INDUCIBLE ANTI-HERBIVORE DEFENCES OF THE ANNUAL SUNFLOWER (*HELIANTHUS ANNUUS*) AGAINST A SPECIALIST SUNFLOWER BEETLE (*ZYGOGRAMMA EXCLAMATIONIS*)

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Dedication

I dedicate this thesis to the many

people who have made it possible for me

to be writing these words

Abstract

This thesis aims to determine the effects of sunflower plant defences on the specialist sunflower beetle and whether sunflower beetles modify their behaviour in response to plant defences as a form of herbivore offence. Chapter 1 begins with a general introduction, reviewing previous literature on inducible plant defences and herbivore offence. Chapter 2 investigates plant defence induction in sunflowers, initiated by sunflower beetle larvae feeding and mechanical damage. No evidence of plant defence induction was found. Chapter 3 investigates the possibility of herbivore offence in sunflower beetles, specifically: selective oviposition on undamaged sunflowers, avoidance of damaged leaves and plants, and gregarious feeding. No evidence of selective oviposition, or avoidance of damaged leaves or plants was found. Evidence of gregarious feeding was found as larval survivorship increased with increasing larval densities. Chapter 4 summarizes the results of Chapters 2 and 3, and proposes future lines of research based on these findings.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
ECI	Efficiency of Conversion of Ingested food
LD	Lower leaf Damaged
RCR	Relative Consumption Rate
RGR	Relative Growth Rate
UD	Upper leaf Damaged

Chapter 1 – General Introduction and Literature Review

Goals of Thesis

This thesis seeks to expand on the body of knowledge of inducible plant defence and its counterpart, herbivore offence, in terms of behavioural responses by herbivores. The goals are to: (1) Determine if effective plant defence responses are inducible in the common annual sunflower (*Helianthus annuus*) against the sunflower beetle (*Zygogramma exclamationis*); (2) Determine if sunflower defences reduce survivorship and/or feeding in sunflower beetle larvae; and (3) Determine if sunflower beetle larvae and adults modify their behaviour in response to host plant defences in terms of oviposition, avoidance, and gregarious feeding.

Literature Review

Herbivory

Herbivory refers to the consumption of plants by animals (reviewed in Herrera and Pellmyr 2002). Herbivores consume varying amounts plant tissue, resulting in the loss of light capture potential, energy, and overall fitness of the plant (Ohnmeiss & Baldwin 2000; Rudgers & Hoeksema 2003). The results of this loss can range from negligible amounts of plant tissue and energy, to the loss of potential offspring (Hochwender et al., 2003) and reduced plant survivorship (Rudgers & Hoeksema 2003).

It is important to note that not all interactions between herbivores and plants significantly reduce plant fitness. In some cases, plants are able to compensate for or mitigate the impacts of herbivory through tolerance or overcompensation and thereby negate negative fitness consequences. Tolerance is the ability of plants to receive herbivore damage but with reduced or negligible fitness consequences (Painter 1958); whereas overcompensation is the phenomenon in which plants produce more biomass (plant tissue or seeds) after experiencing herbivory (Herrera and Pellmyr 2002).

Despite this, herbivory generally has negative effects on plant fitness (Herrera & Pellmyr 2002; Hochwender et al. 2003; Dyer et al. 2004). For this reason, plants have evolved a variety of strategies to reduce or mitigate the impacts of herbivory (Herrera & Pellmyr 2002; Heil 2010).

Plant Defence

In the interaction between plants and herbivores, many studies have reported a negative outcome from the perspective of plants (Herrera & Pellmyr 2002; Hochwender et al. 2003; Dyer et al. 2004); these outcomes have been measured through seed number and mass (Rudgers & Hoeksema, 2003), and through the asexual performance of daughter plants (Dyer et al, 2004). The ability to escape, reduce, or tolerate herbivory is advantageous to plants if these strategies prevent or reduce the loss of plant tissues (Herrera & Pellmyr 2002; Llandres et al. 2010). For this reason, plants have evolved a large variety of plant defences (Heil, 2010). These defences can be categorized several ways. One method is by the nature of their expression: whether plant defences are expressed all the time (constitutive defences); or only when a prerequisite cue, such as damage or exposure to herbivore saliva, is present (inducible defences). Another categorical method is by determining whether the form of defence falls within the categories of: direct defence, indirect defence, or tolerance. These categories can be further subdivided by the mechanism of defence.

Direct Defence

In direct defence, the plant protects itself through direct interaction with potential herbivores, typically by means of physical barriers or chemical deterrents (Heil 2010). Physical barriers refer to corporeal defence that prevent herbivores from reaching plant tissue that they would otherwise potentially consume. Examples of this form of defence include thorns (Belovsky et al., 1991), and trichomes (Rowe et al. 2012).

Chemical deterrents refer to the chemical-based defences that prevent or reduce herbivore consumption of plant tissue. This can result in the reduced palatability of the plant tissues, and the death or deterrence of the herbivore, thereby reducing future herbivory potential. Examples of this form of defence include: the production of nicotine in tobacco plants (*Nicotiana sylvestris*) (Baldwin 1996); the production of coumarin and its secondary compounds scopoletin and ayapin in the common annual sunflower, *Helianthus annuus*, against the sunflower beetle, *Zygogramma exclamationis* (Olson and Roseland, 1991); and the production of dictyol E and secondary metabolites, such as pachydictyol A, by *Dictyota menstrualis* when grazed by *Ampithoe longimana* (Cronin and Hay, 1996). Not all chemical barriers require ingestion on the part of the herbivore. Konstantinidis &

Bennington (2010) demonstrated that the floral tissues of *Passiflora incarnata* produce a chemical barrier that prevents ants from exploiting their floral nectars.

Indirect Defence

Indirect defence refers to the recruitment of other organisms to defend the plant from herbivores (Heil 2010). In many cases, this takes the form of the recruitment of ants or parasitic wasps that attack herbivores within the vicinity of the plant (Beattie 1985). Much of the time, extrafloral nectar (Katayama & Suzuki 2004; Koptur 1992), Beltian bodies (Janzen 1966), and/or shelter (Hocking, 1970) are utilized by plants as incentives for ant solicitation. Many ant species are aggressive and territorial by nature and so will attack any herbivore they come across (Katayama & Suzuki, 2004). In some cases this relationship is highly coevolved, as is the case of the mutualism between acacia trees and acacia ants, in which acacia trees provide homes and food for acacia ant colonies and the ants defend the trees to the point of excluding even large herbivores (Huntzinger et al. 2004). In all of these cases, indirect defence is best understood as a form of mutual exploitation. This has been demonstrated by the nutrient content of extrafloral nectar in barrel cactus (*Ferocactus wislizeni*), which is rich in carbohydrates, but relatively poor in proteins (Ness et al. 2009). Ness et al. (2009) have shown that colonies of desert ants that only have access to supplemental carbohydrates have greater aggression to herbivores than control desert ant colonies or desert ant colonies that were provided protein in addition to the carbohydrates.

Another form of indirect defence involves the attraction of female parasitic wasps via the release of volatile organic compounds (Turlings et al., 1990). These wasps use herbivores, typically caterpillars, as hosts for their eggs and larvae. The process of parasitism kills the host herbivore, relieving the plant of herbivore pressure and so indirectly defending it.

Tolerance and Over-Compensation

Painter (1958) defines tolerance as the ability of plants to receive herbivore damage without severe fitness consequences. Belsky et al. (1993) further elaborates this by describing tolerance as a physiological adaptation in which plants possess organs and physiological traits that enable plants to regrow and replace tissues that have been damaged. They continue to state that tolerance is not just a response to herbivory, but can also be applied to other forms of damage (fire, trampling, wind, etc.). Unlike direct and indirect plant defence, tolerance generally has limited negative fitness effects on herbivores (Herrera & Pellmyr 2002).

Strauss and Agrawal (1999) describe five primary mechanisms of tolerance: 1) increasing the rate of net photosynthetic production after damage; 2) greater relative growth rates; 3) increased branching or tillering after release of apical dominance; 4) carbon storage in roots for above-ground reproduction prior to damage; and 5) ability to shunt carbon stores to shoots following damage. Each of these mechanisms serve to reduce fitness loss due to herbivory and damage. For example, the increase in relative growth rates decreases the time during which the plant suffers from the loss of productive plant tissues.

In addition to tolerance, plants may also “overcompensate” for herbivore damage. Overcompensation is also known by “vigorous regrowth” and “herbivore optimization” (Belsky et al. 1993), as it refers to the phenomenon in which browsed plants produce more plant tissues or seeds than their un-browsed neighbours (Herrera and Pellmyr 2002). However, a plants ability to overcompensate is limited by nutrient availability, increasing plant-plant competition, and the timing of herbivory (Maschinski & Whitham 1989).

A plant defence strategy utilizing tolerance and overcompensation is beneficial because it is a general response that can reduce fitness loss, even with multiple species of herbivores or non-herbivore damage. Plants may also be ecologically, genetically or physiologically constrained from evolving other forms of defence (Belsky et al. 1993).

Constitutive Defence vs. Induced Defence

Constitutive defences are expressed at all times, whereas inducible defences are expressed following herbivore damage. The hypothesized selective pressure underlying the evolution of inducible defences is that plant defences are costly; and so it is advantageous to only express plant defences when the plant is likely to experience future herbivore pressures, opposed to squandering resources maintaining plant defences against fictional herbivores. For example, Baldwin (1996) determined that the induction of nicotine-based defence through the direct application of methyl jasmonate to tobacco plants (*Nicotiana sylvestris*) decreased overall plant biomass compared to plants that had not been induced. Another study by Baldwin et al. (1998) determined that tobacco plants, grown in nitrogen-starved soil and treated with methyl jasmonate, allocated significantly less nitrogen to seeds. This was a result of having to allocate nitrogen to plant defences as opposed to increasing seed quality. Another line of evidence for costly defence is the finding that defences may be resource-limited. Mondor et al. (2006) demonstrated that the expression of induced extrafloral nectaries was significantly greater in broad bean plants (*Vicia faba L.*) which had been grown in growth medium with 6g or 12g of 14:14:14 Nitrogen:Phosphorus:Potassium (N:P:K) fertilizer than those broad bean plants grown in growth medium without any fertilizer added.

For this reason, many plants preferentially express defences after a plant is exposed to general or specific forms of stimuli. In response to these stimuli, the plant up-regulates its defences (Karban & Myers 1989). Such defences are said to be inducible. These defences may take many forms including, for example: the increased expression of the toxin coumarin and its derivatives, scopoletin and ayapin, in the leaf tissues of the common annual sunflower (*Helianthus annuus*) (Olson & Roseland 1991); the release of terpenoid volatiles in corn (*Zea mays*) to dissuade continued herbivory and to attract parasitic wasps, including *Cotesia marginiventris* (Turlings et al., 1990); and the increased expression of extrafloral nectar to attract ant bodyguards in the broad bean plant (*Vicia faba*) (Mondor & Addicott, 2003). However, constitutively expressed plant defences would be favourable in the event that the plant is very likely or certain to experience herbivory and/or these plant defences are relatively inexpensive to express (Karban 2011).

Non-linear Relationship between the Strength of the Induction Cue and the Magnitude of Plant Defence Expression

The magnitude of the signal of defence induction and/or the effectiveness of the defence does not necessarily have a linear relationship with the extent of herbivore damage or the magnitude of the prerequisite trigger. Indeed there is often a non-linear response to these cues. Baldwin (1996) found that the application of 45 μg of methyl ester jasmonic acid in hydroponic solutions to flowering tobacco (*Nicotiana sylvestris*) was able to induce a significantly greater nicotine-based defence throughout the plant than in control plants. However, when these tobacco plants were treated with varying numbers of applications of methyl ester jasmonic acid, the magnitude of the response was not significantly different among treatments. This demonstrates a relationship between the defence response and the cue, which is binary in terms of initiation and decelerating in terms of response.

Cronin and Hay (1996) observed that *Dictyota menstrualis* collected from sites with higher grazing intensities were less susceptible to further herbivory by *Ampithoe longimana*. However, it was noted that palatability and chemical defences of *Dictyota menstrualis* did not always differ between the sites surveyed over the years of the experiment. Cronin and Hay (1996) go on to suggest that there may be a grazing threshold required before the induction of defences occurs. This suggests that plant defence may require a certain magnitude of stimulus before it will mount an inducible defence. Cronin and Hay (1996) suggest that is possible that variation in nutrient abundance between sites may also play a role in this.

In terms of defence effectiveness, Katayama and Suzuki (2004) found that indirect defence by extrafloral nectary induction in *Vicia faba* to attract pugnacious ant bodyguards (*Tetramorium tsushimae*) against first-instar silkworm larvae (*Bombyx mori*) was positively correlated with the number of ants on the plant. The amount of time a silkworm larva was allowed to remain on the plant was negatively correlated with the number of ants visiting the broad bean plant. However, when there were fewer than four ants per broad bean plant, the defence was described as "ineffective". In trials with ant densities higher than four ants per plant, the silkworm larva was removed in far less than the 100-minute allotted time per trial. However, when there were fewer than four ants per plant, the silkworm was allowed to persist for far greater time; in most cases, the silkworm was simply not excluded. This study demonstrates that in the case of some plant defences, there are both thresholds and non-linear relationships of plant defence effectiveness against herbivores.

Herbivore Offence

Several studies have shown that plant defences result in lower rates of herbivore consumption of leaf material (e.g., Olson and Roseland 1991), lower rates of survival of the herbivore (e.g., Rogers and Thompson 1980) and herbivore exclusion from habitat (e.g., Katayama & Suzuki 2004). Though this is beneficial for plants, it is generally detrimental to the herbivore. Therefore, herbivores would be able to increase their fitness if they were able to counter, avoid, or mitigate the effects of plant defences (Karban & Agrawal 2002). Such strategies are termed herbivore offence.

The function of herbivore offence is not necessarily to destroy or harm the host plant itself, but to increase the herbivore's own feeding success and fitness. Specifically, herbivore offence describes herbivore traits that allow herbivores to increase their ability to feed on and utilize plants for the benefit of the herbivore (Karban & Agrawal 2002). For this reason, it may be more accurate to describe them as "herbivore counter-defensive strategies"; as the term "offence" may lead one to believe that it is a form of herbivore attack. It may be that the term "offence" is used to describe direct opposition to the term "defence" in the term plant defence. This thesis will continue to use the term "herbivore offence" as it has been commonly used in previous peer-reviewed literature.

Herbivore offence takes a variety of forms, as it has evolved in response to plant defences herbivores encounter. They have resulted from an evolutionary arms race between plants and herbivores in which plants avoid being eaten and herbivores attempt to eat (Dawkins & Krebs 1979). For this reason, these offences can be very general if they are to counter a common defence held by many plant species; or very specific, if the herbivore is a specialist species and so must counter a very specific and unique form of plant defence. Examples of herbivore offences explored in this thesis include: selective oviposition, the avoidance of plant defences, and gregarious feeding.

Selective Oviposition

Selective oviposition is the host or site selection by parental organisms for the purpose of depositing their eggs. Selection pressures for the evolution of selective oviposition may result from the fitness benefits of increased egg/larvae/offspring survivorship (Damman 1987, Encalada & Peckarsky 2006), and discrete patches of resource availability to offspring (Ruiz-Durbreuil et al. 1994).

Selective oviposition does not just account for premium host, patch, or tissue selection; it also can help parental organisms avoid unfavourable hosts or environments that may result in increased mortality. Sato et al. (1999) found that *Dacne picta* avoided shiitake mushrooms (*Lentinula edodes*) that had begun to decay. They found that the mortality of these larvae was greater in decaying mushrooms than non-decaying ones. They then suggest that chemical inhibitors and nutritional deficiencies might be significant factors in this increased mortality and that because the decay process of the mushrooms caused the mushrooms to harden, it inhibited the ability of the larvae to consume the mushrooms themselves. In summary, selective oviposition can protect parental organisms from ovipositing on unfavourable hosts.

Avoidance of Plant Defences

Avoidance of plant defence refers to the active evasion of plant defences by herbivores to mitigate fitness loss. An example is the avoidance of glucosinolates in the *Arabidopsis thaliana* rosette leaves by cotton bollworm larvae (*Helicoverpa* *armigera*) (Shroff et al. 2008). Shroff et al. (2008) determined that glucosinolate, a class of anti-herbivore chemical used in plant defence, was most concentrated along the midvein and leaf periphery through MALDI-TOF Imaging and HPLC analysis. By allowing cotton bollworm larvae to feed on *Arabidopsis* leaves, they determined that the larvae almost exclusively fed on the inner lamina of the leaf and avoided the midvein and periphery of the leaf. This was further reinforced by a significant feeding preference of the larvae for leaf disks from the inner lamina of a leaf when also presented with a leaf disk from the leaf periphery. Taken together, this is an example of herbivore avoidance of plant defences.

