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# Hommage á Trois: A Tale Of Some Very Hungry Grammia Spp. Caterpillars

Katherine Hernandez

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HOMMAGE Á TROIS: A TALE OF SOME VERY HUNGRY *GRAMMIA* SPP.  
CATERPILLARS

by

Katherine Hernandez  
Bachelor of Science, Truman State University, 2011

A Thesis

Submitted to the Graduate Faculty  
of the  
University of North Dakota  
in partial fulfillment of the requirements

for the degree of  
Master of Science

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May  
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This thesis, submitted by Katherine Hernandez in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota, has been ready by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

---

Dr. Rebecca Simmons

---

Dr. Alena Kubatova

---

Dr. Diane Darland

---

Dr. Brian Darby

---

Wayne Swisher  
Dean of the School of Graduate Studies

---

Date

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Caterpillars

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May 2016

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This thesis is dedicated to my family and my caterpillars

## ABSTRACT

Larvae of the Arctiinae use an impressive arsenal of defenses to protect themselves against natural enemies. Several species within this subfamily have been studied for their defensive capabilities, specifically the use of plant secondary metabolites. Life history and sequestration patterns of secondary metabolites have been well documented for *Grammia incorrupta*; however, little information exists for other species of *Grammia*. Furthermore, information on sequestration patterns during the immature stages of several *Grammia* spp. is largely unknown. To address these gaps in knowledge, I am conducting a comparative study of the chemical ecology and life history traits for the virgin tiger moth (*Grammia virgo*), the little virgin tiger moth (*Grammia virguncula*) and the figured tiger moth (*Grammia figurata*) throughout larval development. I divided larvae into four treatment groups: one on white clover (*Trifolium repens*), one on broadleaf plantain (*Plantago major*), one on narrowleaf plantain (*Plantago lanceolata*), and one on a wheat-germ based artificial diet. I collected data on development time for each instar, larval weight at each instar and overall survivorship. A subset of larvae was also harvested at each instar; these samples were prepared for chemical analysis to determine presence or absence of aucubin and catalpol at different instars. Aucubin and catalpol are found in one or both of the *Plantago* species used in this study. Here I will present results of the feeding trials and the chemical analyses of the

*Grammia* tiger moths and discuss implications of these results on the understanding the chemical ecology and development of these species.

CHAPTER I  
TIGER MOTHS, CHEMICAL ECOLOGY AND PLANT ASSOCIATIONS

INTRODUCTION

Tiger moths are fantastic organisms that serve as models to answer questions in evolutionary biology, chemical ecology, animal-plant interactions, among many other areas of interest. Tiger moths have unique behaviors and traits in several habitat types, from tundra to the tropical rainforests. The stark and dramatic differences in habitats that tiger moths can inhabit make them suitable for any field of biology. Due to the diversity of habitats that tiger moths live in, there is a great array of tiger moth phenotypes in terms of coloration, size and behaviors. Most importantly, tiger moth feeding, reproductive and defensive strategies have made these species unique among insects.

For *Grammia* and other tiger moths, development is intimately linked to larval relationships with their host plants. The interplay between tiger moth caterpillars and their host plants involves complex chemical defenses (on the part of the plant) and adaptive herbivory (on the part of the caterpillars). These adaptations allow caterpillars to circumvent some of the defensive strategies implemented by the plant(s). Plant-insect interactions have been well-studied (Erich & Raven 1964, Ryan & Byrne 1988; Strong et al., 1984; Rosenthal & Berenbaum, 2012), and continue to be a source for researchers to study the coevolution of plants and insects. It is these relationships that have sparked a

dramatic and an extraordinary amount of diversity in how insects and plants survive and reproduce in different habitats (Wesseling et al 1997; Ellers & Van Alphen, 1997; Wajnberg et al., 2012). Plants have been highly influential in increasing insect diversity over time (Southwood et al., 1979). The behaviors and metabolic mechanisms that have been elicited from insects to circumvent the defenses of plants are fascinating, complex and deserving of further research efforts.

### **Butterflies and Moths as Systems to Study Plant-Animal Interactions**

The order Lepidoptera is extremely diverse with approximately 20,000 described species of butterflies and over 150,000 described species of moths (Weller et al., 1999; Brock & Kaufman, 2006). The diversity observed in this order is defined by magnificent colors and patterns and their ability to inhabit a variety of ecosystems. Their ability to adapt to different ranges and climates throughout the globe makes them one of the most successful organisms in terms of fitness and reproduction on the planet (Gullan & Cranston, 2004; Ferro & Romanowski, 2012).

