard greens, "Georgia southern") in 30.5cm cube mesh cages in a greenhouse with a 16L:8D photoperiod (18-27° C, 40% RH). Adult parasitoids were kept in 946-mL plastic deli cups in Sanyo MIR-554 environmental chambers set at 15° C (16L:8D photoperiod, 30% RH). They were provided a water cup with a cotton wick and fed pure clover honey smeared on the side of the cup.

5.3.2 Preparation of fluids for injection

Oviposition fluids (venom and calyx fluids) were obtained from laboratory reared females kept at 15° C as described previously. Female wasps were first anaesthetized with carbon dioxide and then the abdomen was separated from the thorax in sterile 1x phosphate-buffered saline (PBS). Using fine forceps, the ovipositor was removed, revealing the ovaries (which includes the calyx region) and venom reservoir. Reproductive structures from groups of five females were pooled together in PBS. Next, venom reservoirs and ovaries were separated from one another and placed into clean drops of PBS. Finally, each group of pooled reservoirs and/or ovaries was placed into a 3µL drop of PBS and broken open (either separately or together depending on treatment) using a small dissecting probe. The fluids were mixed thoroughly with the PBS before being drawn into the injection needle. Injection needles were made from pulled glass capillaries (I.D. 0.02 mm, Drummond Scientific) using a Sutter Instruments P-97 needle puller. A Drummond Nanoject III mounted on a micromanipulator was used for all injections. Caterpillars were injected with 30nL each, resulting in 0.05 female equivalent per injection (from five reservoirs and/or ovaries in 3uL PBS). One female equivalent is defined as the full oviposition fluid volume held in one adult wasp.

5.3.3 Experimental design

Second instar *P. rapae* were first parasitized by placing individual caterpillars on small pieces of collard leaves in a 60-mm petri dish with a mated female *C. glomerata* and observing oviposition. Only caterpillars for which parasitoid oviposition was observed were used for the experiment and larvae were removed immediately after oviposition to avoid superparasitism. Parasitized caterpillars were then reared on collard plants in an environmental chamber set at 25° C (16L:8D photoperiod, 30% RH) until treatment the following day. The day after parasitism, caterpillars were either injected with 30nL of venom only (n = 27), calyx fluid only (n = 28),

calyx fluid and venom (n = 30), or PBS only (n = 31). Caterpillars receiving both oviposition fluids were injected with 0.05 female equivalent of each in the same total volume (30nL) of PBS. Prior to injection, caterpillars were placed into a -20° C freezer for approximately 10 minutes to slow movement and reduce bleeding from the needle wound. Caterpillars were injected by piercing the ventral abdomen with the needle while squeezing with forceps to allow easier entry. A set of *C. glomerata* parasitized caterpillars (n = 26) was left uninjected after initial parasitism to serve as a control against the effects of injection. Another set of *C. glomerata* parasitized caterpillars (n = 30) was parasitized naturally by *C. rubecula* instead of being injected. These caterpillars were parasitized individually by *C. rubecula* with the same set up as used previously for *C. glomerata.* All caterpillars were placed on a collard plants after treatment and reared in an environmental chamber at 25° C (16L:8D photoperiod, 30% RH) for two days until dissection.

Caterpillars from all treatments were dissected two days following treatment (three days after initial parasitism by *C. glomerata*). *Cotesia rubecula* hatches approximately two days after oviposition and *C. glomerata* typically hatches three days after oviposition (D. Vyas and R. Paul pers. obs.), so both would be near hatching at the time of dissection. Caterpillars were cut open on the ventral side using fine scissors (Fine Science Tools), the gut was removed, and the cuticle was scraped to remove all parasitoid larvae/eggs. For caterpillars parasitized by *C. rubecula*, the presence of the solitary parasitoid larva confirmed successful oviposition. All caterpillars were assessed for the presence of encapsulation and caterpillars with no evidence of encapsulation or parasitoid brood were assumed to be unsuccessfully parasitized and excluded from analysis. Unencapsulated parasitoid individuals were scored based on development stage (undeveloped egg, egg with larva, or larva) and whether development appeared normal, deformed, and/or coated in a film (Figure 4.9). Larvae covered in film were distinguished from encapsulation as