Avoidance may be a response to selective defence of tissues, as predicted by optimal defence theory, in that when the plant is resource-limited, more valuable plant tissues will have more plant defence effort allocated to them (Herrera and Pellmyr 2002, McCall & Fordyce 2010). As a result, herbivores can avoid these tissues in their own form of optimal forging, in which they selectively consume plants and tissues with higher nutrient contents while attempting to minimize their exposure to plant defences and so maximizing their own net benefit of foraging. This often puts plants and herbivores at odd with one another and is an underlying condition in the maintenance of an evolutionary arms race (Dawkins & Krebs 1979); and may result in a zero-sum game between conflicting species allowing for each to co-exist, as described by Van Valen (1973) in the regards to the "Red Queen Hypothesis".

Group Feeding and Gregarious Feeding

Gregarious feeding can be defined as the tendency of organisms to feed in groups, as opposed to feeding alone (Allen 2010). This behaviour may result in increased feeding efficiency and growth (e.g. Denno & Benrey 1997), recruitment (e.g. Lopez et al. 1989), and protection or survival (e.g. Damman 1987; Damman 1994).

Damman (1987) demonstrated an effect of group feeding on survivorship in pyralid moth larvae (*Omphalocera munroei*) on *Asimina* spp. Larvae of this moth species feed on older plant leaves, as they tend to have higher rigidity and a lower likelihood of wilting. For this reason, older leaves can be folded into a defensive shelter to protect the larvae from predators. By keeping to these older leaves, the larvae are able to feed in relative safety. Damman (1987) first demonstrated that larval survivorship was significantly greater within a leaf roll than on the surface of either the top or the bottom of a leaf. He then demonstrated that groups of approximately 20 larvae had significantly greater survivorship than those groups of 1, 3, 5, or 10 larvae. This was a result of the inability of pyralid moth larvae to create a leaf fold with group sizes of less than about 20 individuals.

In terms of increased defence efficiency, Damman (1994) investigated the effects of group size on survivorship in the gregarious leaf-mining beetle, *Microrhopala vittata*, when reared on goldenrods (*Solidago altissima* and *Solidago rugosa*). He determined that increasing group size allowed quicker establishment of leaf-mines, which decreased the time the larvae were vulnerable to predation.

Denno and Benrey (1997) observed *Chlosyne janais* larvae feeding in aggregations on *Odontonema callistachyum* plants. They determined that there was a significant positive relationship between larval growth and aggregation size, though were unable to determine the exact mechanisms behind this. However, they also found that there was no relationship between larval growth and aggregation size when the larvae were raised on excised leaves. Because it was unlikely that a systemic plant defence response could be mounted in an excised leaf as it was removed from the plant, Denno & Benrey (1997) suggested that aggregations allow larvae to suppress or overwhelm plant defences. Their results supported the idea that these aggregations helped increase larval feeding efficiency and facilitated larval growth.

However, gregarious feeding has other consequences. These consequences could include increased resource competition (Wise et al. 2006), decreased growth (Damman 1994, Wise et al. 2006) and even increased cannibalism (Mertz and Robertson 1970, Via 1999). In several species of beetles, it is not uncommon for larvae from the same species or even the same brood to cannibalize their neighbours or siblings as other larvae represent an easily accessible and nutritious alternative form of food (Damman 1994), especially when in the presence nutrient stress (Via 1999) and high population densities (Mertz & Robertson 1970). Therefore, whether gregarious feeding is selected for or against will naturally depend on the balance of its positive and negative effects.

Study Species

This thesis will use the close relationship between the common annual sunflower, *Helianthus annuus* L. (Asteraceae), and the specialist herbivore sunflower beetle, *Zygogramma exclamationis* Fabricius (Chrysomelidae), to examine the relationship between plant defence and herbivore offence.

The Common Annual Sunflower (Helianthus annuus)

The common annual sunflower (*Helianthus annuus* L.) is an annual plant that is native to the Great Plains region of North America (Rogers 1977). The sunflower emerge from a seed with a pair of cotyledons and then develop a series of leaf pairs that then develop multiple lateral shoots and unpaired leaves. Flower heads are produced at the terminal ends of the lateral shoots, thereby producing multiple inflorescences.

The common annual sunflower contends with many herbivore species such as the sunflower beetle (*Zygogramma exclamationis*) (Rogers 1977), the sunflower moth (*Homoeosoma electellum*) (Chen & Welter 2003); and the sunflower stem weevil (*Cylindrocopturus adspersus*) (Barker 1990). To counter these threats, sunflowers have evolved an array of defences. These include trichome defences (Hausen & Spring 1989) and chemical defences (Olson & Roseland 1991).

Sunflowers have been shown to produce the toxin coumarin and its derivatives: scopoletin and ayapin. Olson and Roseland (1991) demonstrated that scopoletin was significantly induced by mechanical damage via leaf puncture, thrip feeding, and sunflower beetle feeding within 9 days of initial damage when compared to undamaged controls. Ayapin was significantly induced 25 days after damage in the sunflower beetle feeding treatment. During a feeding preference experiment, sunflower beetles were given a choice between a leaf disk sprayed with either coumarin, scopoletin, or ayapin dissolved in distilled water, and a control leaf disks sprayed with only distilled water. Sunflower beetles significantly preferred leaf disks sprayed with only distilled water over leaf disks sprayed with these chemicals at a concentration of 20μg/g. This was also true at a concentration of 100μg/g for ayapin and scopoletin. However at a concentration of 1000μg/g, there was no significant preference. The authors suggested that the beetles had become acclimated to high levels of coumarins in the assay chamber. Lastly, a survival assay was performed on sunflower beetles. It was found that there was no significant difference in terms of mortality between sunflower beetles feeding on control leaves and leaves sprayed with concentrations of 1mM and 10mM coumarin, scopoletin, or ayapin.

Roseland and Grosz (1997) investigated the induction of the coumarin derivatives scopoletin and ayapin among various wild populations of sunflower across 11 states in the USA. Under UV irradiation stress, acetyl salicylic acid application and topical treatment of mercuric chloride ($HgCl_2$), 107 populations of sunflowers showed an induction of scopoletin within 72 hours. Only 18% of these showed an induction of ayapin. They then compared feeding preferences of sunflower beetles between unstimulated leaves and stimulated leaves of eleven sunflower populations that had previously shown induction of scopoletin and ayapin. In 8 of 11 populations, plants were significantly less preferred when stimulated than in unstimulated controls. This study demonstrates variation in coumarin induction and variation in deterrence effectiveness of sunflower beetle feeding between sunflower populations.

The Sunflower Beetle (Zygogramma exclamationis)

The sunflower beetle (*Zygogramma exclamationis*) is a specialist herbivore and major defoliator of many species of sunflowers (Rogers & Thompson 1980), including the common annual sunflower (*Helianthus annuus*). It can be found throughout the Great Plains region of North America, and has been reported as an economic pest to sunflower crops in both Canada and the United States of America (Brewer and Charlet 2004).

Adult sunflower beetles over-winter by burrowing into the soil. They tend to emerge during late May in the prairie provinces of Canada and begin mating within 2 days after emergence (Gerber et al. 1979). Within a week of mating, female sunflower beetles begin laying eggs.

Rogers (1977) determined that sunflower beetles gathered from Bushland (High Plains) and Chillicothe (Rolling Plains) Texas in laboratory conditions at 27°C ±2°C, and under a 14:10 Light:Dark photoperiod had the following bionomics pattern. These collected sunflower beetle adults were found to mate and begin laying eggs with a mean pre-oviposition period (± standard error) of 15.6 ± 0.9 days, a mean oviposition period of 75.6 ± 10.5 days. Female adults lay an average of 14.8 ± 1.1 eggs per day. The eggs then hatch into larvae after 5.4 ± 0.1 days. Larvae consume leaf tissue on their host sunflower plant to develop through four instars [first instar: 4.5 ± 0.1 days; second instar: 3.5 ± 0.1 days; third instar: 3.3 ± 0.1 days; fourth instar 3.8 ± 0.2 days], a prepupa [4.8 ± 0.2 days] and pupa stage [6.7 ± 0.2 days] (Rogers 1977). The larvae occur on the sunflower plant throughout all four instars. At the beginning of the prepupa stage, the pupa leaves the sunflower and buries itself 2-5 cm under the surface of the soil and remains there until the end of the pupal stage (Rogers 1977). At the end of the pupal stage the adult sunflower beetle emerges from the soil and feeds on sunflowers until it over-winters. In southern latitudes of its range, the sunflower beetle may undergo a second reproductive cycle per year (Rogers 1977); though within Canada, sunflower beetles tend to undergo only a single reproductive cycle per year (Gerber et al. 1979), possibly due to a shorter summer season.

Structure of Thesis and Goals of the Chapters

Chapter 2

This chapter describes an experiment investigating the possible induction of sunflower plant defences through leaf damage by sunflower beetle larvae feeding and by mechanical damage application. This induction is measured through consequences to sunflower beetle larvae (i.e., a bioassay approach): survivorship, Relative Growth Rate, Relative Consumption Rate, and the Efficiency of Conversion of Ingested Food.

Chapter 3

This chapter uses four experiments to investigate behavioural strategies sunflower beetles may use to reduce the impacts of sunflower defences. The first of these experiments will determine if sunflower beetle adults preferentially oviposit on undamaged sunflower plants when presented with damaged and undamaged plants in a field setting. The second experiment will examine if sunflower beetle larvae avoid sunflower leaves that have been damaged within a single plant individual. The third experiment will examine if sunflower beetle larvae avoid the leaves of sunflower plants that have been damaged when presented with the leaves of a damaged and an undamaged plant. The fourth experiment examines if gregarious feeding increases survival rates in sunflower beetle larvae.

Chapter 4

Chapter 4 is a general discussion of the thesis that aims to summarize the thesis and integrate the experimental chapters (2 and 3) into the literature on plant defence and herbivore offence explored in the present introductory chapter. Chapter 4 will then discuss prospective avenues of future research.

Chapter 2 – Survival and Growth of Sunflower Beetle Larvae on Damaged Sunflowers

Abstract

In this chapter I investigate the induction of plant defences in the common annual sunflower (*Helianthus annuus*) through mechanical and sunflower beetle (*Zygogramma exclamationis*) larva feeding damage. I applied mechanical damage, sunflower beetle larva feeding damage, or no damage (control) to sunflowers and allowed 10 days for plant defence induction. Larvae were removed and a new sunflower beetle larva was applied to each plant regardless of treatment and allowed to feed for 7 days. This larva was used to assess the effects of plant damage on several sunflower beetle larval traits: survivorship, relative growth rate (RGR), relative consumption rate (RCR), and the efficiency of conversion of ingested food (ECI).

The experiment was performed in the summer of 2011 and resulted in the non-significant effects of treatment on RGR, RCR and ECI. However, the effect of treatment on larval survivorship was marginally non-significant, with the greatest difference in mortality rates occurring in larvae between 1.5mg and 2.5mg. For this reason, the experiment was repeated in the summer of 2013 with the modification of doubling the sample size to increase statistical power. No significant effects of treatment were found on RGR, RCR, ECI, and larval survivorship. These results suggest that we were unable to induce plant defences through mechanical and larval

feeding damage; or that because the larvae used in 2013 were under 1.5mg in weight, that we were unable to detect effects of sunflower plant defences.

Introduction

Herbivory generally has a negative impact on plant fitness (Ohnmeiss & Baldwin 2000; Rudgers & Hoeksema 2003). This fitness loss can take the form of the loss of potential offspring (Hochwender et al. 2003) and decreased survivorship (Rudgers & Hoeksema 2003). Plants have evolved a variety of defences to reduce this loss of plant fitness by preventing or discouraging herbivore feeding (Herrera & Pellmyr 2002; Heil 2010). Many of these plant defences are costly to produce or maintain (Baldwin 1996) and so the expression of these defences is initiated or up-regulated after the plant suffers damage or receives a specific cue, a phenomenon known as inducible defence (Olson & Roseland 1991; Mondor & Addicott 2003, Heil 2010).

The sunflower beetle (*Zygogramma exclamationis*) is a specialist insect herbivore; adults and larvae consume the leaf tissue of the common annual sunflower (*Helianthus annuus*) (Rogers 1977), and other closely related sunflower species. In response to this, and to predation by many other generalists, a number of defences have evolved in sunflowers. These defences include physical (trichomes) (Hauen & Spring 1989; Rowe et al. 2012), chemical (coumarins) (Olson & Roseland 1991), and additional unidentified (Rogers & Thompson 1980) defences.

Olson and Roseland (1991) demonstrated that sunflower defences are inducible via mechanical damage and sunflower beetle larvae damage. Specifically, they determined that the coumarin derivative, scopoletin, is initially expressed in significantly greater concentrations than in the undamaged controls following the application of mechanical damage or thrip feeding damage; and the coumarin derivative, ayapin, is initially expressed in significantly greater concentrations than in the undamaged control following the application of mechanical damage, thrip feeding damage, or sunflower beetle feeding damage. Roseland and Grosz (1997) were also able to induce coumarin expression by the application of mercuric chloride to sunflower plant leaves.

Olson and Roseland (1991) further demonstrated that sunflower beetle adults showed a significant avoidance to coumarin, scopoletin, and ayapin. They did this by presenting sunflower beetle adults with two sunflower leaf disks, one covered in coumarin, ayapin, or scopoletin diluted in distilled water and the other covered in only distilled water (control). Sunflower beetle adults significantly preferentially fed on the leaf disk covered in only distilled water. The authors suggested that sunflower beetle adults avoid coumarin and its derivatives.

It is from these results by Olson and Roseland (1991) that we hypothesized that sunflower beetle larvae would be discouraged from consuming damaged sunflower leaf material, and so would suffer fitness costs in terms of larval growth, quantity of leaf consumption, and the efficiency of conversion of ingested food into body mass. When measuring these, it is necessary to correct for initial larval mass, as it affects the rate at which they consume plant tissue and grow. The formulae for relative growth rate (RGR), relative consumption rate (RCR), and efficiency of conversion of ingested food (ECI), which assume constant, continuous growth are as follows (Waldbauer 1968, Scriber & Slansky 1981):

Relative Growth Rate (RGR) $\left(\frac{mg}{mg*days}\right)$

$$RGR = \frac{\ln(M_A) - \ln(M_B)}{t}$$

Relative Consumption Rate (RCR) $\left(\frac{mm^2}{mg*days}\right)$

$$RCR = \frac{\ln(M_A) - \ln(M_B)}{t(M_A - M_B)} * L$$

Efficiency of Conversion of Ingested food (ECI) $\left(\frac{mg}{mm^2}\right)$

$$ECI = \frac{M_A - M_B}{L}$$

Where:
M_A= Mass of larva after feeding (mg)
M_B= Mass of larva before feeding (mg)
t = time of larval feeding period (days)
L = Leaf Area Consumed by larva (mm^2)

RGR is the product of RCR and ECI. Thus, a decrease in larval growth implies that at least one of RCR or ECI also decreased. RCR allows us to determine if larvae are dissuaded from feeding on plant tissue, which may be the case if toxins such as coumarin have been produced and are present in damaged plant material (Olson & Roseland 1991, Roseland and Grosz 1997). ECI allows us to determine the efficiency larvae are able to digest plant material and use it to grow. A decrease in ECI may suggest that larvae are having a harder time digesting ingested plant material, possibly due to a form of plant defence in a manner similar to the effect of protease inhibitors found by Scott et al. (2010) in the tomato mutants Def-1 (jasmonic acid deficient mutant/defence compromised) and Prosys (jasmonate over expresser/over expressed defence) on 5th instar cabbage looper larvae (*Trichoplusia ni*).

In short, the objective of this chapter and these experiments is to determine if sunflower beetle larva survivorship, RGR, RCR, and ECI are affected by previous sunflower damage. We will also be able to determine if this effect on sunflower beetles is a triggered by general mechanical damage, or if herbivore feeding damage is required. Herbivore feeding damage may be required as plant damage experienced in a natural setting may not be the result of herbivore feeding. Simple environmental factors may result in plant damage, such as trampling by large fauna, weather such as hail, and flooding; all of which were observed in during the conduction of the experiments within this thesis. The expression of plant defences in response to these other sources of damage would not benefit the plant.