Coevolution is an evolutionary process wherein two organisms interact so closely that they evolve together in response to shared or antagonistic selection pressure (Knowles & Markow, 2001; Connell, 1980). For plants and insects, the phenomenon of coevolution has been the foundation of their relationships over millions of years. As described often in the literature (Karban & Anurag, 2002), host plants have evolved toxic secondary metabolites to deter herbivory. Countering this strategy, many insect groups have developed ways to overcome these chemical hurdles. All of the major arctiid clades sequester pyrrolizidine alkaloids, cardiac glycosides, or other secondary metabolites from their host plants (Weller et al., 1999; Zaspel et al., 2014). These secondary metabolites

are inextricably linked to the life history of these herbivores, without them development is often unsuccessful, mating success decreases, and mortality due to external forces has a greater impact (Conner, 2009).

The bella moth (*Utetheisa ornatrix*) typically feeds on *Crotalaria* species that contain toxic alkaloid compounds (Conner, 2009). These compounds make the plants unpalatable to most herbivores, making pyrrolizidine alkaloids (PA) a valuable resource. Caterpillars sequester these toxins and use them as deterrent for predators (Conner, 2009). When larvae hatch, they feed on leaves during their first instar enter seedpods and in the second or third instar. The seeds have the highest concentrations of PAs in the plant (Conner, 2009). Larvae will compete with one another in order to colonize seedpods. Unsuccessful larvae obtain PAs from the leaves; these caterpillars obtain lower levels and thus, are more susceptible to predation (Kelly et al., 2012). In order to try and decrease chances of predation, caterpillars will resort to cannibalism to obtain PAs to increase their PA loads during development (Bogner & Franz, 1996). The bella moth larvae are not negatively affected by PAs (Conner, 2009). Their ability to detoxify PAs in their bodies is due to the gene pyrrolizidine-alkaloid-N-oxygenase found in their bodies (Cogni et al., 2012). Cogni et al. (2012) have also shown the expression of the gene is upregulated when the amount of PAs in their diet increases. The caterpillars that survive immature development and successfully metamorphose into adult moths carry the PAs with them (Conner, 2009). The sequestered PAs render the bella moths unpalatable to many of its predators (Eisner & Eisner 1991; Hristov & Conner, 2005).

Pyrrolizidine alkaloids also play an important part in bella moth reproductive strategies. Females will mate with multiple males and each male will give her a nuptial

gift, a spermatophore, which contains sperm, nutrients and alkaloids (Kelly et al., 2012). Females of this species select males based on their body size, their load of pyrrolizidine alkaloids and by the glandular content of the courtship pheromone hydroxydanaidal (Iyengar et al., 2001). Courtship consists of females releasing a sexual pheromone to lure males and they emit these chemicals in short pulses. Bella moths are unique, the courtship process has the females compete with other females to lure males. Females from the same brood will often engage in collective pheromone release termed “female pheromonal chorusing”, allowing females to increase their attractiveness of genetic relatives and their indirect fitness (Lim et al., 2007). After copulating with several males, the females go through a post-copulatory selective process to choose which male’s sperm will have access to the eggs (LaMuyon & Eisner, 1993). The sperm chosen is based on the intensity of the pheromone hydroxydanaidal and the intensity is directly proportional to the amount of alkaloids sequestered by the males (Kelly et al., 2012).

In contrast to the bella moth, are many larval arctiids that exhibit polyphagous feeding. Many of these polyphagous species eat plants that contain toxic phytochemicals, and there it seems that these generalists have reduced fitness compared to host plant specialists (Cornell & Hawkins, 2003). Arctiid larvae like *Grammia incorrupta* are in fact, “specialized generalists” and are a unique combination of ecological generalism and physiological specialization (Singer & Bernays, 2009). *Grammia incorrupta* has been recorded grazing on more than 80 plant species from nearly 50 families (Singer and Stireman, 2001). The diet breadth of this species is impressive, but misleading. Despite the taxonomic diversity of their host plants, *Grammia incorrupta* caterpillars feed



preferentially on plants that contain toxic secondary metabolites like PAs and iridoid glycosides (IGs) (Singer & Stireman, 2001; Hartmann et al., 2004b).