they were still alive or remained transparent (in the case of eggs) and did not cluster together. Encapsulation forms opaque/cloudy clusters of eggs (Brodeur and Vet 1995) and rarely occurs on *C. glomerata* larvae (R Paul, pers. obs.). Brood size was counted as the number of unencapsulated individuals remaining in the host, including any that were malformed. A subset of individuals from each brood was photographed and measured to obtain egg and larva length and areas using a Nikon SMZ18 dissecting microscope equipped with a DS-Fi2 camera and Nikon Elements software.

5.3.4 Statistical analyses

All statistical analyses were performed using R Studio (R v. 3.6.1). Since not all replicates were performed on the same date, date was used as a fixed effect in analyses, but was not significant and thus dropped from the model. All proportions were analyzed using logistic regression (glm function in R with binomial family) with treatment as the predictor. Pairwise comparisons of odds ratio were performed using the emmeans package and all p-values were adjusted using Tukey HSD. Post-hoc contrasts were used to compare groups combining treatments containing either venom or calyx fluid using a Sheffe adjustment. For brood size, a linear model was constructed using encapsulation and treatment as fixed effects (interaction was not significant). For analyses of development success, uninjected caterpillars were first compared with PBS injected treatments performed on the same day. Upon finding no significant difference, uninjected controls were dropped from this analysis and further comparisons were made using PBS injected caterpillars as the control with experiment date as a random effect. Sizes of parasitoid individuals inside the host was analyzed using a mixed model including treatment and deformed (yes or no) as fixed effects with a random effect for the host caterpillar. Size was analyzed separately for eggs and larvae. All pairwise comparisons between treatments were

made using a one-way ANOVA with a Tukey adjustment. All significant differences were based on significance level of 0.05. Results are given as means with standard error.

5.4 RESULTS

Development of *C. glomerata* was inhibited by presence of both calyx fluid and venom (together or separately). Venom fluid reduced success of initial development while calyx fluid had a more visually apparent effect, causing deformities to older *C. glomerata* eggs and early larvae. Broods experiencing venom containing treatments had lower odds of developing beyond the egg stage (Figure 4.12), with no visible larval development inside. Significantly more *C. glomerata* individuals were deformed (Figure 4.9) in treatments containing calyx fluid than those without (Figure 4.10) and a larger number of *C. glomerata* individuals were surrounded by a film (Figure 4.11). This film was distinctly different in appearance than encapsulation and affects larvae and eggs of *C. glomerata* in similar proportion, whereas encapsulation was limited to eggs. Physical combat in *C. rubecula* parasitized caterpillars was only actively observed once (Figure 4.9) and evidence of combat could not be distinguished from deformed *C. glomerata* larvae.

Introduction of venom affected the developmental success of *C. glomerata* during the early stages of development (Figure 4.12). Injection of venom alone decreased the odds of successful larval development in the eggs by 1.9 times compared to the control while calyx and venom injection decreased odds of successful development by 2.5 times. Parasitism by *C. rubecula* decreased odds of successfully developing by 1.7 times compared to PBS injections. Broods with more undeveloped eggs were more likely to experience encapsulation (LR χ^2 = 46.32, df = 1, $p < 0.0001$), although the addition of venom itself did not significantly affect

encapsulation ($z = 1.861$, $p = 0.0627$). Interestingly, calyx fluid injections alone increased development odds by 2.1 times.