Methods

Study Species

The common annual sunflower (*Helianthus annuus* L.) is a plant native to North America that expresses several forms of plant defence, including trichomes (Hausen & Spring 1989) and the production of coumarin and its derivatives scopoletin and ayapin (Olson & Roseland 1991). The sunflower beetle (*Zygogramma exclamationis* Fabricius) is a chrysomelid beetle that specializes on species in the genus *Helianthus* in both its adult and larval stages (Rogers 1977). This species has been shown to induce plant defences in *Helianthus annuus* (Olson and Roseland 1991) and to be affected by *Helianthus* spp. plant defences in turn (Rogers & Thompson 1980, Olson & Roseland 1991, Roseland & Grosz 1997).

Experimental Preparation

The experiment was completed twice: once in the summer of 2011 and again in the summer of 2013. Sunflowers emerging with their first true leaf-pair and cotyledons were collected from naturally occurring populations located within the untended areas of the University of Lethbridge campus on May 6, 2011 and May 21, 2013. They were carefully removed from the soil with a trowel, with consideration to keep their root system as undamaged and intact as possible. The sunflowers were planted into 12 x 6 cells gardening trays in a 5cm deep black tray (a "flat") with no holes, filled with Sunshine Mix #1 Growth Medium (Sun Gro Horticulture Canada) (sphagnum peat moss, perlite, dolomitic lime, "starter nutrient charge" (with Gypsum) and "wetting agent") and placed in the University of Lethbridge Biological Sciences Experimental Greenhouse.

The sunflowers were allowed to grow until May 26, 2011 and June 5, 2013. They then were transplanted again into 1-Trade Gallon Pots (16.5 cm Diameter x 17.8 cm High; 2.78 L) filled with Sunshine Mix #1 Growth Medium. The sunflowers were then randomly assigned a treatment (Larval Damage [n_L = 30], Mechanical Damage [n_M = 30], and Control [n_C = 30]).

On June 24, 2011 and July 2, 2013, the sunflowers of the Mechanical Damage treatment had four 3 mm wide holes hole-punched into their topmost two leaves. This procedure was repeated on the new topmost two leaves on June 30, 2011 and July 6, 2013. Because sunflowers produce new leaves from the apical meristem as they grow, the second round of damage was to leaves that had not been previously damaged. This damage regime roughly approximated the extent of damage experienced by those in the Larva Damage treatment.

Sunflower beetle larvae used in this study in summer of 2011 were collected from the same population of sunflowers used as our sunflower samples described above. In the summer of 2013, due to a reduced number of sunflower beetles in this sunflower patch, a patch in Indian Battle Park in the Lethbridge river valley, and a patch along Bridge Dr. W, Lethbridge were used to supplement the numbers of larvae collected. First and second instar sunflower beetle larvae were collected on June 24, 2011 and July 2, 2013 from source populations as above. These were collected the same day that they were placed on the experimental sunflower plants and kept briefly in Fisherbrand Free-Standing Microcentrifuge 2.0mL Tubes with Screw Caps during transportation and larvae measurement. These sunflower beetle larvae are referred to as our "Initial Larvae". A single larva was then randomly assigned to each sunflower plant of the larva damage treatment. These initial larvae were allowed to feed on the sunflowers for 10 days. They were then carefully removed on July 4, 2011 and July 12, 2013, respectively.

Experimental Sunflower Beetle Larvae Feeding

On July 6, 2011 and July 14, 2013, 1st and 2nd instar sunflower beetle larvae were collected from the populations of sunflowers and sunflower beetle on the University of Lethbridge campus that have been previously discussed. These larvae are referred to as the "Experimental Larvae". They were collected and measured using the same procedure used to collect the Initial Larvae. The Experimental Larvae were then individually numbered and randomly assigned to a sunflower of one of the three treatment groups to determine their response to variation in prior damage.

The Experimental Larvae were allowed to feed on the sunflowers for 7 days before they were carefully removed on July 13, 2011 and July 21, 2013, for the two experimental runs, respectively. Larvae were individually weighed to determine larval growth.

Leaf Area Consumed Image Analysis

Leaf images were taken immediately before and immediately after the addition and removal of the Experimental Larvae to determine damage existing prior to Experimental Larvae addition and damage done by Experimental Larvae. Leaf images were taken using a 5-megapixel (2560 x 1920) iSight high-definition camera and a HP Scanjet G4010 Photo Scanner (4800 x 9600 dpi).

Images were then edited and processed using MS Paint (Version 6.1 (Build 7601: Service Pack 1) Copyright © 2009 Microsoft Corporation) and Paintbrush (Version 2.1.1 (20101020) Copyright © 2007-2010 "Soggy Waffles"). These images were edited by colouring in the holes produced by the Experimental Larvae (black: 0:0:0 RBG). Images were manually coloured as opposed to automatically processed due to fecal matter left behind by sunflower beetle larvae, discolouration of leaves due to leaf senescence, and leaf tissue collapse around leaf edges or along leaf veins.

The images were then processed in ImageJ (version 1.46r) to determine the leaf area damaged by the Experimental Larvae. The scale was set using a ruler that had been placed in view of the image beside the leaf. Leaf area damaged was measured by first setting the image to "Type" 8-bit. The image "Threshold" was then set to a min of "0" and max of "0". This would leave only the coloured in areas to be measured as the only pixels remaining would be 0:0:0 RBG. The pixels remaining were analyzed to reveal the damaged area.

Analysis (Survivorship, RGR, RCR, and ECI)

Survivorship was determined by recovery of sunflower beetle larvae after the feeding period on the experimental sunflower plants. Any sunflower beetle larva that was not recovered was assumed to have died. When determining the effect of treatment on larval survival, a Generalized Linear Model (GLM) with a binomial distribution was created, using larval survivorship as the response variable and treatment crossed with initial larval mass as explanatory variables. This was used to determine whether there was a significant effect of treatment on survivorship; subsequently, GLMs consisting of the two treatment pairings involving the control treatment (Mechanical Damage – Control; and Larval Damage – Control) were used as planned contrasts to determine which treatments were significantly different from one another.

RGR, RCR, and ECI were determined using the formulae described previously. Those sunflower beetle larva that did not survive the full experiment duration (7 days) were excluded from this part of the analyses as it was not possible to determine sunflower beetle larva final mass and so impossible to determine their individual RGR, RCR, and ECI. Only larvae in the Larval Damage treatment raised on plants on which both the initial larva and the experimental larva survived were used to determine RGR and its components, as much of the time initial larva that did not survive until the end of that phase of the experiment produced no or limited damage to the plant. It was then determined if there was an effect of treatment (Mechanical Damage, Larva Damage, and Control) on these measures (RGR, RCR, and ECI) using ANOVAs.

All statistical analyses were performed in R (Version 3.0.0) in RStudio (Version 0.97.335 – © 2009-2012 RStudio, Inc.).

The experiment was originally performed in the summer of 2011 and then repeated in the summer of 2013. This is because the survivorship of sunflower beetle larvae raised on mechanically damaged plants was marginally non-significantly different from control plants (i.e., the p-value was only slightly above the threshold of α = 0.05 for planned orthogonal contrasts). This suggested a possibility of mechanical damage as a trigger for the induction of plant defences in sunflower. To be prudent, the experiment was repeated in the summer of 2013 with the modification that the sample size was doubled of that from the summer of 2011 to increase statistical power (i.e., initial ($n_L = n_M = n_C = 60$).

Results

Larval Survivorship

In the 2011 version of the experiment, there was a significant effect of treatment on larval survivorship (Figure 2.1a; GLM with binomial distribution; χ^2 = 11.15; p = 0.0038, df = 2, $n_L = n_M = n_C = 30$). There was also an effect of initial mass on larval survivorship (Figure 2.1a; GLM with binomial distribution; χ^2 = 5.87; p = 0.015, df = 1, $n_L = n_M = n_C = 30$), but there was not a significant treatment by initial larval mass interaction (Figure 2.1a; GLM with binomial distribution; χ^2 = 2.14; p = 0.34, df = 2, $n_L = n_M = n_C = 30$).

Due to the significant effect of treatment on larval survivorship, a reduced GLM based on a subset of the data was used to contrast treatment pairs (Mechanical Damage – Control, Larval Damage – Control). There was a marginally non-significant difference between the Mechanical Damage and Control treatments (Figure 2.1a; GLM with binomial distribution; χ^2 = 3.22; p = 0.073, df = 1, $n_L = n_M = 30$). There was also no significant difference between the Larval Damage and Control treatments (Figure 2.1a; GLM with binomial distribution; χ^2 = 2.64; p = 0.10, df = 1, $n_L = n_M = 30$). However, post hoc (i.e., not a planned contrast), it was determined that there was a significant difference between the Larval Damage and Mechanical Damage treatments (Figure 2.1a; GLM with binomial distribution; χ^2 = 10.91; p = 0.00096, df

= 1, $n_L = n_M = 30$), indicating that this difference drove the significant treatment effect in the whole model.

In the 2013 version of the experiment, no significant effect of treatment was found on survivorship (Figure 2.1b; GLM with Binomial Distribution; $\chi^2 = 1.88$, p = 0.39; df = 2; $n_L = n_M = n_C = 60$). There was no significant effect of initial mass on larval survival survivorship, in contrast to 2011 (GLM with Binomial Distribution; $\chi^2 = 0.032$; p = 0.86; df = 1; $n_L = n_M = n_C = 60$). Lastly, there was no significant Treatment x Initial Mass interaction on larval survivorship (GLM with Binomial Distribution; $\chi^2 = 0.12$; p = 0.94; df = 2; $n_L = n_M = n_C = 60$). Unlike in 2011, because no significant effect of treatment was detected, contrasts based on reduced GLMs were unnecessary.

RGR, RCR, and ECI

The experiment carried out in 2011 determined that there was no significant effect of treatment on relative growth rate (RGR) (Figure 2.2a; ANOVA: F = 0.36, p = 0.70, ndf = 2, ddf = 40, $n_L = 12$, $n_M = 12$, $n_C = 19$); relative consumption rate (RCR) (Figure 2.3a; ANOVA: F = 0.94, p = 0.40, ndf = 2, ddf = 40, $n_L = 12$, $n_M = 12$, $n_C = 19$); or efficiency of conversion of ingested food (ECI) (Figure 2.4a; ANOVA: F = 0.013, p = 0.99, ndf = 2, ddf = 40, $n_L = 12$, $n_M = 12$, $n_C = 19$).

In the summer of 2013, no significant effect of treatment was found on relative growth rate (RGR) (Figure 2.2b; ANOVA: F = 1.034, p = 0.36, ndf = 2, ddf = 102, $n_L = 31$, $n_M = 35$, $n_C = 39$); relative consumption rate (Figure 2.3b; ANOVA: F = 0.50, p = 0.61, ndf = 2, ddf = 102, $n_L = 31$, $n_M = 35$, $n_C = 39$); and efficiency of conversion of ingested food (Figure 2.4b; ANOVA: F = 0.52, p = 0.60, ndf = 2, ddf = 102, n_L = 31, n_M = 35, n_C = 39).

Discussion

This experiment did not detect a significant effect of previous leaf damage by mechanical application or insect feeding, in terms of sunflower beetle larvae survival, relative growth rate, relative consumption rate, and efficiency of conversion of ingested food. These results contradict several other studies, which have shown reduced survival rates of sunflower beetles and larvae (and beetle feeding aversion) due to induced plant defences following damage from both mechanical sources and previous feeding by sunflower beetle larva (Rogers and Thompson 1980, Olson and Roseland 1991, Roseland & Grosz 1997). This reduced survival rate was as great as 0% survivorship in experimental sunflower beetle larvae (Rogers & Thompson 1980). However, Olson and Roseland (1991) used 50 sunflower beetle adults to damage sunflower plants for one day and measured coumarin, scopoletin, and ayapin concentrations in ground-up leaf material over the 25 days following damage; whereas I allowed a single sunflower beetle larva to continuously feed on a sunflower plant for a week and measured the larval survival, RGR, RCR, and ECI of another larva feeding over a week. The main differences between this study and my own are the length and severity of damage exposure (acute damage induction vs. chronic damage induction). Therefore, this could imply that I did not exceed a required damage threshold within a short enough timespan in order to induce an increased expression of sunflower plant defences.

My study also found that sunflower beetle survivorship over a period of seven days was also not significantly different between the different treatments. In the summer of 2011, sunflower beetle larva survivorship was marginally non-significantly different on sunflowers that had been mechanically damaged compared to the undamaged control sunflowers. However, in the summer of 2013 when the experiment was repeated with a much larger sample size, there was no significant difference in survivorship between the different treatments. It is possible that sunflower beetle larvae are capable of inhibiting sunflower defence initiation similar to the Colorado potato beetle (*Leptinotarsa decemlineata*) against tomato plants (*Solanum lycopersicum*) (Chung et al. 2013); or that no sunflower defences were induced during the experiment, possibly due to not exceeding a damage-induction threshold.

The non-significant difference between sunflower treatments in terms of RGR, suggests that sunflower plant damage and possible induced sunflower defences did not affect the ability of sunflower beetle larvae to grow (Scriber & Slansky 1981). In terms of RCR, this non-significant difference between sunflower treatments, suggests that sunflower plant damage and possible induced sunflower defences do not discourage sunflower beetles from consuming leaf tissue (Scriber & Slansky 1981), which we hypothesized may have occurred due to decreased palatability (Gomez et al. 2008) or increased threat of toxicity (Olson & Roseland 1991) due to possibly induced sunflower defences. Lastly, non-significant differences between damage treatments in terms of ECI suggest that sunflower beetle larvae experience no significant decrease in their ability to digest ingested leaf material (Scott et al. 2010).

Figure 2.1a demonstrates that the greatest difference in larval survivorship between experimental treatments occurred within the initial larva mass range of 1.5mg and 2.5mg. It is also possible that because those larvae in 2013 were almost entirely drawn from initial mass range of 0.5mg to 1.5mg, that any difference in larval survivorship may not have been observed. Therefore, this may imply that induced plant defences may more strongly affect larger larvae which may have consumed more plant tissue and accumulated plant defences within their bodies and tissues over time; or that because larger larvae consume leaf material at a greater rate, that they are also exposed to greater amounts of plant defences within a shorter period of time and so may experience increased toxicity.

However, it must be considered that this study may be inherently biased in terms of RGR, RCR, and ECI, as only sunflower beetle larvae that were able to survive the full 7 days of the experiment were used in the analysis of RGR, RCR, and ECI. And further, in the Larva Damage Treatment, only those plants whose initial larvae survived were included. This may bias the results in two non-exclusive scenarios. The first scenario is that only sunflower beetles that are more resistant to sunflower defences were able to survive. If this is true, then the data used to determine sunflower beetle RGR, RCR, and ECI would come from a "resistant" subpopulation of the sunflower beetle larva population. Therefore, these data would only represent those sunflower beetle larvae that experienced limited consequence of induced sunflower defence. The second scenario is that only sunflower beetle larvae that were, by chance, raised on sunflowers that were not able to induce a significant defence were able to survive. In either scenario, very few sunflower beetle larvae experiencing a decrease in RGR, RCR, and ECI would be observed at the end of the experiment, as they would be already dead by the time these metrics could be calculated.

Mondor & Addicott (2003) demonstrated that plant defences in the form of extrafloral nectaries on the broad bean plant (*Vicia faba* L.) were inducible through leaf damage. Mondor et al. (2006) further demonstrated that these defences were not just damage dependent, but also resource dependent. That is to say that the absence of 14:14:14 NPK fertilizer significantly reduced the induction of extrafloral nectaries. Likewise, it may be useful in future experiments to add fertilizer to the growth medium in which the sunflowers are grown.

Conclusion

In conclusion, this study did not detect any significant differences in larval survivorship, RGR, RCR, or ECI among sunflower beetle larvae raised on sunflowers previously damaged by other sunflower beetle larvae, mechanical damage, or left undamaged, in either 2011 or 2013.

Figure Captions

Figure 2.1 – Survivorship of sunflower beetle larvae (*Z. exclamationis*) individually raised on sunflower plants (*H. annuus*) that had been previously damaged by sunflower beetle larva feeding, mechanically damaged using a 3mm diameter hole-punch, or left undamaged (control), after 7 days of feeding. Bubble size represents number of individuals in each 0.5 mg bin. This experiment was performed over the summer of 2011 (a) and the summer of 2013 (b).

Figure 2.2 – Relative Growth Rate (RGR) of sunflower beetle larvae (*Z. exclamationis*) individually raised on sunflower plants (*H. annuus*) that had been previously damaged by sunflower beetle larva feeding, mechanically damaged using a 3mm diameter hole-punch, or left undamaged (control), after 7 days of feeding. Symbols indicate means ± SEMs. This experiment was performed over the summer of 2011 (a) and the summer of 2013 (b).