The caterpillars' acceptance or rejection is strongly influenced by the profile of the plant secondary metabolites and not as strongly to the plant's nutritional content (Singer & Bernays, 2009). The PAs and IGs, among other compounds that stimulate feeding and make host plants acceptable to *G. incorrupta* caterpillars (Singer & Bernays, 2009). Physiological specialization for PAs has been demonstrated and electrophysiological studies of the taste system of *G. incorrupta* have found taste cells that detect PAs (Bernays et al., 2000, 2002a, b; Bernays & Chapman, 2001). The sensitivity of the taste receptors that respond to PAs can be at concentrations as low as  $1 \times 10^{-12}$  M (Bernays et al., 2002b). Bernays et al. (2000) also found similar responses in another phagostimulatory cell to IGs in *G. incorrupta*.

PA-sensitive neurons in *G. incorrupta* also display plasticity. Bernays et al. (2003b) found that a change in sensitivity can occur within hours and the possibility of a loss of response by the PA cell after excessive ingestion of PAs. This response may mediate a behavioral switch to a non-PA plant, which may allow the caterpillar to avoid a PA overdose. This response from the PA cell and possibly from the cell that responds to IGs could have a large role in the foraging behavior of *G. incorrupta* and of other arctiine species.

Polyphagy is beneficial for the survival of generalist arctiines. The benefits of polyphagy include increased food options; thus, the ability to optimize finding plants with higher nutritional value. Plants tend to be low in nitrogen, which is required for growth of larval herbivores. New growth tends to have higher nitrogen concentrations

than older leaves, necessary for the growth of these larvae (Kursar & Coley, 1991). Further, younger leaves have a higher concentration of secondary metabolites, reinforcing the observation that these metabolites are necessary for *Grammia* larvae (Samuelson, 2000). *Grammia* may prefer young leaves because of increased nitrogen, increased amounts of secondary metabolites, or both. Additionally, polyphagy could reduce the risk of autotoxicity during herbivory. Host plant switching by *G. incorrupta* limits overloading on PAs by switching to PA-free host plants (Singer et al., 2002).

Polphagy, which allows for sequestration of a diverse array of secondary chemicals, could possibly limit the amount of risk involved with predators and parasitoids with the constant movement between plants and self-medication (=pharmacophagy; Boppre 1984). *Grammia incorrupta* larvae are highly mobile and are able to cover a lot of ground during feeding (Singer et al., 2002), limiting exposure to predators and parasitoids within a given space. There is a positive association between resistance against parasitoids and the PAs sequestered in *G. incorrupta* caterpillars (Singer et al., 2004a). In addition, *G. incorrupta* displays adaptive plasticity in self-medication. Bernays and Singer (2005) compared the gustatory responsiveness of the taste neurons from *G. incorrupta* caterpillars that were parasitized and un-parasitized.

They found the parasitized caterpillars had a higher gustatory responsiveness (rate of action potentials fired) to PAs and catalpol (=IG). A parasitized caterpillar would be more likely to eat PAs, catalpol and possibly other deterrent compounds in order to combat endoparasitism. Singer et al., (2004a) conducted experiments to test whether parasitized caterpillars that feed on PA loaded plants have a higher resistance versus parasitized caterpillars that do not feed on PA loaded plants. Singer et al., (2004a) found

that a diet with PA plants provided resistance against parasitoids and the caterpillars survived and continued development. There may be other species that utilize similar strategies like that of *G. incorrupta*, but little is known of the larval feeding behaviors across Erebidae.

### **Dark and Mysterious: Why Are Moths So Unique?**

The family Erebidae encompasses a large and diverse group of moths, including the tiger moths (Arctiinae). Approximately 11,000 arctiine species have been described, representing about 6% of lepidopteran species diversity worldwide (Weller et al., 1999; Conner, 2009). The genus *Grammia* contains 37 species with 36 occurring in North America. Schmidt et al. (2009) has noted that the greatest diversity of *Grammia* occurs in the grasslands of the Great Plains and the southern Rocky Mountain region, making North Dakota an ideal location for this project.

*Grammia* are predominately orange and black on the hind wings and abdomen; this contrasting coloration is most likely used to deter predators. Bright colors are typically associated with high levels of toxicity that can result in bitter tasting prey or induced harmful effects on the predator (Conner, 2009). For many tiger moths, mimicry of poisonous insects may be enough of a deterrent, but Hartmann (1999) suggests members of *Grammia* feed on plants containing secondary metabolites that are deterrents to predators. Understanding whether species in *Grammia* are able to sequester secondary metabolites from their host plants is important in order to identify the trade-offs they endure to increase survivorship.