Overall, 64.8% (57/88) of broods experiencing calyx fluid had at least one deformed *C. glomerata*. Only 8.4% (7/83) of total broods in the treatments without calyx fluid had any deformed *C. glomerata* individuals. Deformities almost exclusive in eggs once larvae had already begun forming or with young hatched larvae. Calyx treatments had approximately twice as many deformed *C. glomerata* eggs as larvae in treatments and the amount of deformities differed between treatments. Caterpillars which were also parasitized by *C. rubecula* showed the most *C. glomerata* deformities, followed by caterpillars injected with calyx fluid alone, and then those injected with both calyx fluid and venom (Figure 4.10). All treatments without calyx fluid showed almost no deformities with only 0.8% (11/1324) total *C. glomerata* deformed in these treatments.

The proportion *C. glomerata* eggs and larvae with film also differed between treatments but only occurred in 0.8% (11/1324) of individuals in treatments without calyx fluid (Figure 4.11). Caterpillars injected with calyx fluid alone had the most *C. glomerata* individuals surrounded by the film followed by those experiencing calyx and venom injected together, and then caterpillars parasitized by *C. rubecula* had the least individuals with film of the calyx treatments.

Injection of caterpillars with PBS alone did not cause significant changes to *C. glomerata* broods compared to uninjected controls. Brood size ($t = 1.858$, $df = 169$, $p = 0.4319$) and rates of encapsulation ($z = 1.045$, $p = 0.9027$) were both similar between injected and uninjected controls. Encapsulation proportions (LR $\chi^2 = 3.46$, $df = 4$, $p = 0.484$) and brood size ($F_{4,140} = 1.32$, $p = 0.267$) also did not differ among injection treatments.

5.5 DISCUSSION

Our results suggest that *C. rubecula* uses multiple mechanisms in tandem to ensure success in multiparasitized hosts. First, venom fluid decreases likelihood of *C. glomerata* eggs developing, possibly by directly killing them. Second, eggs which do begin developing are subjected to calyx fluid which causes deformation of eggs, which often rupture, and breaks down or helps encapsulate (formation of film) larvae which do manage to hatch. Finally, physical combat becomes important for *C. rubecula* to kill remaining *C. glomerata* that resist the effects of oviposition fluids. We found that *C. rubecula* oviposition fluids can inhibit development success of *C. glomerata*, even in the absence of *C. rubecula* larvae providing one of the only explicit examples of oviposition fluids from the adult parasitoid aiding in competition for developing offspring. We provide an example of several mechanisms of interference competition occurring between multiple life stages of parasitoids.

Competitive superiority of solitary parasitoid larvae has largely been attributed to their physical combat ability (enlarged mandibles and high mobility) against gregarious species (Laing and Corrigan 1987, Magdaraog et al. 2012, Harvey et al. 2013). Previous investigations of intrinsic interactions between *C. glomerata* and *C. rubecula* found the gregarious larvae shrunken alongside the *C. rubecula* larvae and were assumed to have died via physical combat (Laing and Corrigan 1987). In our study, definite evidence of physical combat in the *C. rubecula* parasitized caterpillar was observed only once and many *C. glomerata* had failed to emerge from eggs at normal hatching time in the presence of *C. rubecula*. At the time of dissections, *C. rubecula* was no more than a few hours old and the suppression of most *C. glomerata* could not have occurred via physical combat or larval secretions. Our findings show that physiological

suppression is an important component in intrinsic competition between these species and can reduce the need for physical combat by eliminating some *C. glomerata* in earlier stages.

Venom may kill eggs direct or may indirectly cause death, such as through disruption of egg coatings which are often important to avoid inducing encapsulation responses (Strand and Pech 1995). Broods with more undeveloped eggs in our experiment were more likely to experience encapsulation. Increased immune susceptibility has also been suggested as a mechanism for *Hyposoter ebeninus* physiological suppression of *C. glomerata* (Poelman et al. 2014). We find it more likely that *C. rubecula* venom is directly toxic to young eggs and leads to encapsulation after the egg is already dead, rather than host encapsulation response leading to egg death. Broods experiencing *C. rubecula* venom had more undeveloped eggs that were not encapsulated as well, supporting the conclusion of venom leads to early egg death. Field collected and lab reared caterpillars parasitized by *C. glomerata* rarely have eggs at different development stages within the same brood (RP and DV pers. obs.), yet this occurred numerous times in caterpillars injected with venom.