Figure 2.3 – Relative Consumption Rate (RCR) of sunflower beetle larvae (*Z. exclamationis*) individually raised on sunflower plants (*H. annuus*) that had been previously damaged by sunflower beetle larva feeding, mechanically damaged using a 3mm diameter hole-punch, or left undamaged (control), after 7 days of feeding. Symbols indicate means ± SEMs. This experiment was performed over the summer of 2011 (a) and the summer of 2013 (b).

Figure 2.4 – Efficiency of Conversion of Ingested food (ECI) of sunflower beetle larvae (*Z. exclamationis*) individually raised on sunflower plants (*H. annuus*) that had been previously damaged by sunflower beetle larva feeding, mechanically damaged using a 3mm diameter hole-punch, or left undamaged (control), after 7 days of feeding. Symbols indicate means ± SEMs. This experiment was performed over the summer of 2011 (a) and the summer of 2013 (b).

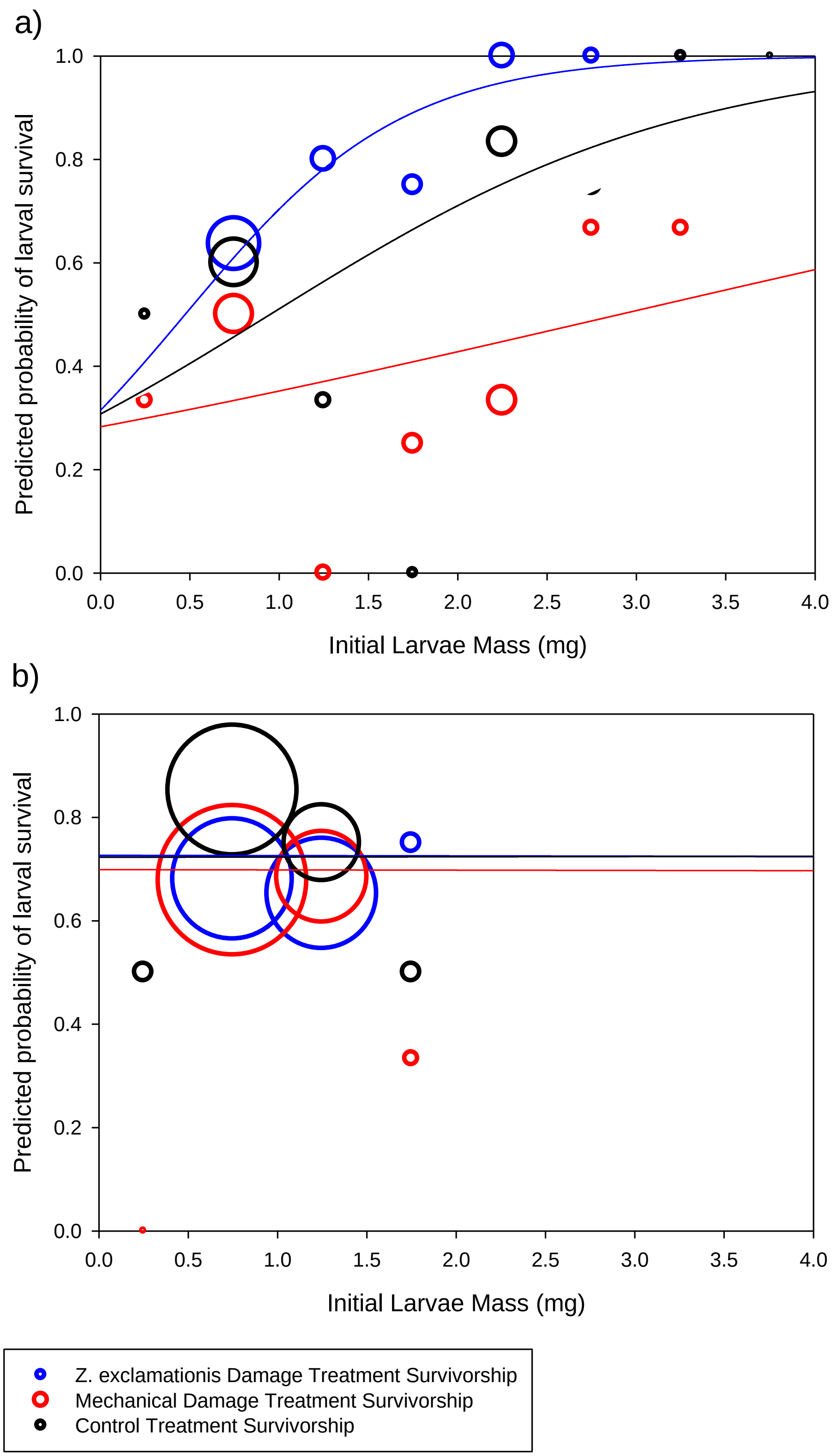

Figure 2.1 – (a. 2011, b. 2013) Larval survivorship of sunflower beetle larvae (*Z. exclamationis*) on larvae and mechanically damaged sunflower plants (*H. annuus*)

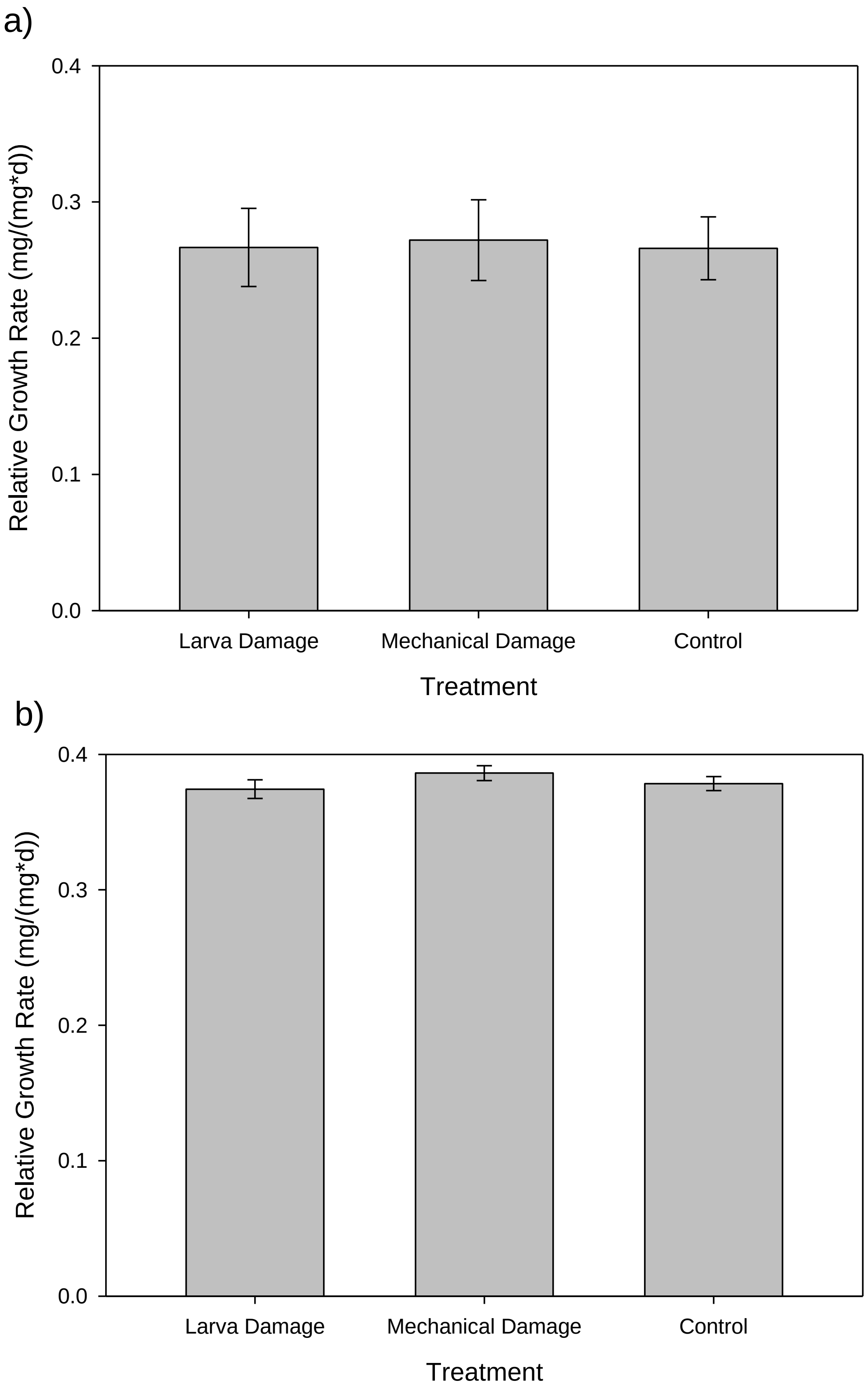

Figure 2.2 – (a. 2011, b. 2013) Relative Growth Rate of sunflower beetle larvae (*Z. exclamationis*) on larvae and mechanically damaged sunflower plants (*H. annuus*)

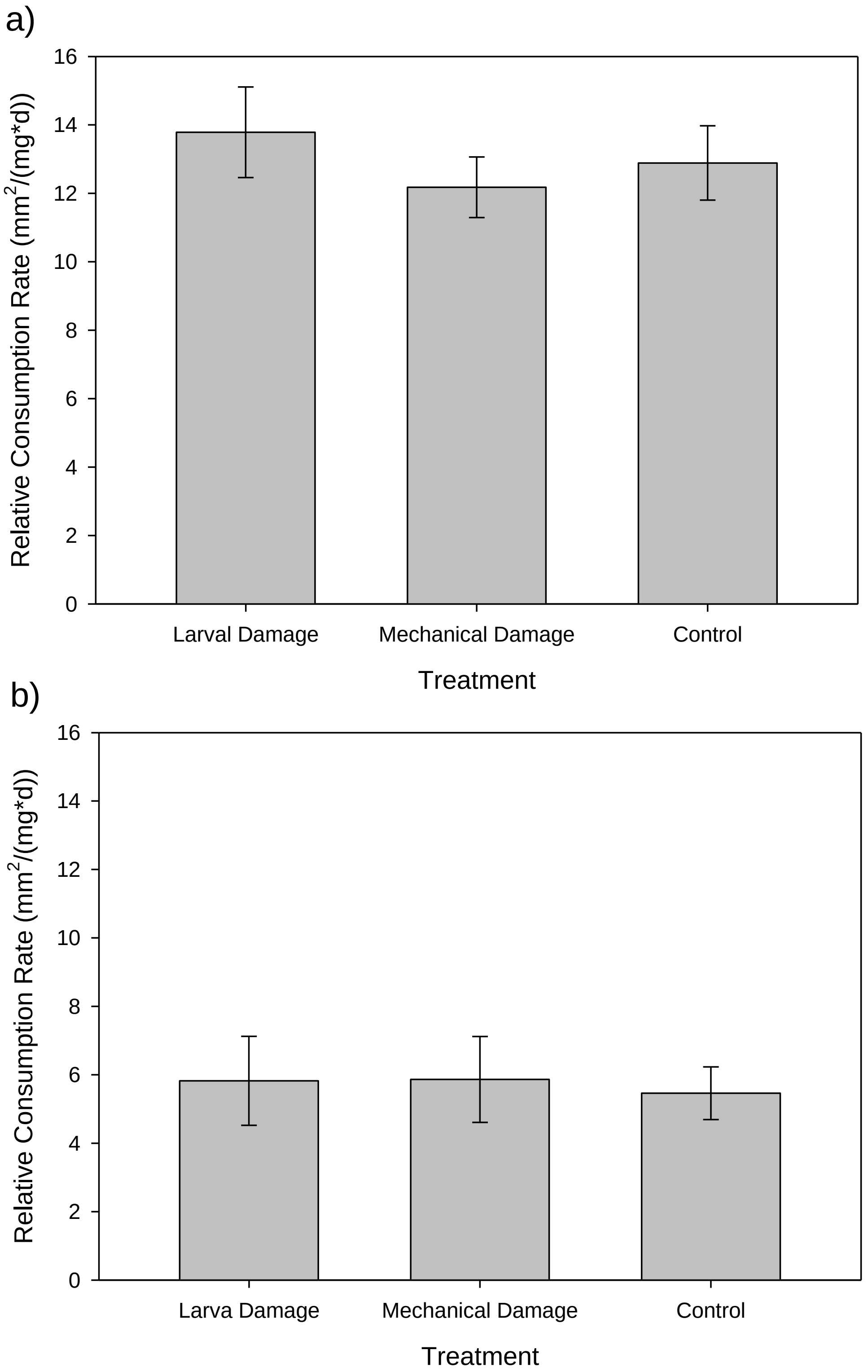

Figure 2.3 – (a. 2011, b. 2013) Relative Consumption Rate of Sunflower Beetle Larvae (*Z. exclamationis*) raised on *H. annuus*.

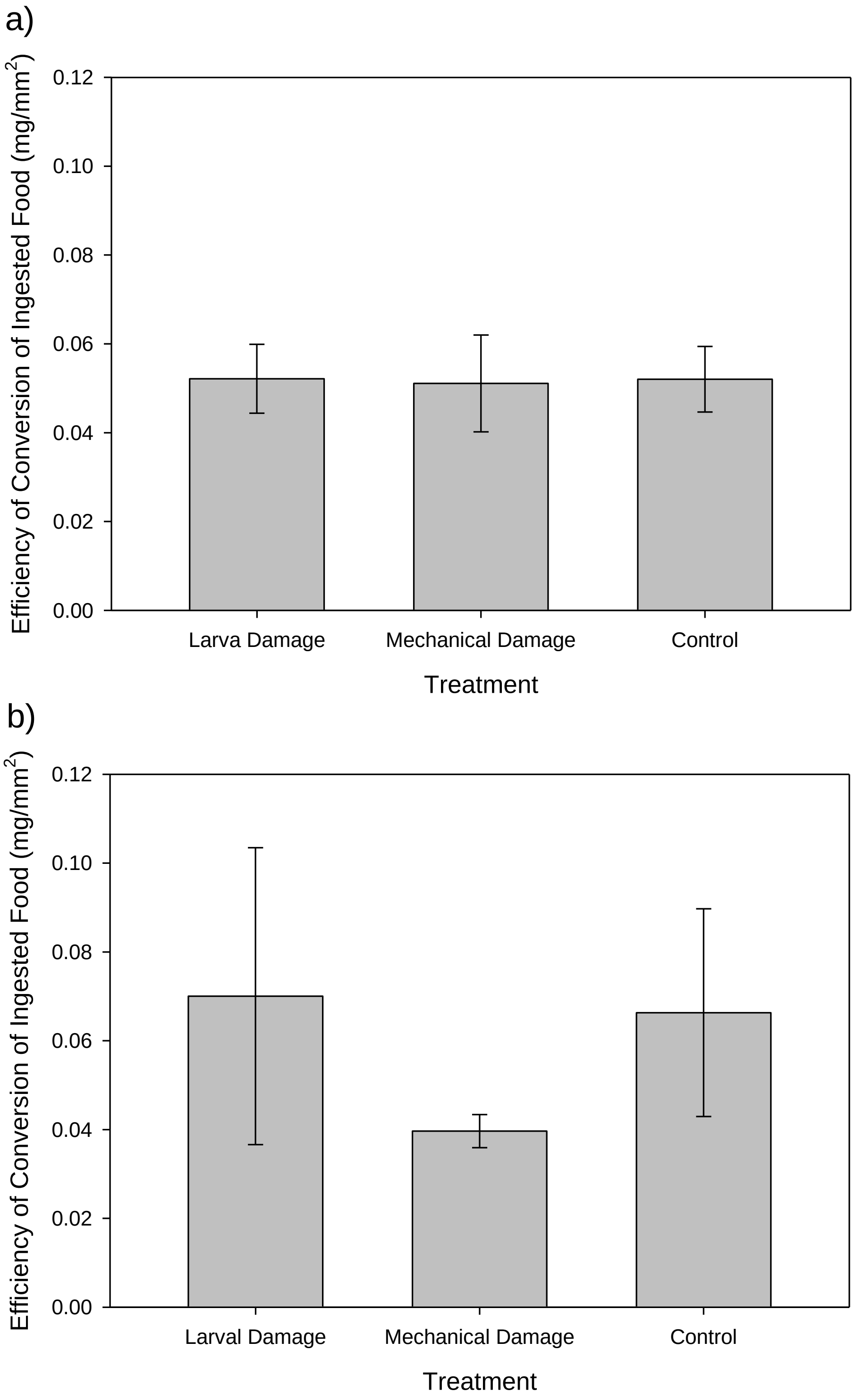

Figure 2.4 – (a. 2011, b. 2013) Efficiency of Conversion of Ingested Food of sunflower beetle larvae (*Z. exclamationis*) on larvae and mechanically damaged sunflower plants (*H. annuus*)

Chapter 3 – Selective Oviposition, Feeding Avoidance, and Gregarious Feeding by Sunflower Beetle Larvae Feeding on Common Annual Sunflower

Abstract

In this chapter, I explore the use of selective oviposition, avoidance, and gregarious feeding, as forms of herbivore offence in response to plant defences and plant damage using the sunflower beetle (*Zygogramma exclamationis*) and the common annual sunflower plants (*Helianthus annuus*). The first experiment investigates the possibility of selective oviposition when sunflower beetles are presented with damaged and undamaged sunflower plants under field conditions. No significant discrimination between damaged and undamaged plants was found in terms of oviposition. However, the number of eggs surveyed on sunflower plants was significantly positively related to the number of leaves on the sunflower, suggesting beetle oviposition choice based on resource abundance opposed to plant defence and damage avoidance.