The life cycle of most species of *Grammia* is unknown; an accurate estimate of instars has yet to be determined for many species. In general, most species of *Grammia* have at least six larval instars during development. Some species of *Grammia* undergo larval diapause during the winter and emerge in the spring to complete development. Diapause is characterized by maintaining the body in a state of suppressed metabolism and as a response to environmental stimuli (Kostal, 2006). Stimuli that can initiate or terminate diapause vary considerably and include changing exposure to sunlight, changing temperature, or contact with water (Weiss et al., 1987; Denlinger, 2002). The larvae presumably undergo at least one more instar before pupation, but this information is not provided in current literature. It is possible that larvae undergo an additional two to three instars after diapause; the timing would coincide with the typical *Grammia* flight time in July. Below (Figure 1.1), is a generalized life cycle of a moth that is univoltine. The lifecycle of *Grammia incorrupta* is well studied and provides a reference point for work that will be conducted on other species with the genus *Grammia*. The work that will be done with *Grammia figurata*, *G. virgo*, and *G. virguncula* will further our understanding of these species and the genus as a whole.

### **Food Is Where the Heart Is...**

Most of the information regarding *Grammia*'s larval host plants is vague. Because these species are generalists, it is difficult to compile a comprehensive list of suitable host plant species. According to several experts (Deane Bowers, pers. comm.; Jerry Fauske, pers. comm; pers. obs.), *Grammia* larvae feed on several lowland plants, including broadleaf plantain (*Plantago major*), narrowleaf plantain (*Plantago lanceolata*) and white

clover (*Trifolium repens*) (see Figure 1.2). If they are not sequestering the metabolites, deactivating these toxins could be a cost for obtaining high nitrogen and other nutrients from their hosts. My research will examine nutritional requirements for larvae, including the importance of secondary metabolites to successful larval development. I will also observe if sequestration of these metabolites incur a cost to larvae.

*Grammia* complete their life cycle on many host plants, many of which are non-native species (Chris Schmidt pers. comm.; Jerry Fauske pers. comm.). *Grammia*'s adaptation to non-native plants indicates that these moths are generalists able to deactivate a range of secondary metabolites in their environment. Both broadleaf and narrowleaf plantain are non-native to North America, but have become important host plants for many *Grammia*. Samuelsen (2000) explains that broadleaf plantain has been used historically for medicinal purposes (e.g. antimicrobial and anti-inflammatory) in several cultures. Both species grow along roadsides, lawns and other areas of disturbance. Their use by *Grammia* may be due to their abundance and wide-distribution, though the fitness costs to *Grammia* make this preference perplexing. Both plantains synthesize secondary metabolites that are ingested by *Grammia* larvae. Some of the most commonly found metabolites in plantain are iridoid glycosides, which are also found throughout the plant kingdom (Fig. 1.1; Deane Bowers pers. comm.). Two of the most studied iridoid compounds in plantain are aucubin and catalpol; both are sequestered by *Grammia incorrupta* (Bernays et al., 2002; Hartmann et al., 2005). I chose *T. repens* plant to serve as a control because it does not contain aucubin and catalpol.

## **Chemicals Are the Spice of Life**

Whether a phytophagous insect accepts or rejects plant characteristics is dependent on their behavioral responses (Dussord & Denno, 1991). These characteristics may be physical or chemical and influence the ability of insects to interact with various plant tissues. Many phytophagous insects will incur the cost of these interactions to acquire the necessary nutrition for survival. All nutrients and any additional substances required for an organism's growth and development are called primary metabolites (Rosenthal & Berenbaum, 2012). There is an enormous array of compounds that are not essential for growth and development and are referred to as secondary metabolites, compounds or chemicals. There are secondary metabolites where the function is unknown, but what we do know is that there are many that are biologically active compounds. There are some that aid in competitive interactions among plants, provide protection from abiotic factors or act as poisons. Several secondary metabolites present in plants are toxic to predators and to the plants themselves (Rosenthal & Berenbaum, 2012). The cost of predation is high enough to make production of secondary metabolites worthwhile to these plants; however, plants are able to store compounds as less toxic metabolites, reducing the risk of auto-toxicity.

A common way for plants to store secondary metabolites is to chemically combine them with sugars, salts or proteins to produce comparatively innocuous compounds (Zangerl & Bazzaz, 1991). The transition to toxic forms of these compounds is a result of tissue damage by pathogens or herbivores. When tissue is damaged, free secondary metabolites are released via enzymatic action or oxidation (Schnepf, 1976); consumers can be faced with concentrated doses within the damage area(s). These

secondary metabolites are numerous and diverse and fall into different classes of chemical compounds. For the two secondary metabolites mentioned in the previous section, they fall into one of the largest and most diverse class of organic compounds found in plants, called terpenoids.