The *C. rubecula* PDVs may be responsible for the disease-like effects observed on *C. glomerata* broods with the addition of calyx fluid. Polydnaviruses act as a symbiont for the wasp, but can perform similarly to a pathogen inside the host (Webb et al. 2006). Similarly, injection of solitary parasitoid *Meteorus pulchricornis* virus-like particles prevented hatching and caused deformed larvae in two competing gregarious parasitoid species (Magdaraog et al. 2016). The exact mechanisms of calyx fluid impacts on eggs in our study are still unclear, but it may weaken the egg chorion, as many eggs had bulging sides or ruptured easily in these treatments. The film observed on many larvae in the calyx fluid treatments shares some similarity to early encapsulation responses. However, normal encapsulation does not typically occur against larvae,

and eggs are generally already dead and opaque when they are observed in encapsulation. The observed film was typically transparent and occurred on larvae which were still actively moving, but appeared to restrict their movement ability. It's possible that the film is the result of increased immune susceptibility for *C. glomerata* similar to physiological suppression mechanisms suggested in other studies (Poelman et al. 2014). Currently, the exact cause or purpose of this film covering is unknown.

Reducing the size of *C. glomerata* broods at earlier stages through physiological suppression lessens the need for *C. rubecula* to find and physically kill *C. glomerata*. Alternatively, the reduced brood size may allow the *C. rubecula* to primarily outcompete the *C. glomerata* from resource use, especially given its far quicker development time and early emergence from the host, as often the earlier developing larva gains the upper hand (Harvey et al. 2011, 2013, Zhu et al. 2011). Successfully emerging *C. glomerata* broods are also very rarely smaller than 10 (Vyas, Paul, and Ode unpublished data). Low numbers of *C. glomerata* may simply be unable to successfully develop to egression or may be easily outcompeted through resource use by *C. rubecula*. In this case, *C. rubecula* would only need to reduce the brood to the point at which *C. glomerata* is no longer a competitive threat.

Our results highlight the importance of interference competition for success of developing parasitoids. We demonstrate interference competition used by a parasitoid against the same competitor through multiple life stages and a secondary role for oviposition fluids in mediating competition inside the host. *Cotesia rubecula* is able to kill *C. glomerata* in a shared host at multiple stages of development, in part mediated by parental oviposition. The evolution of multiple mechanisms of competing with another species of parasitoid larva at once indicates the importance of competition as a selective force on parasitoid traits. It is unclear whether the

properties of oviposition fluids contributing to competition are a byproduct of host immune functions or a separate set of traits. Further investigations of competitive mechanisms could help reveal traits in other organisms which serve a secondary role in competition.