The second and third experiments investigate sunflower beetle larvae avoidance of plant defences. The second experiment specifically investigates the avoidance of a damaged and an undamaged leaf on a single sunflower plant individual; whereas the third experiment specifically investigates the avoidance of plant defences between a damaged and an undamaged sunflower plant. Neither experiment showed significant preference for damaged or undamaged sunflower leaves or plants. Instead, the second experiment determined that larvae have a tendency to feed on upper leaves regardless of the presence or absence of damage. This may be a result of the tendency of sunflower beetle larvae to

descend the plant every night and consume leaf material from the first leaves it encounters.

The fourth experiment investigates possible benefits of gregarious feeding in sunflower beetle larvae. Sunflower beetle larvae fed for 1 week at varying larval densities. The survivorship of sunflower beetle larvae feeding at varying larval densities significantly increased with increasing sunflower beetle larvae densities. In the absence of predation, this suggests that there may an advantage to gregarious feeding behaviour in sunflower beetle larvae that extends beyond a simple “Dilution Effect” defence.

Introduction

Plant defences have evolved in response to the fitness costs of herbivory with the function of reducing or mitigating these fitness costs (Herrera & Pellmyr 2002, Heil, 2010). These defences may function through the direct production of chemical toxins (Olson & Roseland 1991), physical barriers (Belovsky et al. 1991), or even indirect recruitment of a predator to the herbivore (Katayama & Suzuki 2004). Regardless of the form of plant defence, these defences have costs associated with them (Baldwin 1996, Baldwin et al. 1998, Mondor et al. 2006). In order to reduce these costs, many plant defences are inducible; that is, their expression is increased once the plant experiences a stimulus that may indicate the likelihood of future herbivory (Olson & Roseland 1991, Herrera & Pellmyr 2002, Mondor & Addicott 2003).

Just as herbivory generally results in fitness costs to plants, many plant defences result in fitness costs to herbivores. These costs vary from the reduction of the absolute magnitude or rate of herbivore feeding (Olson & Roseland, 1991) to far more extreme consequences such as herbivore death (Rogers & Thompson, 1980). Just as plants have responded to the fitness costs related to herbivory with plant defences, herbivores have responded to the fitness costs associated with plant defences with what Karban & Agrawal (2002) have termed “herbivore offence”. Specifically, they define herbivore offence as herbivore traits that increase their ability to feed on and use plants for their own benefit. They argue that herbivore offence is an important addition to the traditional perspective of plant fitness in plant-herbivore interactions. Thus, studies of herbivore offence is concerned with the fitness costs and benefits experienced by herbivores, typically balanced with those costs and benefits experienced by plants. Herbivore offence can take a variety of forms and traits; these traits can be general or very specific to a certain type of defence or host plant species. Examples of these traits can range from the avoidance of sunflower plants (*Helianthus annuus*) or plant tissues that are less palatable, possibly due to the presence of toxins such as coumarins (Olson & Roseland 1991) or nicotine in tobacco plants (*Nicotiana sylvestris*) (Baldwin 1996); the selective oviposition of eggs on preferential hosts to increase offspring survivorship (Ruiz-Dubreuil et al. 1994, Encalada & Peckarsky 2006); and gregarious feeding to produce collective protective shelters (such as leaf mines) (Damman 1994), overwhelm induced allelochemical plant defence responses, or induce nutrient sinks (Denno & Benrey 1997).

Avoidance is a category of herbivore behaviour in which the herbivore avoids or removes a plant defence to effectively circumvent the effects of plant defence. It terms of selective feeding, this can be thought of as the selection of less costly food. The effect of this is the maximization of the difference between the benefits and the costs of the selected food. As plant defences are a form of these costs, in many cases it is beneficial to avoid plant defences. For example, Barker (1990) demonstrated that sunflower stem weevil (*Cylindrocopturus adspersus* LeConte) removed sunflower trichomes; while Roseland and Grosz (1997) demonstrated that sunflower beetle adults (*Zygogramma exclamationis*) selectively consume leaf disks covered in distilled water over leaf disks covered in coumarin and its derivatives, scopoletin and ayapin, diluted in distilled water.

Another more specific version of avoidance and selective behaviour in herbivores manifests in the behavioural form of selective oviposition. In this form, it is selection on the part of the parental generation for the offspring generation. For example, Sato et al. (1999) demonstrated that the parental selection by the fungus beetle (*Dacne picta* Coleoptera) of shiitake mushrooms (*Lentinula edodes*) without decay improved larval survival, thereby improving the fitness of the offspring generation and so improving the intrinsic fitness of the parental generation as well.

A choice made by the herbivore parental generation is not just the selective oviposition on a suitable host, but also the number of offspring to oviposit. Once hatched, larvae may benefit from gregarious feeding. These benefits range from increased feeding efficiency and growth (Denno & Benrey 1997), increased recruitment (Lopez et al. 1989), and increased protection and survival (Damman 1987). However, they must also consider the negative consequences inherent with gregarious feeding. A few of these consequences may include increased resource competition (Wise et al. 2006), decreased growth (Damman 1994, Wise et al. 2006), and cannibalism (Mertz & Robertson 1970, Via 1999).

In this chapter, I discuss four experiments, using the common annual sunflower (*Helianthus annuus*) and sunflower beetle larvae (*Zygogramma exclamationis*), I explore selective oviposition on undamaged plants, herbivore avoidance of feeding on previously damaged leaves, herbivore avoidance of feeding on previously damaged plants, and gregarious feeding in larvae.

Methods

Study Species

The common annual sunflower (*Helianthus annuus* L.) is a plant native to North America that expresses several forms of defence, including the production of coumarin and its derivatives scopoletin and ayapin (Olson & Roseland 1991). Sunflower plants used in the greenhouse experiments (Experiment 2, 3, and 4) were collected from the University of Lethbridge campus.

The sunflower beetle (*Zygogramma exclamationis*) is a chrysomelid that specializes on species of the sunflower genus (*Helianthus* spp.) in both its adult and larval stages (Rogers 1977). This species has been shown to induce defences in *Helianthus annuus* (Olson and Roseland 1991) and to be affected by *Helianthus* spp. defences in turn (Olson and Roseland 1991, Rogers and Thompson 1980, Roseland and Grosz 1996). Sunflower beetle larvae used in the greenhouse experiments (Experiment 2, 3 and 4) were collected from the same population of sunflowers used as our sunflower samples described above. These were collected the same day that they were placed on the experimental sunflower plants and kept briefly in Fisherbrand* Free-Standing

Microcentrifuge 2.0mL Tubes with Screw Caps during transportation and larvae measurement.

Experiment 1: Selective Oviposition of Sunflower Beetles between Damaged and Undamaged Sunflower Plants in a Natural Setting

Experiment 1 took place during the summer of 2011 in and around the natural preserve of Dinosaur Provincial Park in southern Alberta. Research and Collection Permits were obtained. The experiment took place during the period of time that sunflower beetle eggs and larvae were observed, between the dates of May 19, 2011 and July 19, 2011.

On May 19, 2011, I searched for suitable populations of sunflowers. Three sites were selected, based on current and previous sunflower abundance, and frequency of undamaged sunflowers. Two of these sites were found within the natural preserve, and another site along Highway 34 just south of where it crosses the Red Deer River. These sites were visited once a week, starting on June 7, 2011 and ending on July 19, 2011, with the exception of July 12, 2011 due to poor weather.

On June 7, 2011, I went to each population and selected sunflower plants that were undamaged and had no sunflower beetle eggs, larvae or adults on them. These plants were randomly assigned to “damaged” and “control” treatments. Sixty plants were selected at each site, with 30 in the damage treatment, and 30 in the control treatment. However, one site had a smaller sunflower population, and so only 26 sunflowers were selected for the damage treatment and another 27 sunflowers for the control treatment.

Plants in the “Damage” treatment were damaged by hole-punching four holes into the two top-most leaves of each plant with a 3mm-diameter hole-punch. If the top-most leaves were too small to hole-punch, the next top-most leaf was used. If the two top-most leaves were too small for 4 holes, as many holes as possible were hole-punched into them (typically three holes). This damage was repeated every two weeks, beginning on June 7, 2011, until the end of the experiment (June 21, 2011, July 5, 2011, and July 19, 2011). Once every week, I surveyed all plants of both treatments at each of the three sites for the number of *Zygogramma exclamationis* eggs, larvae, and adults on each plant.

Additional sites were surveyed, but were excluded from the study as no sunflower beetle eggs, larvae, or adults were found at them. Therefore, 30 damaged and 30 undamaged sunflowers were present at the first usable site, and 26 damaged and 27 undamaged sunflowers were present at the second usable site. Due to trampling, erosion, or large herbivore grazing, 6 damaged and 5 undamaged sunflowers across both sites were lost before the end of the experiment.

Experiment 2: Larvae Feeding Avoidance of Damage Leaves

Sunflower plants were collected from the University of Lethbridge campus on May 14, 2012. On June 20, 2012, all plants gathered had their two top-most leaves isolated by applying Tanglefoot Insect Barrier © around the main stem of the sunflower, above and below the two top-most leaves. The sunflower plants were then randomly divided into two treatments: Upper Damage (UD) (n = 26), and Lower Damage (LD) (n = 21). Due to larval death throughout the term of the experiment, the final sample size for the experiment decreased to: UD (n = 9) and LD (n = 7).

Plants were damaged by hole-punching four 3mm holes into their leaves with a 3mm diameter hole-punch. Those plants of the UD treatment had only their top-most leaf damaged, leaving the second top-most leaf undamaged. Those plants of the LD treatment had only their second top-most leaf damaged, leaving the top-most leaf undamaged.

One week later, on June 27, 2012, sunflower beetle larvae were gathered and had their weight measured. A single sunflower beetle larva was then carefully added to the bottom of the isolated stem segment of each plant. The larva was allowed to feed for one week before it was removed, at which point it was re-weighed.

An image was taken of both focal leaves of every plant, every day of the experiment. These images included a scale bar used to calibrate area within the image and so was used to determine the leaf area consumed by the larva. This was used to determine larval feeding preference between damaged and undamaged leaves, and upper leaves and lower leaves.

Experiment 3: Larvae Feeding Avoidance of Damaged Plants

Sunflower beetle larvae do not typically transfer between sunflower plants when the plants are at low densities, as the larvae do not typically move across the ground between plants. However, I observed that if sunflower plants were in direct contact, sunflower beetle larvae were able to move fairly easily from the leaf of one sunflower plant to the leaf of another sunflower plant. For this reason, this experiment was designed to determine if sunflower larvae are able to avoid damaged plants that may have induced defences.

Sunflower plants were collected from the University of Lethbridge campus on May 14, 2012. Sixty sunflower plants were randomly paired to form 30 pairs of plants. The two top-most leaves were paired and levelled by elevating the shorter plant so that both leaves were at an equal height. One plant of each pair was randomly selected to be the damage treatment plant and the other to be the control. Starting with the second-top-most leaf, the damage treatment plant then had four 3mm holes hole-punched into every other leaf. Note that the top-most leaf, paired with the control plant, was left undamaged. Three larvae died at the beginning of the experiment resulting in a final sample size of 27 larvae and sunflower plant pairs.

One week later on July 4, 2012, the leaves were isolated from the rest of the plant by cutting a slit into a small 15cm Styrofoam plate and punching a hole in the center. The plate was then placed around the stem of the leaf and “Tanglefoot Insect Barrier” was applied to the leaf stem and plate. The leaf stems were then placed in contact with one another and crossed over one another. A single sunflower beetle larva collected from the same sunflower plant patch that the sunflowers were obtained from, was weighed and placed where the leaf stems crossed, oriented in a neutral direction to each plant. A picture of each leaf was taken every day for a week to measure leaf area eaten, as in the previous experiment. The larvae were then removed after a week and re-weighted.

Experiment 4: Gregarious Feeding of Sunflower Beetle Larvae

Sunflower plants (n = 25) and sunflower beetle larvae (n = 155) were collected from the University of Lethbridge campus on July 11, 2012. Twenty-five sunflower plants were randomly divided into five treatment groups: 1, 2, 4, 8, and 16. Sunflower plants within these treatment groups received 1, 2, 4, 8, or 16 larvae per plant, respectively. Larvae were recovered after one week to determine overall survivorship.

Leaf Area Consumed Image Analysis

Leaf images were taken once every day for one week during the experimental week of Experiments 2 and 3 to determine the daily amount of leaf area consumed by the sunflower beetle larvae on each of the two available leaves. Leaf images were taken using a 5-megapixel (2560 x 1920) iSight high-definition camera.

Images were then edited and processed using MS Paint (Version 6.1 (Build 7601: Service Pack 1) Copyright © 2009 Microsoft Corporation) and Paintbrush (Version 2.1.1 (20101020) Copyright © 2007-2010 Soggy Waffles). These images were edited by filling holes produced by the experimental larvae (black: 0:0:0 RBG). Images were processed before analysis to manually correct for the presence of fecal matter left behind by sunflower beetle larvae, discolouration of leaves due to leaf senescence, and leaf tissue collapse around leaf edges or along leaf veins.

The processed images were then analyzed in ImageJ (Version 1.46r; National Institutes of Health) with a scale bar to determine the leaf area damaged by the experimental larvae. Images were converted to 8-bit colour format and contrasted. This contrast allowed ImageJ to calculate the amount of leaf area consumed by the larvae.

Analysis

Experiment 1 was analyzed using a full-factorial repeated-measures ANOVA composed of plant treatment (damaged or undamaged), site (individual sunflower populations), and date (date when damage was surveyed), with plant individual nested within treatment and site as a random effect, and the number of leaves as a co-variate. Both the number of eggs surveyed and the number of larvae surveyed on experimental sunflower plants were $\log_{10}(x + 1)$ transformed to improve homoscedasticity, and were used as the dependent variables in these analyses.

Experiment 2 was analyzed using repeated-measures ANOVA, with a full factorial analysis of leaf damage (experimentally damaged or undamaged leaf), leaf position (whether the leaf was the upper or lower of the two experimental leaves), and date (date when damage was surveyed); with plant included as a random effect. The cumulative leaf damage of each leaf was $\log_{10}(x + 1)$ transformed to improve homoscedasticity, and used as the dependent variable.

Experiment 3 was analyzed using repeated-measures ANOVA, using the factors of treatment (damaged or undamaged plant), date (date of which damage was surveyed), and the treatment by date interaction. The analysis also included the larvae individuals as a random effect, and plant individuals nested within treatment as a random effect. Unlike Experiment 2, it is necessary to treat larva as a separate random effect as each larva is associated with both the damage and undamaged treatments, whereas plant individuals are only associated with a single treatment and so are nested within treatment and treated as a random effect. (In Experiment 2, a single larva is associated with a single plant and so cannot be treated as a separate random effect.) The cumulative leaf damage was $\log_{10}(x + 1)$ transformed to promote homoscedasticity, and was used as the dependent variable.

Experiment 4 used a generalized linear model assuming a binomial distribution (i.e., logistic regression) to determine the relationship between the larval density per plant and the proportion of larvae surviving per sunflower plant, weighted by the number of observations (larvae) per plant.

Statistical analysis was performed in JMP (Version 7.0.2 Copyright © 2007 SAS Institute Inc.) and R (Version 3.0.0) in Rstudio (Version 0.97.335 – © 2009-2012 Rstudio, Inc.).

Results

Experiment 1: Selective Oviposition of Sunflower Beetles on Undamaged Plants

Of the 3 sites surveyed over the summer of 2011, *Z. exclamationis* eggs, larvae, and adults were only found at 2 of these sites. *Z. exclamationis* eggs appeared June 21, 2011 through to July 19, 2011 (Figure 3.1). Larvae appeared on June 28, 2011, through to beyond the end of the experiment on July 19, 2011 (Figure 3.3). Adults were present within the sunflower populations surveyed, though no more than 2 in an entire site at a time, and typically none at all. As a result of this, only 2 of the 3 sites were used in the analysis, and only $\log_{10}(x + 1)$ transformed number of eggs and larvae were used as dependent variables.