Terpenoids always sequester in specific sites within plants, the sites can vary depending on the species or genus but there is consistency (Gershenzon & Croteau, 1992). In some plants, single cells are modified and become nonliving cells that harbor the secondary metabolites. Other plants, like cotton, will have secretory pockets in which the terpenoids will accumulate (Harborne, 1991). Many other species have glandular trichomes where different terpenoids will sequester and be contained for the duration of the plants life or until damage occurs (Harborne, 1991). With over 15,000 characterized so far, terpenoids all share a common biosynthetic origin with a fusion of a five-carbon unsaturated hydrocarbon (isoprene units) (Harborne, 1991). Terpenoids are lipid soluble and found in the cytoplasm or in other specialized areas mentioned above (Harborne, 1991; Gershenzon & Croteau, 1992). In the case of aucubin and catalpol, both are considered to be monoterpenoids but specifically iridoids and have an additional lactone (5-membered ring) and occur as glycosides.

Iridoid glycosides (Figure 1.3) are a group of terpene-derived compounds. Bowers (1991) has found that all plant iridoids via the mevalonic acid pathway *de novo*. Ronsted et al. (2000) published a biosynthetic pathway that has aucubin as a precursor to catalpol (Figure 1.3). The biosynthetic pathway was first developed by Jensen et al., (unpublished) in their study of *Plantago major* as the species of interest. This pathway is particularly important in larval feeding, aucubin to catalpol concentrations could shift

after ingestion by a larva during feeding bouts. The iridoid glycosides are produced by plants primarily as a defense against herbivores or against infection by microorganisms (Bowers, 1981; Dinda et al., 2007). Iridoid glycosides have been discussed in detail to be a deterrent or toxic to several generalist insects and lepidopteran larvae (Bernays and DeLuca, 1981; Bowers and Puttic, 1988, 1989).

Secondary metabolites or their derivatives (Hartmann et al., 1990; Weller et al., 1999; Hartmann, 2005) can be stored in several places within larvae and adults of tiger moths, but information is restricted to a few species (and often one life stage). Secondary metabolites have been found in the larval and adult integument, stored in hemolymph, stored within the larval setae, or exuded via secretions from the adult cervical gland (Dethier, 1939; Weller et al., 1999). While there has been substantial research looking at plant secondary metabolites functioning in defense of the plant against herbivores (Fraenkel, 1959, 1969; Jazen & Rosenthal, 1979; Campbell, 1989), more work is necessary to elucidate the roles iridoids have in insect-plant interactions, specifically for insects that sequester these chemicals.

*Trifolium repens* does not contain aucubin or catalpol; thus, this species was chosen to serve as the plant control for all experiments. While *T. repens* lack in those two iridoid glycosides, they do produce cyanogenic glycosides (Conn, 1979). Specifically, *T. repens* produces linamarin and lotaustralin and widespread and occur together in the plant kingdom (Conn, 1979). Both linamarin and lotaustralin produce hydrogen cyanide (HCN), which is a respiratory poison and stored in the vacuoles of plants in a nontoxic form, usually by combining with a sugar. The common catalyst for HCN to be removed from the compound is through tissue damage. The damage made will allow the



cyanogenic glycoside to make contact with cytoplasmic hydrolyzing enzymes (Conn, 1979). The sugar gets removed and what is left become unstable and eventually breaks down to give HCN (Conn, 1979). The HCN is usually ingested by the herbivore that created the damage via feeding and has proven to be toxic and potentially fatal to vertebrates and invertebrates.

## RESEARCH OBJECTIVES

The overview discussed above provides the framework for the major areas that will be the focus of my thesis, 1) growth and development of caterpillars on different diets and 2) chemical ecology of caterpillars at different developmental stages. The study systems for this research will involve three tiger moth species, the figured tiger moth *Grammia figurata* (Drury 1773) (Erebidae), the virgin tiger moth *Grammia virgo* (Linnaeus 1758) (Erebidae) and the little virgin tiger moth *Grammia virguncula* (W. Kirby 1837) (Erebidae). All three species have ranges within the United States and *G. virgo* and *G. virguncula* both have local populations within North Dakota.

These three species exhibit bright orange and black coloration on their bodies with striking striped and spotted patterning on the wings. The life history and chemical ecology of *G. incorrupta* has been well documented by several research labs (Bernays & Chapman 2001; Hartmann et al., 2005; Bernays & Singer 2005; Singer et al., 2009; Smilanich et al., 2011). *Grammia incorrupta* caterpillars consume alkaloid-laden leaves to fight off parasitism by fly larvae (Singer 2009). Pyrrolizidine alkaloids and iridoid glycosides being found in late instar *G. incorrupta* caterpillars (Hartmann et al., 2005;

Smilanich et al., 2011). Pankoke et al., 2012 found that plant iridoid glycosides impair larval development in *G. incorrupta*.