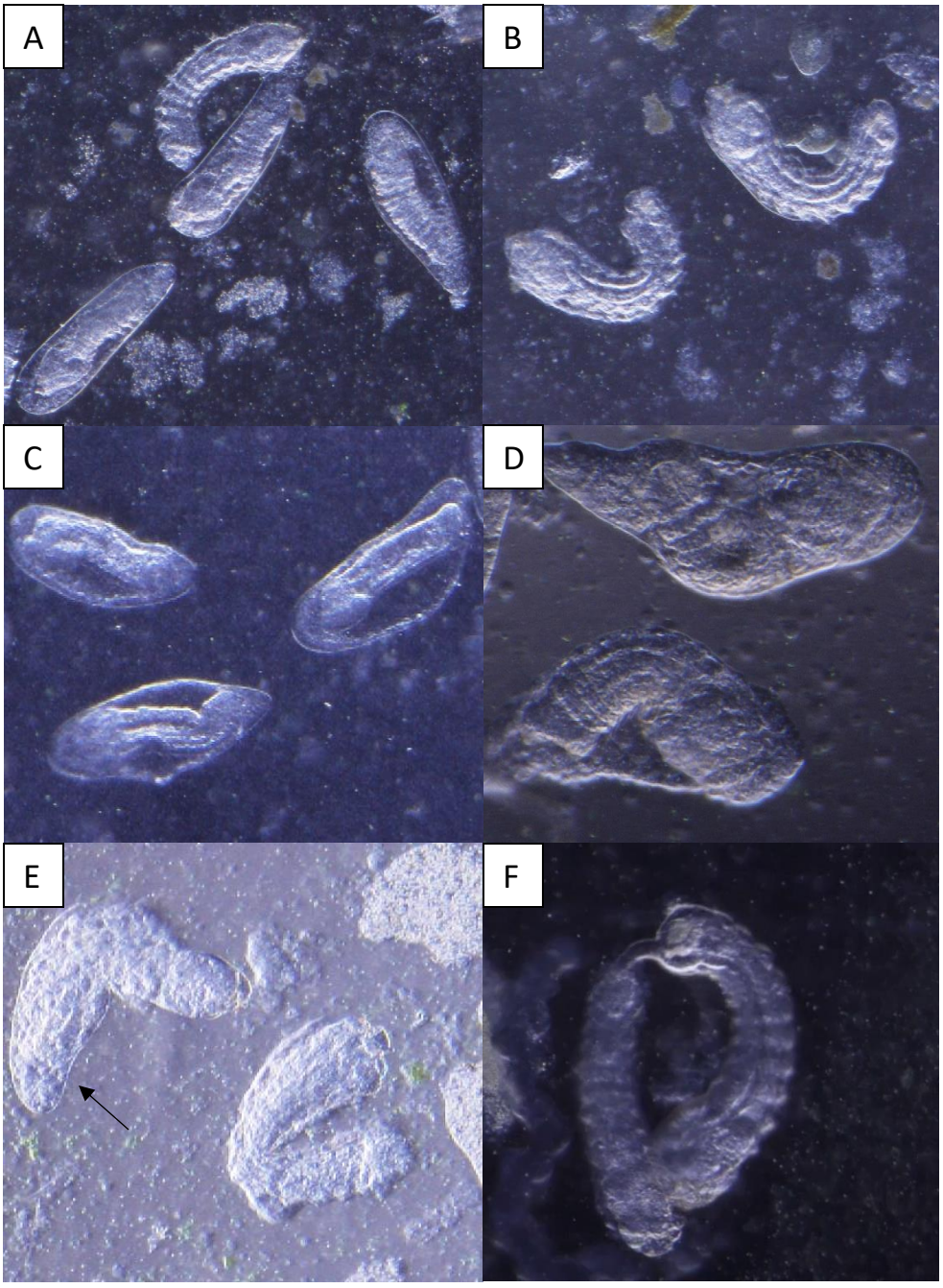

Figure 4.9 Dissected parasitoid larvae from different injection treatments. (A) *C. glomerata* eggs and larva showing normal development from uninjected control caterpillars. Larvae fill the inner egg space and egg margins are well defined smooth lines. (B) Normally developing larvae from a venom injected caterpillar. (C) Deformed eggs showing shriveled developing larvae and irregular egg shape from calyx fluid injection. (D) Irregular *C. glomerata* egg shapes and degenerate developing larva from a caterpillar multiparasitized by *C. rubecula*. (E) Deformed larvae from a caterpillar injected with calyx fluid. Larval features are difficult to recognize and body form is irregular. The arrow indicates the eggshell still on the larva from hatching. (F) *C. rubecula* larva (right, with anal spike) with mandibles imbedded in *C. glomerata* larva (left). Deformed *C.*

glomerata eggs were around 62% larger in area than normal eggs ($t = 4.40$, $df = 124.56$, $p < 0.0001$). Deformed larvae were typically smaller in length by 18.6% on average ($t = 10.75$, $df = 199$, $p < 0.0001$).