Sunflower beetle adult females did not significantly discriminate between damaged plants and control plants in terms of the number of eggs oviposited on the sunflower plants (Figure 3.1; Table 3.1), but there was there a significant difference between sites of sunflower populations (Table 3.1). There was a significant effect of survey date on the number of eggs found (Figure 3.1; Table 3.1). The number of eggs surveyed began and ended very low, but peaked around June 28th, 2011. There were no significant interactions between site, treatment, or date, other than a site x date interaction (Table 3.1). The number of eggs oviposited was significantly positively related to the number of leaves per plant (Figure 3.2; Table 3.1).

In terms of the number of sunflower beetle larvae found on experimental sunflower plants, there was no significant difference between the number of larvae found on damaged plants and control plants (Figure 3.3; Table 3.2). However, there was a significant effect of both date and site on the number of larvae surveyed (Figure 3.3; Table 3.2). There were no significant interaction effects in terms of treatment x site, treatment x date and the three-way interaction between treatment x site x date; but there was a significant interaction effect of site x date, (Table 3.2). Lastly, the number of leaves per plant was significantly related to the number of larvae surveyed (Figure 3.4; Table 3.2).

Experiment 2: Larvae Feeding Avoidance of Damaged Leaves

Sunflower beetle larvae did not significantly discriminate between damaged and undamaged leaves in terms of cumulative leaf area consumed ($\log_{10}(x + 1)$ transformed; Figure 3.5; Table 3.3). However, leaf position (upper or lower leaf), date, and the leaf position x date interaction had significant effects on cumulative leaf area consumed (Figure 3.5; Table 3.3). Specifically, upper leaves were consumed more than lower leaves, and this difference increased as time went on. All other interactions did not have significant effects (Table 3.3).

Experiment 3: Larvae Feeding Avoidance of Damaged Plants

There was no significant discrimination of damaged plants by sunflower beetle larvae in terms of leaf area consumed ($\log_{10}(x + 1)$ transformed; Figure 3.6; Table 3.4). There was a significant effect of date on the amount of leaf area consumed by the sunflower beetle larva (Figure 3.6; Table 3.4). Lastly, there was no significant treatment x date interaction found (Figure 3.6; Table 3.4).

Experiment 4: Gregarious Feeding of Larvae

A generalized linear model, assuming a binomial distribution, of the proportion of sunflower beetle larvae surviving per plant at varying larval population densities, revealed a significant increase in sunflower beetle larval survivorship with increasing sunflower beetle larval densities (Figure 3.7; Logistic Regression: $\chi^2 = 4.58$, $p = 0.032$, $df = 1$).

Discussion

Experiment 1 – Selective Oviposition on Undamaged Sunflower Plants

I had predicted that, with the induction of sunflower plant defences (Rogers & Thompson 1980, Olson & Roseland 1991, Roseland & Grosz 1997) and intraspecific competition (Mertz & Robertson 1970, Damman 1994, Via 1999), sunflower beetle females would avoid laying eggs on damaged sunflower plants. These predictions assume that because sunflower defences and intraspecific competition would decrease the fitness of sunflower beetle offspring and thereby decrease the intrinsic fitness of the female sunflower beetles, these female sunflower beetles would instinctively oviposit on undamaged sunflower plants.

However, this experiment provided no evidence that female sunflower beetle adults discriminated between damaged and undamaged sunflower plants in terms of the number of eggs oviposited on the sunflowers plants (Figure 3.1). Instead, the number of eggs oviposited on sunflower plants was positively related to the number of leaves on the sunflower plants. This result could be interpreted as a selective discrimination of plants based on varying resource (food) abundance (Figure 3.2).

As sunflower beetle larvae hatch from sunflower beetle eggs, it is unsurprising that the relationship between the number of larvae surveyed on sunflower plants and whether the sunflower plant was damaged or undamaged, follows a similar pattern as that with the number of sunflower beetle eggs surveyed (Figure 3.3); that is, there was no significant effect of plant damage on the number of larvae surveyed per plant. However, the number of sunflower beetle larvae surveyed (Figure 3.4) was significantly positively related to the number of leaves on the sunflower plants.

The effects of date on the number of sunflower beetle eggs and larvae surveyed on sunflower plants is a result of the experiment beginning before sunflower beetle females began ovipositing, rising to a peak on June 28th, 2011, and dropping off towards the end of the experiment as eggs hatched in to larvae (Figure 3.1); or in the case of larvae, persisting beyond the end of the experiment (Figure 3.3).

This experiment did not detect any significant discrimination between damaged and undamaged sunflower plants in terms of the number of eggs oviposited (Figure 3.1). However, it did detect a significant positive relationship between the number of eggs oviposited and the number of leaves on a sunflower plant (Figure 3.2). This suggests that sunflower beetle females: do not selectively oviposit on undamaged sunflower plants; cannot detect plant damage; or selectively oviposit on sunflower plants that offer greater quantity and/or quality resources for their offspring. Alternatively, Hilker and Meiners (2006) discussed the induction of plant defences by insect egg oviposition. Therefore, female sunflower beetle oviposition may induce plant defences, and so plant discrimination based on the current presence of plant defences would be redundant, as oviposition would induce defences, whether or not plant defences had been previously induced.

This may have also resulted from the uncontrolled nature of field experiments, as both damaged and undamaged sunflower plants showed insect herbivore damage as the experiment progressed. This damage would have decreased differences between the two treatments, whether through induction of sunflower plant defences or through visual cues of leaf damage, and so limit the ability of the sunflower beetle females to discriminate between the two treatments.

A final interesting result is the significant effect of site on the number of sunflower beetle eggs and larvae surveyed (Table 3.1; Table 3.2). It is possible that this result may be attributed to the difference in terrain, wind cover, sun exposure, nutrient availability, and surrounding vegetation. However, this is mere speculation and further study is required before any conclusions can be made.

Future lines of research for this experiment include repeating it in a controlled environment in which a sunflower population, isolated from external damage and environmental factors, is exposed to a number of sunflower beetle adults and surveyed for eggs and larvae to determine if sunflower beetles do avoid damaged sunflower plants. However, this field experiment is useful in that it is a representation of natural conditions and pressures influencing sunflower beetle adult host choice, as opposed to creating a highly artificial situation, which may be useful in breaking down behaviours individually, but is nonetheless a reductionist representation of host selection in sunflower beetle adults.

Experiment 2 – Larvae Feeding Avoidance of Damaged Leaves

This experiment determined that sunflower beetle larvae did not significantly discriminate between sunflower leaves that had been damaged or left undamaged (Figure 3.5). However, sunflower beetle larvae did have a significant tendency to consume leaf tissue from the upper leaf of the two leaves accessible to them (Figure 3.5). Sunflower beetle larvae have a tendency to travel towards the terminal ends of the sunflower shoots during the day as they provide hiding places (e.g., bracts) in which they can protect themselves from foraging predators; during the night, the sunflower beetle larvae leave the terminal ends of the sunflower shoots to consume leaf material (Rogers 1977). Sunflower beetle larvae may simply consume sunflower leaves that they come across first, which due to this behaviour would result in the consumption of higher leaves. Another possibility is that the higher leaves of many species of plants have a tendency to be of higher nutritional value (Ahmad et al. 2012). In addition to this, sunflower plants utilize leaf senescence in order to reallocate nutrients from lower leaves so that they may recycle these nutrients in the production of new leaves nearer to the apical meristems of the plant as it grows (Aguera et al. 2010). However, several studies suggest that various plants disproportionately allocate greater plant defences to these more valuable leaves (Van Dam et al. 1996, Ohnmeiss & Baldwin 2000, McCall & Fordyce 2010). If higher sunflower leaves have a tendency to be of higher quality and possess greater potential for nutrients, and herbivore benefits gained from increased nutrient access out-weighted the possible consequences of increased plant defence expression, it would beneficial for sunflower beetle larvae to selectively consume these higher value leaves.

Experiment 3 – Larvae Feeding Avoidance of Damaged Plants

I had predicted that if sunflower beetle larvae were able to detect induced sunflower plant defences, they would discriminate between plants with or without induced plant defences (Olson & Roseland 1991). However, this experiment determined that the presence or absence of damage on sunflower plants did not affect the amount of leaf area consumed by sunflower beetle larvae, when presented with the choice between the undamaged leaves of a damaged and an undamaged plant (Figure 3.6). However, if sunflower beetle larvae were able to detect gradients of sunflower defences, some feeding preference for the leaf of the undamaged sunflower plant should have been detected. This result contradicts previous studies that have determined that sunflower beetles are capable of detecting and avoiding sunflower defences (Olson & Roseland 1991), and so the more probable explanations are that: this experiment was unable to detect sunflower beetle larva avoidance behaviour of induced sunflower defences; or no significant difference in sunflower plant defences were induced between the damaged and undamaged sunflower plants, possibly due to lack of damage induction.

Experiment 4: Gregarious and Group Feeding of Larvae

Prior to Experiment 1, I had observed that sunflower beetle larvae tended to oviposit several eggs on a single sunflower plant, rather than distribute them singularly among several plants. This is a curious observation, as gregarious feeding may result in intraspecific competition among the larvae, whether through increased resource competition (Wise et al. 2006), decreased growth (Damman 1994, Wise et al. 2006), or cannibalism (Mertz & Robertson 1970, Via 1999). This experiment may suggest one reason why this is so. Sunflower beetle larvae had significantly greater probability of survival when living on sunflower plants with a greater population density (larvae/sunflower) than those on sunflower plants of a lower population density (Figure 3.7). Denno & Benrey (1997) observed similar results, in that they found that *Chlosyne janais* larvae fitness increased when feeding in large aggregations opposed to small ones. However, they measured fitness through larval weight gain opposed to the probability of larval survival. Their study suggested that this increase in larval fitness was due to the larvae overwhelming plant defence responses or due to the larvae creating a nutrient sink as this increase in larval weight gain was not found when larvae were fed excised leaves.

Future direction for this study would be to determine the exact mechanism of the increase in sunflower beetle larvae survivorship. If sunflower beetle larvae decrease plant defence induction, it would be useful to track coumarin concentrations in the damaged and undamaged leaves of the sunflower plant using the methods described by Tal and Robeson (1986) and used by Olson & Roseland (1991).

Conclusion

The results of the first experiment in this chapter suggested that female sunflower beetle adults did not selectively oviposit on undamaged sunflower plants when presented with undamaged and damaged sunflower plants. However, they did appear to selectively oviposit relative to the number of leaves on sunflower plants. This implied that female sunflower beetle adult selection is based on varying resource abundance.

The second and third experiments of this chapter suggested that sunflower beetle larvae did not avoid damaged sunflower leaves and plants. However, the second experiment determined that sunflower beetle larvae significantly favoured consuming leaf material from the leaves higher on the sunflower stems available to them.

The final experiment determined that sunflower beetle larvae had a significantly higher survival rate when at higher densities of larvae per sunflower than at lower densities. The mechanisms behind this increased survival rate has not yet been determined.

Table 3.1 - Experiment 1 – Repeated-measures ANOVA of number of sunflower beetle eggs surveyed ($\log_{10}(x + 1)$ transformed), in Dinosaur Provincial Park, Alberta between the weeks of June 7, 2011 and July 19, 2011.

Source	NDF	DDF	F	p
Site	1	112.5679	22.55561	< 0.0001
Treatment	1	109.6645	0.027594	0.868373
Date	5	537.6306	9.553201	< 0.0001
Site*Treatment	1	109.6525	0.824739	0.36579
Site*Date	5	530.0886	5.390801	< 0.0001
Treatment*Date	5	529.2611	0.650882	0.660948
Site*Treatment*Date	5	529.4912	1.335262	0.247726
Leaves	1	432.6726	118.9002	< 0.0001

Table 3.2 – Experiment 1 – Repeated-measures ANOVA of number of sunflower beetle larvae surveyed ($\log_{10}(x + 1)$ transformed), in Dinosaur Provincial Park, Alberta between the weeks of June 7, 2011 and July 19, 2011.

Source	NDF	DDF	F	p
Site	1	112.340205	21.5209121	< 0.0001
Treatment	1	109.556641	0.11625796	0.73378203
Date	5	539.063638	36.219995	< 0.0001
Site*Treatment	1	109.549543	0.04217802	0.83766194
Site*Date	5	531.14395	16.5061128	< 0.0001
Treatment*Date	5	530.304823	0.67614827	0.64168972
Site*Treatment*Date	5	530.546827	0.75375774	0.58356384
Leaves	1	338.201691	169.308933	< 0.0001

Table 3.3 – Experiment 2 – Repeated-measures ANOVA of the cumulative leaf area consumed by sunflower beetle larvae (mm^2) ($\log_{10}(x + 1)$ transformed).

Source	NDF	DDF	F	p
Leaf Position	1	381.8	158.8598	< 0.0001
Date	6	390.6	27.0697	< 0.0001
Treatment	1	381.8	1.8749	0.1717
Leaf Position*Date	6	381.8	3.0002	0.0071
Leaf Position*Treatment	1	51.1	0.0336	0.8553
Date*Treatment	6	381.8	0.1587	0.9872
Leaf Position*Date*Treatment	6	390.6	0.2465	0.9606

Table 3.4 – Experiment 3 – Repeated-measures ANOVA of cumulative leaf area consumed by sunflower beetle larvae (mm^2) ($\log_{10}(x + 1)$ transformed).

Source	NDF	DDF	F	p
Treatment	1	26.59	0.2806	0.6007
Date	6	253.4	90.0466	< 0.0001
Treatment*Date	6	251.1	0.8238	0.5524

Figure Captions

Figure 3.1 – Average number of *Z. exclamationis* eggs surveyed per experimentally damaged (open symbols; n = 56) or undamaged (closed symbols; n = 57) *H. annuus* plants ($\log_{10}(x + 1)$ transformed), at two sites (Site 1, circles; n = 60; and Site 2, triangles; n = 53) in Dinosaur Provincial Park, Alberta between the weeks of June 7, 2011 and July 19, 2011. Symbols indicate means ± SEMs.

Figure 3.2 – Scatterplot with regression line of the number of *Z. exclamationis* eggs oviposited *H. annuus* plants relative to the number of leaves.

Figure 3.3 – Average number of *Z. exclamationis* larvae surveyed on experimentally damaged (open symbols; n = 56) or undamaged (closed symbols; n = 57) *H. annuus* plants ($\log_{10}(x + 1)$ transformed), at two sites (Site 1, circles; n = 60; and Site 2, triangles; n = 53) in Dinosaur Provincial Park, Alberta between the weeks of June 7, 2011 and July 19, 2011. Symbols indicate means ± SEMs.

Figure 3.4 – Scatterplot with regression line of the number of *Z. exclamationis* larvae surveyed *H. annuus* plants relative to the number of leaves present.

Figure 3.5 – The cumulative upper and lower leaf area consumed (mm^2) by sunflower beetle larvae (*Z. exclamationis*) on two isolated leaves on a single sunflower (*H. annuus*), with a $\log_{10}(x + 1)$ transformation, when: only the upper leaf was damaged (UD)

(triangles; n = 26); or only the lower leaf was damaged (LD) (circles; n = 21). Lower leaves are represented by open symbols. Upper leaves are represented by closed symbols.

Figure 3.6 – The cumulative leaf area consumed (mm^2) by sunflower beetle larvae (*Z. exclamationis*) (n = 30) on two isolated leaves (using Tanglefoot© and Styrofoam plates) from paired sunflowers (*H. annuus*), with a $\log_{10}(x + 1)$ transformation. In each sunflower pair, one sunflower was damaged (D) (closed symbols; n = 27) and the other was undamaged (C) (open symbols; n = 27).

Figure 3.7 – Predicted probability of sunflower beetle larval (*Z. exclamationis*) (n = 155) survivorship per plant at varying larvae population densities (1, 2, 4, 8, 16 larvae per plant) (n = 155) on sunflower plants (*H. annuus*) (n = 25). The experiment was analyzed using a generalized linear model (binomial distribution) between the larvae density per plant and the proportion of larvae surviving per sunflower plant. Overlapping points are staggered. Symbol area is proportional to the number of larvae per plant.