Though much is known about *G. incorrupta*, there have not been any studies examining the chemical ecology of any of the *Grammia* spp. being used in this study. To date, there are no records of any study looking at the chemical ecology of an insect throughout its larval development. In Chapter 2, I describe a no-choice feeding trial using all three *Grammia* species to examine their response to unconfirmed host plants. All three species have extensive host plant lists but the validity of the lists have been untested with experiments, these experiments are the first to examine the *Grammia* caterpillars' weight gain and overall development. The information presented will provide some interesting insights into the lives of *Grammia* spp. caterpillars. Finally, in Chapter 3, I explore the chemical ecology of these tiger moths and examine their sequestration patterns on various diet treatments. I use various techniques in the sample preparation of these species and utilize gas chromatography and mass spectrometry to identify and eventual quantification of target analytes.

## GENERALIZED METHODS

### **Study sites**

Between 01 June and 20<sup>th</sup> of July, 2014 I searched for gravid female tiger moths along roadsides in eastern North Dakota (Counties: Cass, Grand Forks, and Ransom) and western Minnesota; I also collected gravid females in Glacial Ridge National Wildlife Refuge (Polk County). The study areas comprised of prairie ecosystems and secondary forests. Field collections were made in the morning (0530-0800 h) and collected females

were kept in plastic containers during oviposition. Wild caught females from eastern ND and western MN were collected with plastic cups with ventilated lids. Moth collecting in the evening (2130-0100 h) with light traps were unsuccessful at attracting females and this approach was eventually abandoned. Gravid females were found on the ground during the daylight hours when they were most docile. The females were not supplied food to feed on because adults of these species have non-functioning mouthparts.

Additional *Grammia virgo* females were obtained from David Wagner (University of Connecticut) and Eric Quinter (American Museum of Natural History). I obtained *G. incorrupta* eggs from Peri Mason and Deane Bowers (University of Colorado-Boulder) from wild caught females and therefore did not observe oviposition.