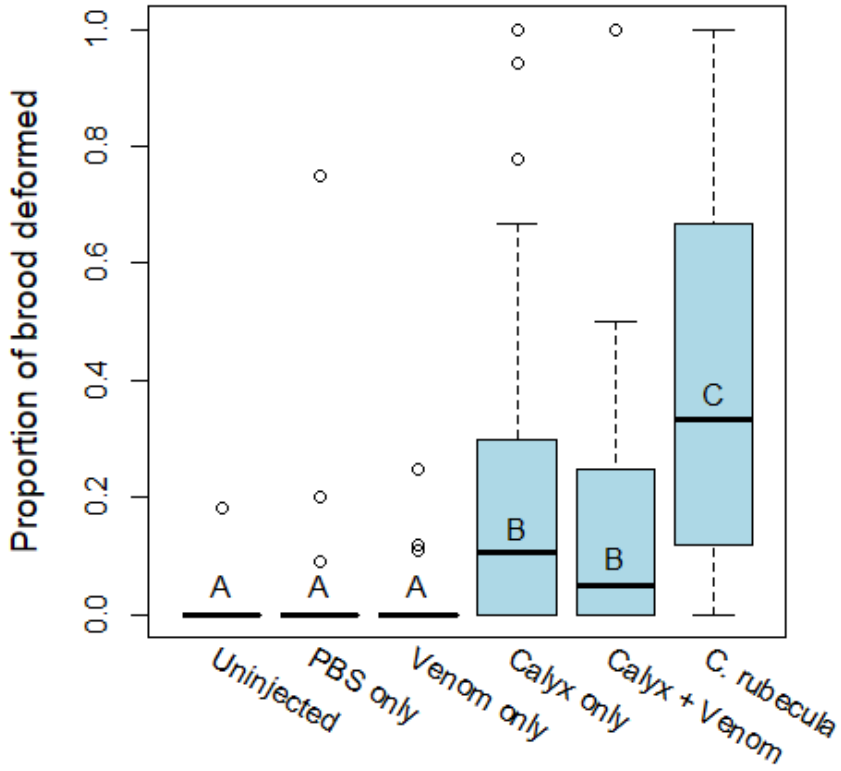

Figure 4.10 Proportion of deformed individuals within each brood. Letters represent significant differences between groups ($p < 0.05$) based on post-hoc pairwise comparisons of odds ratios with Tukey adjustment (likelihood $\chi^2 = 474.22$, $df = 5$, $p < 0.0001$). Odds of deformities were five orders of magnitude higher with the presence of calyx fluid (post-hoc contrast: $z = 10.74$, $p < 0.0001$)