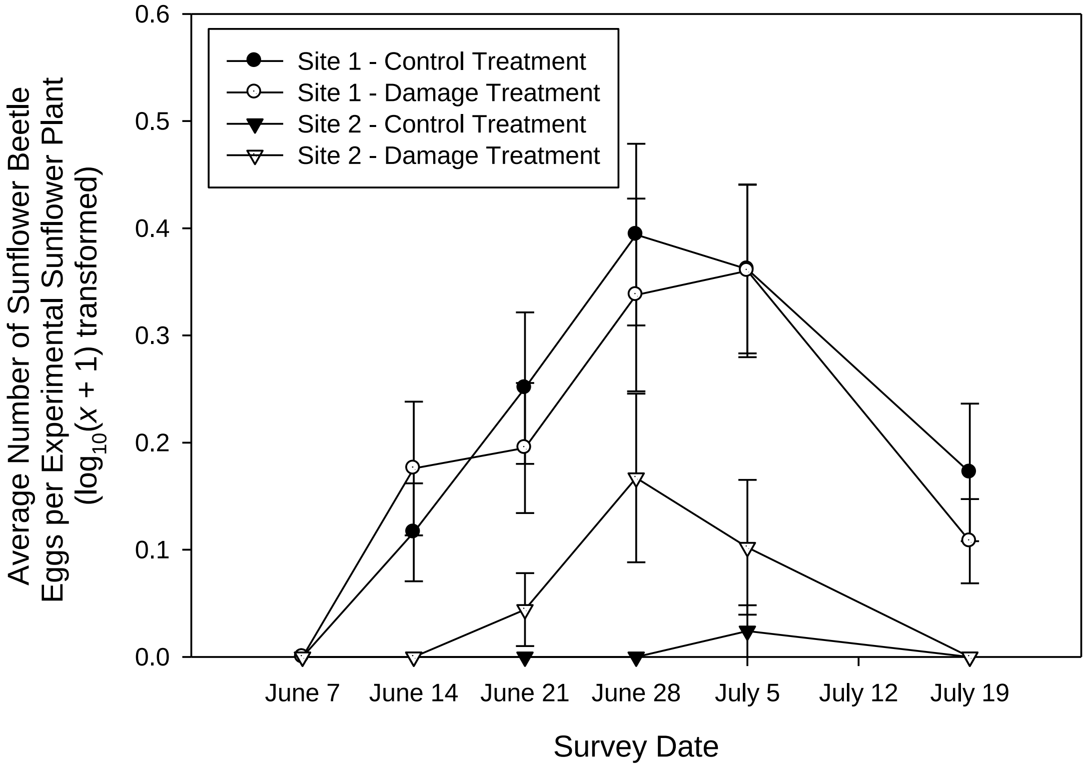

Figure 3.1 – Average number of sunflower beetle eggs (*Z. exclamationis*) eggs surveyed on experimentally damaged and undamaged sunflower plants (*H. annuus*) in Dinosaur Provincial Park

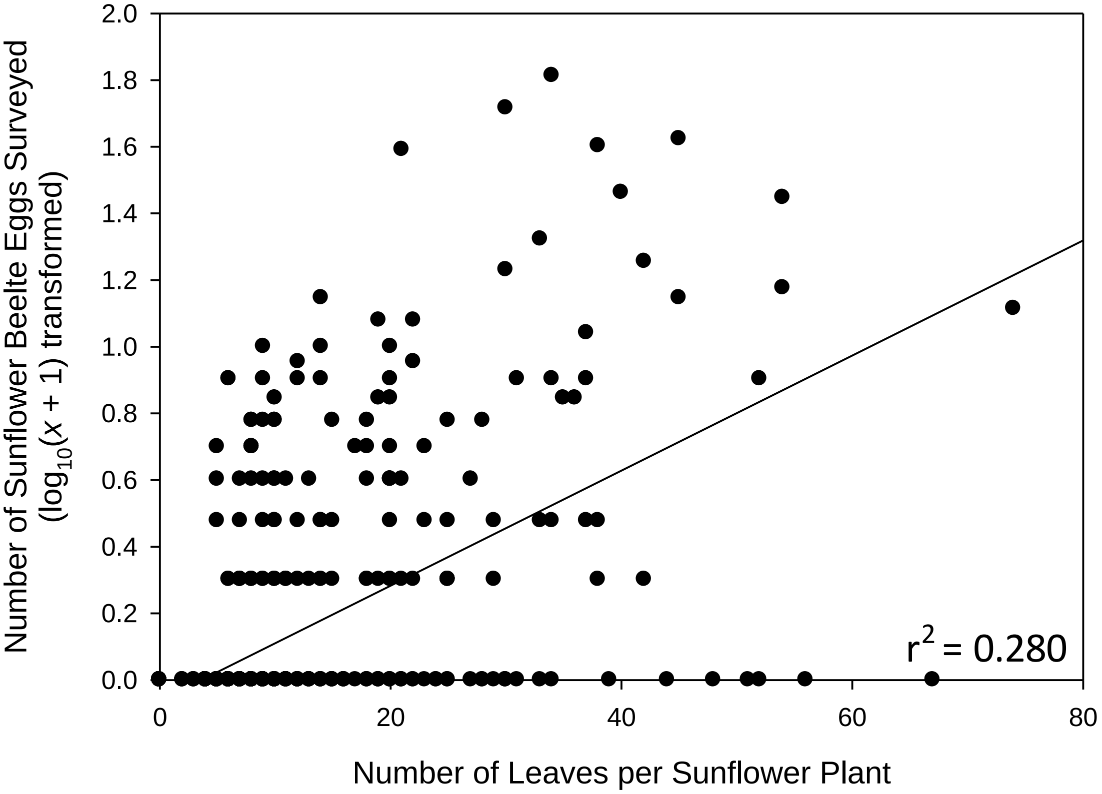

Figure 3.2 – Scatterplot with regression line of sunflower beetle (*Z. exclamationis*) eggs surveyed on experimentally damaged and undamaged sunflower plants (*H. annuus*) in in Dinosaur Provincial Park

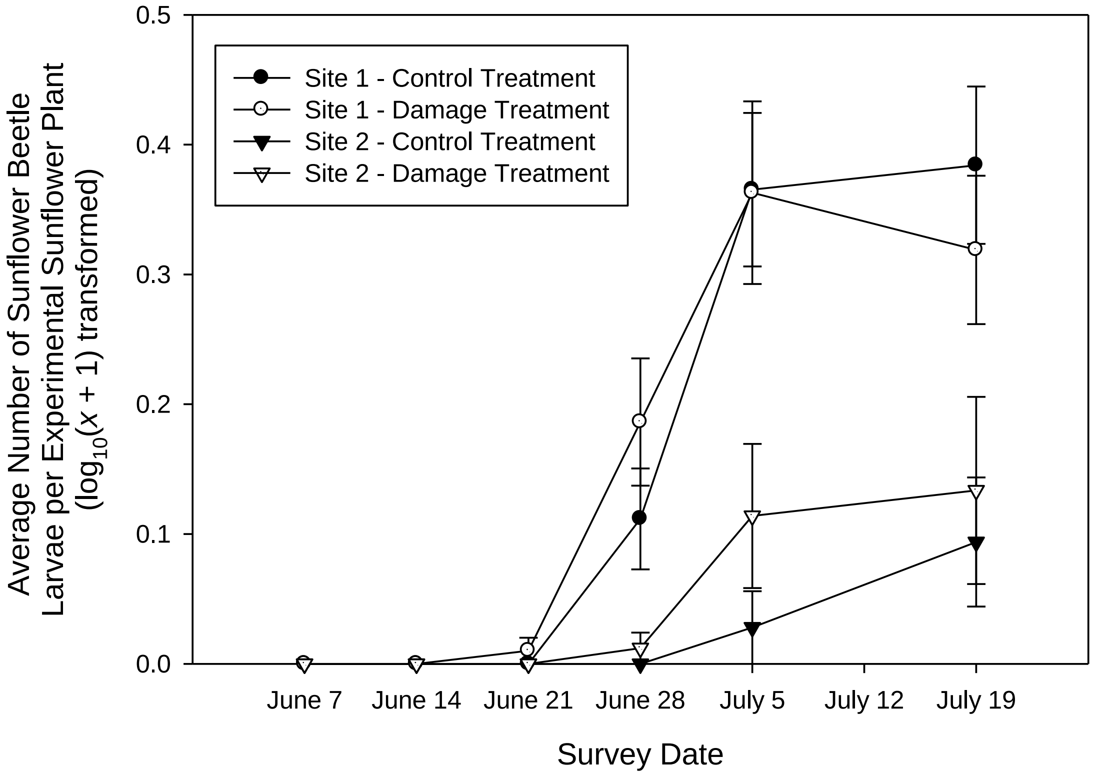

Figure 3.3 – Average number of sunflower beetle (*Z. exclamationis*) larvae surveyed on experimentally damaged and undamaged sunflower plants (*H. annuus*) in Dinosaur Provincial Park

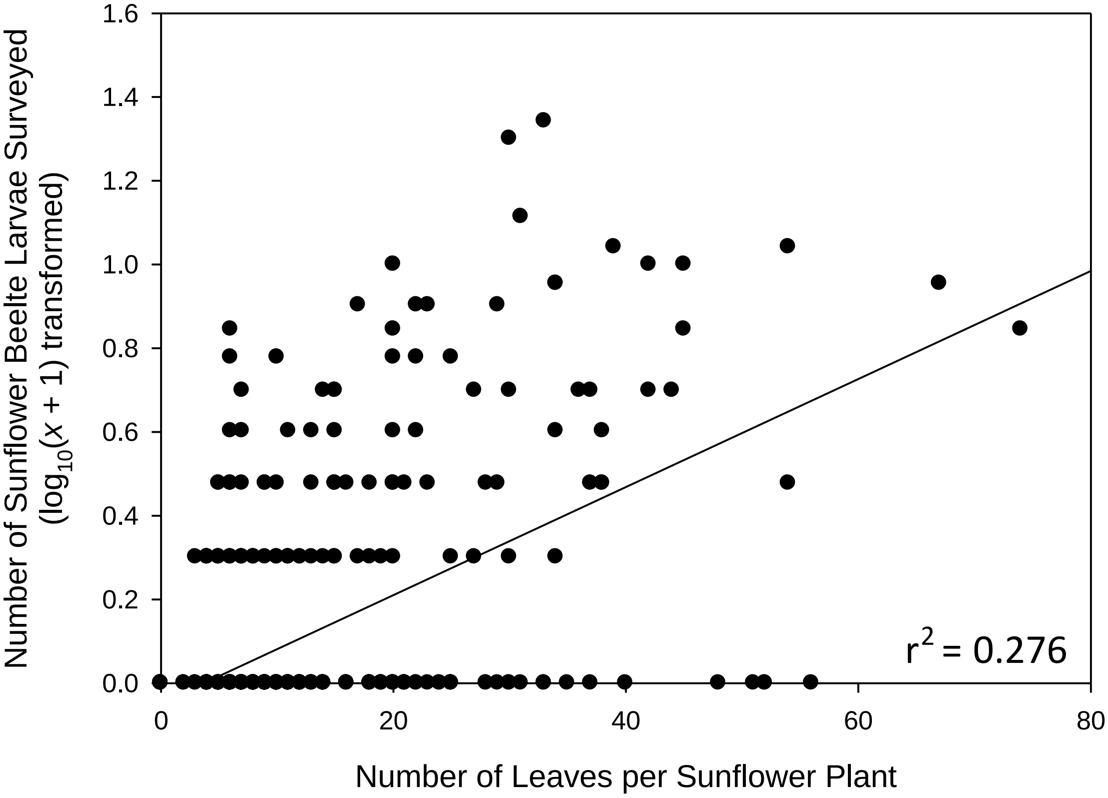

Figure 3.4 – Scatterplot with regression line of sunflower beetle (*Z. exclamationis*) larvae surveyed on experimentally damaged and undamaged sunflower plants (*H. annuus*) in in Dinosaur Provincial Park

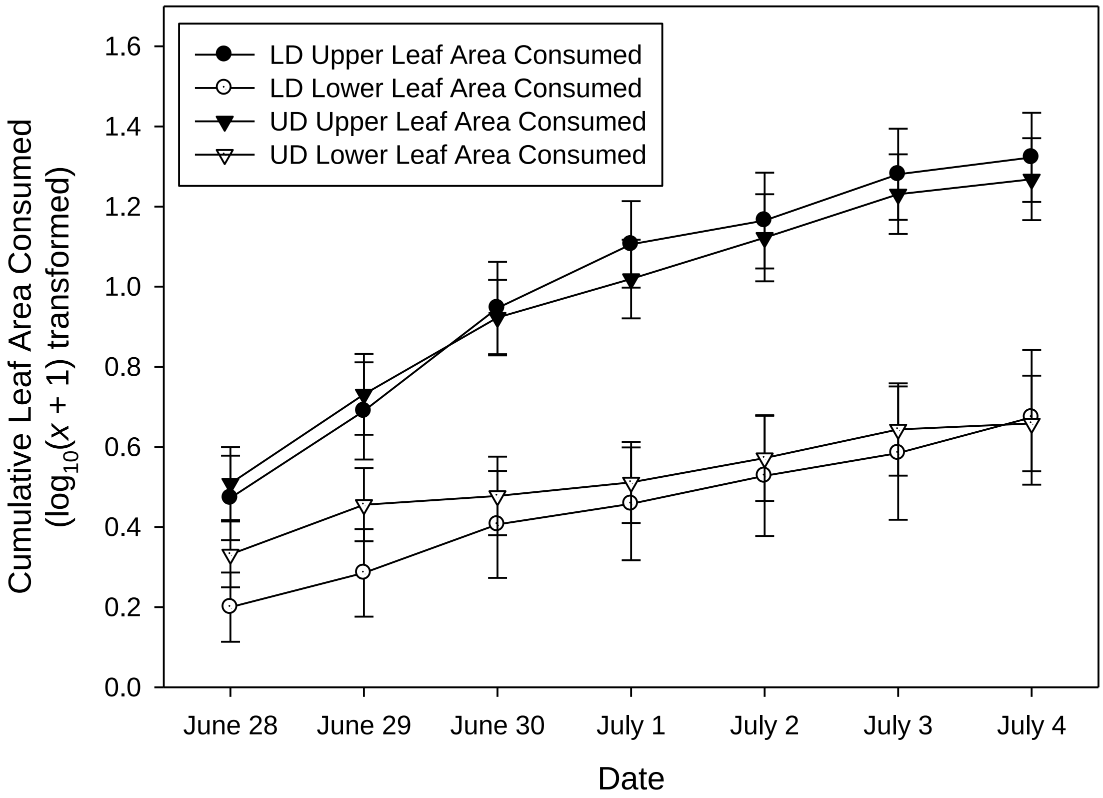

Figure 3.5 – Cumulative area of upper and lower leaf consumed by sunflower beetle larvae (*Z. exclamationis*) on leaves of sunflower plants (*H. annuus*) with either upper of lower leaf damage

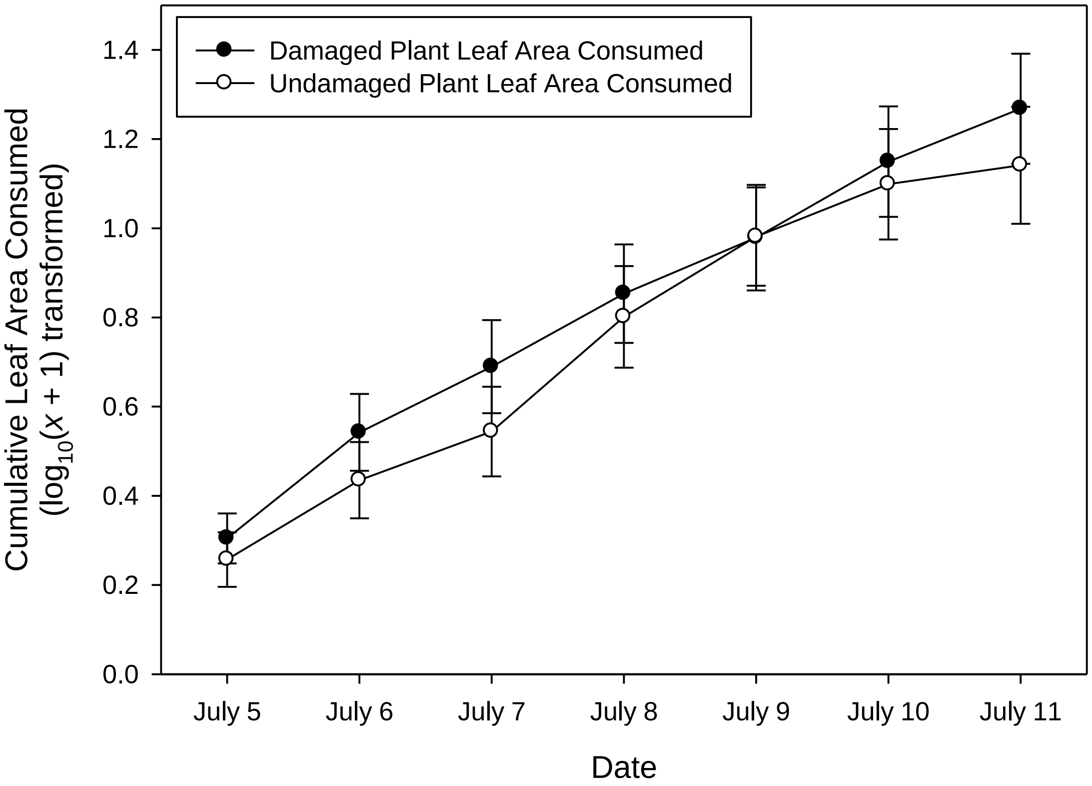

Figure 3.6 – Cumulative leaf area consumed by sunflower beetle larvae (*Z. exclamationis*) on leaves of damaged and undamaged sunflower plants (*H. annuus*)