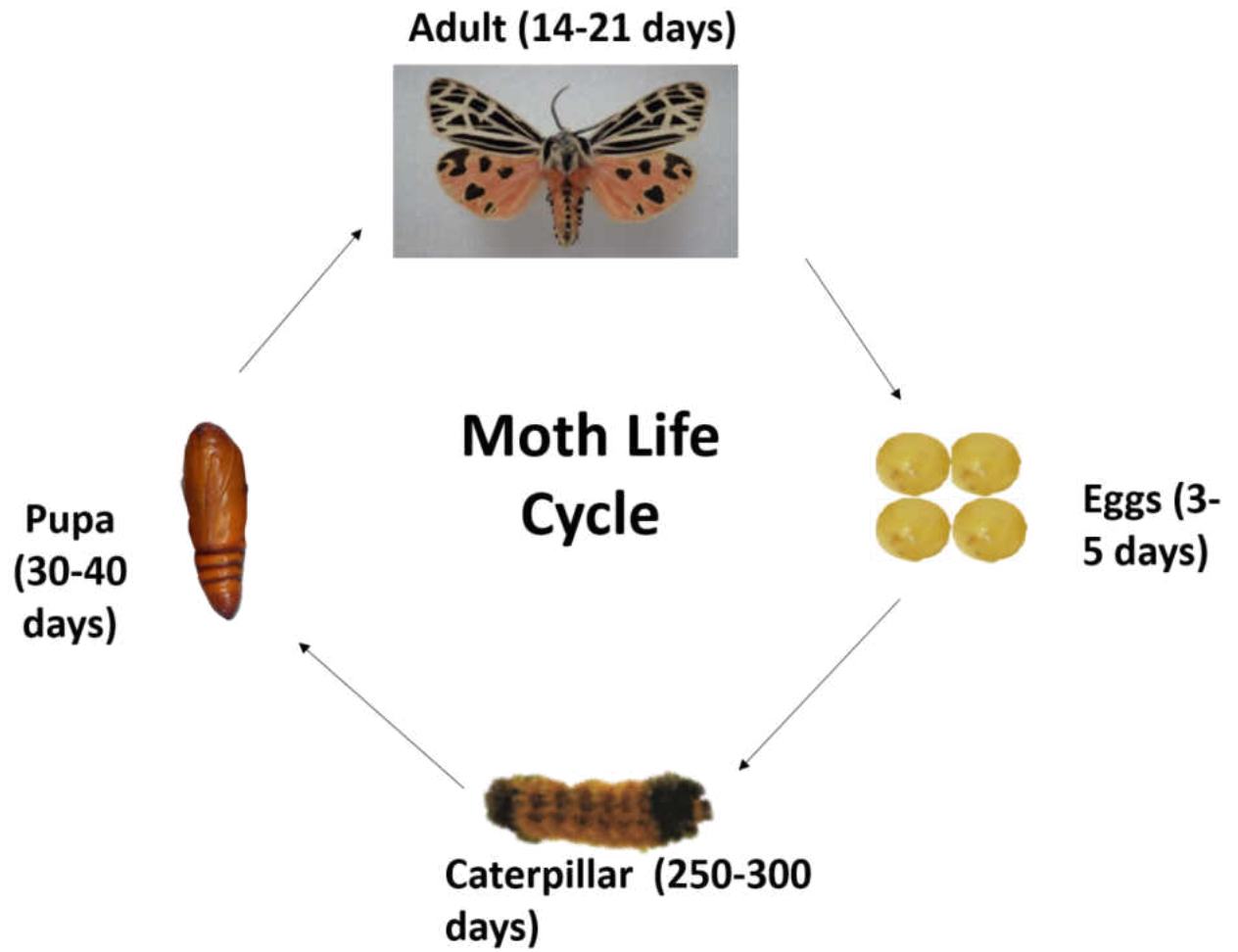


Figure 1.1: Generalized life cycle of a *Grammia* species.



Figure 1.2: Plants being used in the no-choice feeding trials: (A) *Plantago major*, (B) *Plantago lanceolata*, and (C) *Trifolium repens*.