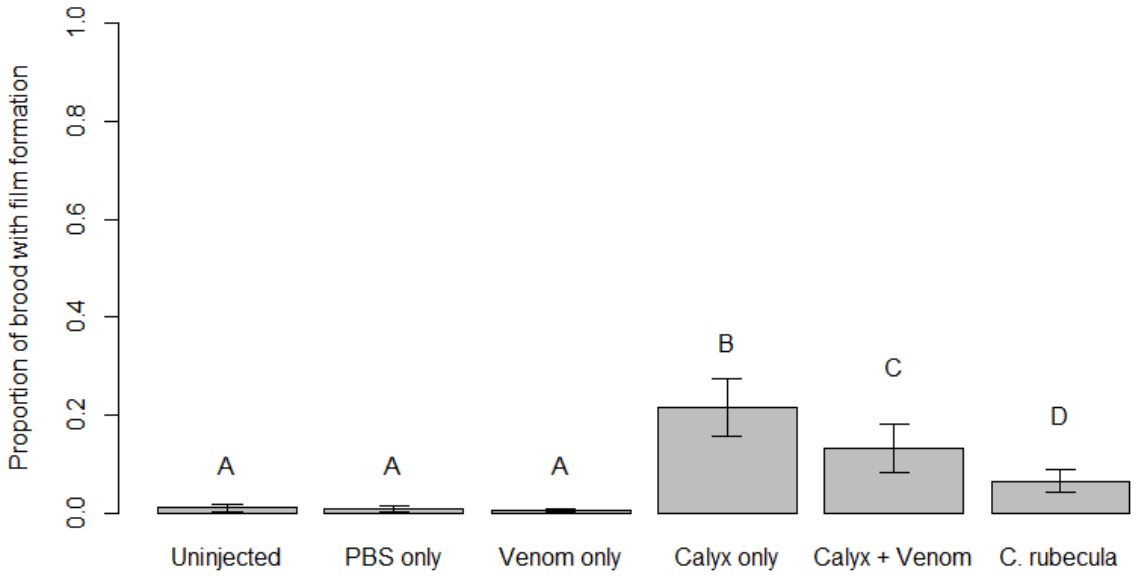

Figure 4.11 Average proportion of individuals in each *C. glomerata* brood with formation of film. Eggs and larvae were pooled in calculating proportion and brood size. Letters above bars indicate significantly different groups ($p < 0.05$) based on pairwise comparisons of odds ratios with Tukey adjustment following logistic regression (LR $\chi^2 = 295.55$, df = 5, $p < 0.0001$). Presence of calyx fluid greatly increased likelihood of film formation ($z = 7.836$, $p < 0.0001$).

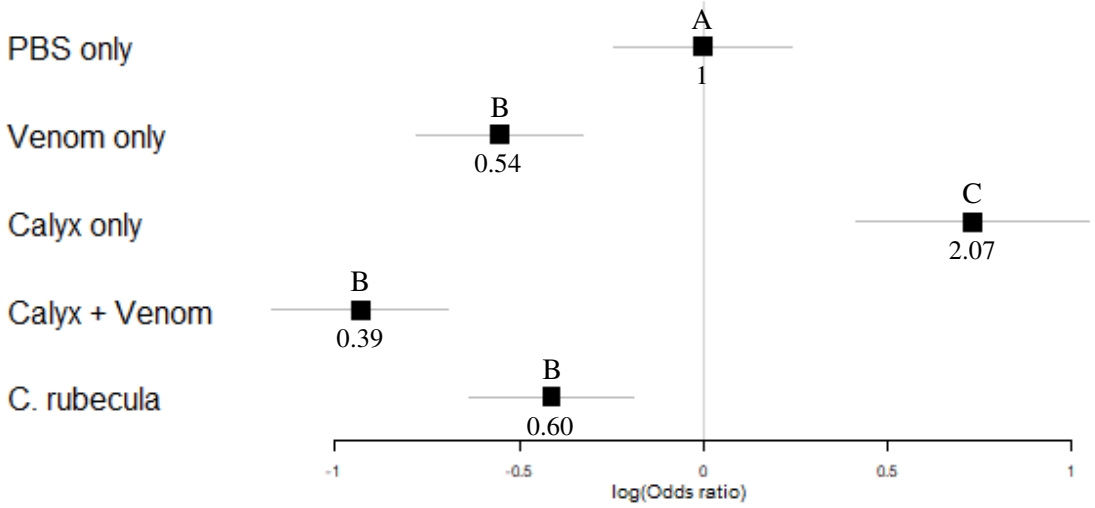

Figure 4.12 Log odds ratios for development of *C. glomerata* eggs in each treatment compared with PBS injected. Values shown below plot points are back-transformed odds ratios. Comparisons are made against the control which is set at log(odds) of zero. Different letters above plot points are significantly different from one another (odds ratios, $p < 0.05$). Odds were calculated based on logistic regression with proportions of undeveloped eggs as a response to treatment (LR $\chi^2 = 691.22$, df = 5, $p < 0.0001$)

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