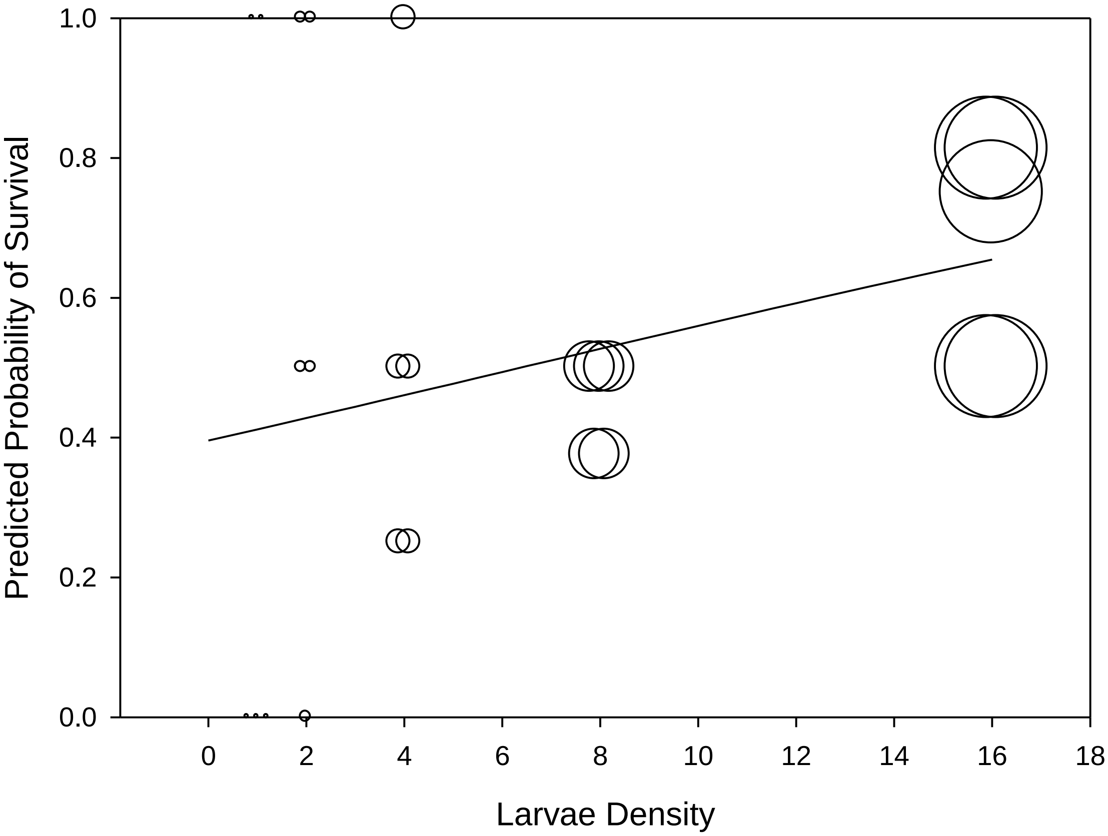

Figure 3.7 – Logistic regression of predicted probability of sunflower beetle (*Z. exclamationis*) larval survivorship on sunflower plants (*H. annuus*) at varying larval densities, weighed by the number of larvae per plant

Chapter 4 – General Discussion

Various studies have examined the effects of herbivory on plant species (e.g., Ohnmeiss & Baldwin 2000, Herrera & Pellmyr 2002, Hochwender et al. 2003, Rudgers & Hoeksema 2003) and the various systems of plant defence used to reduce the fitness consequences of herbivory (Baldwin 1996, Herrera & Pellmyr 2002, Katayama & Suzuki 2004, Heil 2010). Because plant defences have metabolic costs associated with them (Baldwin 1996, Mondor et al. 2006, Heil 2010), many are inducible as opposed to express constitutively. These induced plant defences are up-regulated once the plant experiences a stimulus such as herbivore feeding or mechanical damage (Karban & Myers 1989, Olson & Roseland 1991, Herrera & Pellmyr 2002).

These plant defences typically reduce the fitness of herbivores (Rogers & Thompson 1980, Herrera & Pellmyr 2002, Katayama & Suzuki 2004). For this reason, herbivores have evolved a variety of physiological and behavioural adaptations described by Karban & Agrawal (2002) as "Herbivore Offences". These herbivore offences are adaptations that allow herbivores to reduce or circumvent the costs that plant defences place on them. Herbivore offences work through a variety of mechanisms, some of which include: selective oviposition (Damman 1987, Ruiz-Durbreuil et al. 1994, Sato et al. 1999, Encalada & Peckarsky 2006), plant defence detection and avoidance (Olson & Roseland 1991, Shroff et al. 2008), and gregarious feeding (Damman 1987, Lopez et al. 1989, Damman 1994, Denno & Benrey 1997).

Briefly, selective oviposition is the host or site selection by parental organisms for the purposes of depositing their eggs and offspring. The result of this selection is to increase the fitness for their offspring and indirectly, the parents' own inclusive fitness (Damman 1987, Encalada & Peckarsky 2006). Parental organisms may select a site or host due to increased resource availability (Ruiz-Durbreuil et al. 1994) and/or to avoid unfavourable hosts or environments (Sato et al. 1999).

Avoidance is a behavioural adaptation that consists of herbivore evasion of plant defences when they are able to detect them (Olson & Roseland 1991, Shroff et al. 2008). Olson and Roseland (1991) demonstrated this through feeding preference experiments in which sunflower beetles were given the choice between leaf disks sprayed with distilled water or one of: coumarin, scopoletin, or ayapin. Shroff et al. (2008) demonstrated this through mapping of chemical defence concentrations within *Arabidopsis* leaves and the areas of the leaves cotton bollworm larvae. They determined that cotton bollworm larvae preferentially consumed leaf tissue from areas of the leaves that expressed significantly lower concentrations of chemical plant defences.

Gregarious feeding refers to the tendency of individuals of a species to feed in groups, opposed to alone (Allen 2010). This behaviour can increase the fitness of the individuals within the group by: increasing feeding efficiency and growth (Denno & Benrey 1997), recruitment (Lopez et al. 1989), and protection or survival (Damman 1987, Damman 1994). The mechanisms behind the benefits provided by group and gregarious feeding varies from situation to situation, but may include the swifter establishment of leaf-mines in leaf-mining beetle larvae (Damman 1994), and the necessity of about 20 pyralid moth larvae to create a leaf fold in which the group can use as a shelter against predators (Damman 1987).

When the leaves of the common annual sunflower (*Helianthus annuus*) are damaged by the specialist herbivore, the sunflower beetle (*Zygogramma exclamationis*), it expresses plant defences (Rogers & Thompson 1980, Olson & Roseland 1991, Roseland & Grosz 1997). Sunflowers have been identified to have resistance against sunflower beetle larvae (Rogers & Thompson 1980), produce toxins and unpalatable secondary compounds, which have been shown to discourage sunflower beetle from consuming leaf tissue (Olson & Roseland 1991).

Summary and Implications

Chapter 2 – Survival and Growth of Sunflower Beetle Larvae on Damaged Sunflowers

This chapter investigated the induction of sunflower plant defences through mechanical damage and sunflower beetle larva feeding damage, by placing a larva on each sunflower plant 10 days following treatment application. Survivorship, Relative Growth Rate (RGR), Relative Consumption Rate (RCR), and the Efficiency of Conversion of Ingested food (ECI), as laid out by Waldbauer (1968) and Scriber & Slansky (1981), were used to determine any effects on sunflower beetle larvae feeding on the treated sunflower plants.

The experiment did not detect any significant effects of previous mechanical damage or sunflower beetle larvae feeding damage on larval survival or RGR, RCR, and ECI. This could result from a lack of damage induction of plant defences, possibly due to certain nutrient limitations (Mondor et al. 2006), creating a situation in which the plant does not have the necessary resources or nutrients in the necessary quantity available to produce plant defences. Another possibility is the inhibition of the induction of sunflower defences, similar to the Colorado potato beetle (*Leptinotarsa decemlineata*) against the tomato plant (*Solanum lycopersicum*) (Chung et al. 2013). Lastly, Roseland & Grosz (1996) determined that wild sunflower populations from various states within the United States of America showed varying degrees of coumarin expression, ranging from 56-fold increases to none at all. This suggests that there are genetic and/or population variations in the levels of sunflower defence induction and expression. It is possible that the wild sunflowers used in this experiment fell along the lower end of the sunflower defence induction and expression spectrum.

Chapter 3 – Selective Oviposition, Feeding Avoidance, and Gregarious Feeding by Sunflower Beetle Larvae Feeding on Common Annual Sunflower

This chapter describes four experiments that investigate the possibility of herbivore responses to plant defences in a manner described by Karban and Agrawal (2002) as "Herbivore Offence". Specifically, these experiments investigate: selective oviposition behaviour in female sunflower beetles on damaged and undamaged sunflower plants in the field; sunflower beetle larvae avoidance of damage sunflower leaves and plants; and the survivorship benefits of gregarious feeding among sunflower beetle larvae. Given that I was unable to induce a significant sunflower plant defence in the experiment described in Chapter 2, it is difficult to suggest that sunflower beetle larvae used in Chapter 3 were not able to detect and avoid induced plant defences in damaged sunflower leaves and damaged sunflower plants through selective oviposition and larval feeding, as sunflower plant defences may not have been induced at all.

The first of these experiments found no evidence that sunflower beetle females significantly selectively avoid ovipositing on sunflower plants that had been experimentally damaged. However, it did determine that sunflower beetle females did significantly oviposit on plants with greater number of leaves. This could suggest that sunflower beetle adults selectively oviposit on sunflower plants according to the quantity and quality of resources offered to their offspring rather than avoiding potential sunflower plant defences and plant damage. Conversely, it could suggest that sunflower beetle females are ovipositing in scale with the number of leaves on each sunflower plant, resulting in a situation in which plants with more leaves receive more eggs. Alternatively, this could be a result of sunflower beetle females ovipositing on sunflower plants they are able to find; that is to say, larger sunflower plants are easier to find, and so are more likely to be oviposited on, as smaller sunflower plants are more discreet in a field environment. Rogers (1977) observed a female sunflower beetle lay 4131 eggs in the span of 185 days. This could suggest that sunflower beetles are more concerned with laying more eggs than wasting time finding the most optimal hosts.

The second experiment determined whether sunflower beetle larvae avoided damaged sunflower leaves when isolated to feed on only the two top-most leaves on a sunflower plant. It found that sunflower beetle larvae did not significantly discriminate between damaged and undamaged sunflower leaves. However, it did find that sunflower beetle larvae significantly preferred to feed on the top-most of the two leaves presented to them. This behaviour may occur due to sunflower beetle larvae's tendency to move towards the sunflower terminal ends during the day, as this provides a safe hiding position from their predators (Rogers 1977), and then consume leaf tissue from the first leaf they come across as they descend the plant stem at night. There may also be a tendency for higher leaves to be of greater nutrient value; as sunflowers utilize nutrient recycling, causing reduced nutrient value and eventually leaf senescence in lower leaves (Aguera et al. 2010, Ahmad et al. 2012). With a lack of damage induction and greater leaf quality in higher leaves, we may not be observing the effects of optimal defence theory in sunflower plants in which they would asymmetrically protect those higher quality leaves, thereby reinforcing the larvae behaviour of the consumption of higher leaves. Lastly, this behaviour may simply be instinctual, driven by selection pressures that favour the consumption of higher quality leaves and decreasing the time they are exposed traveling along the plant stem. If this is true, then we may not observe a difference in behaviour if sunflower plant defences were significantly induced.

The third experiment was similar to the second, but instead investigated sunflower beetle larvae avoidance of damaged sunflower plants as opposed to leaves. By allowing access to only the top-most undamaged leaf of a pair of sunflower plants, the sunflower larvae were given the opportunity to discriminate between a damaged and an undamaged sunflower plant in terms of leaf area consumed. Based on feeding preference experiments by Olson & Roseland (1991), it was predicted that sunflower beetle larvae would avoid sunflowers with induced sunflower plant defences. However, there was no significant feeding discrimination between damaged and undamaged sunflower plants by sunflower beetle larvae detected. Based on the lack of plant defence induction during the experiment performed in Chapter 2, it is possible that sunflower defences were not induced in damaged plants, and so sunflower beetle larvae would have no means of discrimination.

The last experiment of this chapter was based on an observation in the first experiment of this chapter. That observation was that sunflower beetle females tended to oviposit several eggs on a single plant, opposed to single eggs on several plants. Due to within-brood competition issues and the possibility of exhausting their food resource, this situation seems to be a paradox. Therefore, this experiment investigated the possibility of survivorship benefits due to gregarious feeding in sunflower beetle larvae. It was found that sunflower beetle larvae did enjoy significantly greater proportions of larval survivorship at higher densities of larvae per plant than at lower densities, though the exact mechanisms are yet to be determined. The absence of sunflower beetle larva predators suggest that these advantages are inherent with feeding, opposed to creating a protective defence against said predators. It also did not appear, though further study is required, that sunflower beetle larvae living in greater densities grew any faster than those living at lower densities. The sunflower beetle larvae also seemed to distribute themselves throughout the sunflower plant. Therefore, this advantage may not be due to the group generation of a nutrient sink to increase growth rate. This gives more weight to the suggestion that gregarious feeding behaviour in sunflower beetle larvae provides the larvae with an advantage against sunflower plant defences; whether through group inhibition of plant defences, or diluting stored and newly produced plant defence throughout the plant instead of allowing it to be concentrated at larval feeding positions. If this is true, then it is possible that sunflower beetle larva resistance to sunflower plant defences lies in gregarious feeding, and so would affect the results of studies examining a single sunflower beetle larva on a sunflower plant. Once again, further study is necessary as this is mere speculation at this point.

Future Directions

It is by the nature of science that when one question is answered, several more are formed. In that, it would be interesting to determine if the addition of fertilizers and nutrients to the growth medium that the sunflowers are raised in would allow for sunflower defences to be observed in terms of RGR, RCR, ECI, and larval survivorship (Mondor et al. 2006). If so, it would then be interesting to determine which specific nutrients are vital to sunflower defences. With the knowledge of vital nutrients required to induce defences, it would be interesting to determine if these nutrients are available to the populations of wild sunflowers I investigated.

Based on the results obtained during the field experiment, it would be interesting to reproduce the experiment in a controlled environment so that the factors involved in female sunflower beetle selective oviposition may be individually studied. Specifically, the factors of involving sunflower height, leaf size, number of leaves, and coumarin experimentally applied to sunflower leaves at varying concentrations (Olson & Roseland 1991), are factors that may be important in sunflower beetle ability to find and select sunflowers for oviposition of the eggs.

Another string of research would be to continue investigating the nature of the potential for an adaptive advantage given by gregarious feeding in sunflower beetle larvae. Using the method for coumarin identification and quantification described by Tal & Robeson (1986) and utilized by Olson & Roseland (1991) and Roseland & Grosz (1997), we would be able to determine if the induction of coumarins had been suppressed. It would be interesting to take sunflower beetle larvae feeding on sunflowers at varying densities and quantify any coumarin build-up in their bodies.

Conclusion

Plant defences and herbivore offences represent opposing forces in the relationship between plants and herbivores, resulting in an evolutionary arms race against one another. Therefore, studies in either topic should consider the effects of the other in a reciprocal manner as each affects the other. This thesis attempts to demonstrate a segment of this relationship between sunflower plants and sunflower beetles, but has only touched the surface of their interactions. Further research will be required to fully understand the coevolution between plants and specialist herbivores.

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