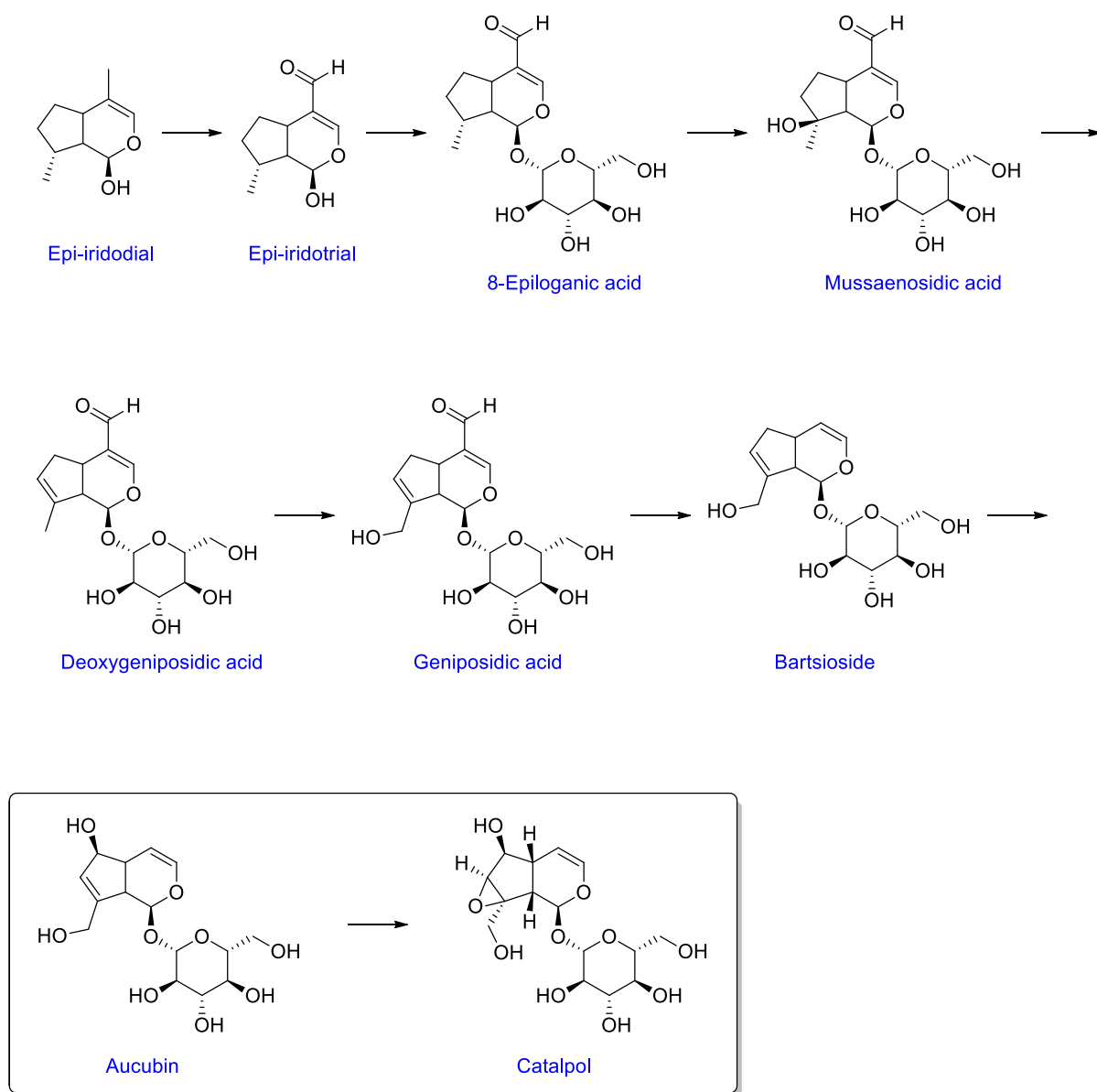


Figure 1.3. Biosynthetic pathway of aucubin and catalpol (Ronsted et al., 2000).

CHAPTER II  
LIFE HISTORY AND TRADE-OFFS OF AWESOME TIGER MOTH (*GRAMMIA* SPP.)  
CATERPILLARS

INTRODUCTION

Feeding is one of the most fundamental and important behaviors for any living organism. For herbivorous insects, feeding on plants requires optimization of strategies to obtain sufficient nutrition while coping with variation in plant quality and defense. Determining adaptive tradeoffs in consumption, allocation and utilization of food has been a central focus of insect nutritional ecology (Slansky & Rodriguez 1987a). Over the past few decades, scientists have begun to elucidate insect adaptations that can reduce the costs and risks associated with plant feeding (Rhoades, 1985; Dussourd & Denno, 1991; Conner, 2008). Understanding tradeoffs and constraints in insect feeding behavior is therefore an important consideration when addressing basic questions in life history evolution.

Caterpillars are an ideal model system to study insect-plant interactions due to their rapid development, simple body plan, and physical changes (weight gain, changes in color, etc.), which can be documented easily. Caterpillars gain energy and nutrients for growth and development, movement, defense and reproduction through consumption of their host plants (Townsend & Calow 1981; Slansky & Rodriguez 1987a).

The life of a feeding caterpillar is not simply about consuming plant material to facilitate growth and maturity. A caterpillar's existence can be best summed up simply and accurately in the following statement by Lawton and McNeill (1979, p. 223): "plant-feeding insects living in a world dominated on the one hand by their natural enemies and on the other by a sea of food that, at best, is often nutritionally inadequate and, at worst, is simply poisonous." Herbivorous caterpillars generally employ one of two strategies to compensate for this situation, generalist or specialist. Both of these strategies have potential costs with most insect species falling somewhere between an extreme generalist and specialist.

Generalism is known to have many benefits; polyphagous caterpillars can feed on several plant species within a variety of genera and families. The ability to switch between different host plants provides caterpillars with an "ecological buffet" within their habitat (Futuyma & Moreno, 1988). This buffet allows caterpillars to be exposed to a wide diversity of plant secondary metabolites and varying degrees of plant quality. Tiger moths, in particular members of genus *Grammia*, are known to be polyphagous and exhibit host plant switching (Singer et al., 2002).

The ability to feed on many plants including those of suboptimal quality can provide a number of benefits. For example, feeding on plants with moderate to high levels of defensive compounds can significantly reduce vulnerability to parasitism (Karban & English-Loeb, 1997). Furthermore, switching to plants of low levels of defensive compounds can avoid overexposure to defensive compounds, which can affect the growth and survival of insects (Karban & English-Loeb, 1997; Van Dam et al., 2005; Singer et al., 2009). Thus, host plant switching enables polyphagous caterpillars to balance the



benefits of defensive compounds in combating natural enemies with the toxicity of these chemical defenses (Singer et al., 2002).

In contrast, specialist feeders are closely associated with their host plant and have adapted to the physical and chemical characteristics of one species (or lineage) in order to complete development. However, a host plant may be of low nutritional quality, which can negatively impact the specialist herbivore (Awmack & Leather, 2002). Despite this lack of nutrition, the plant may be locally abundant, or the local habitat may lack plant species diversity and can force herbivores to specialize (Morris et al., 2014). This association is displayed by the monarch butterfly (*Danaus plexippus*) and its host, species of milkweed (*Asclepias* spp.). These caterpillars have a close association with milkweed plants, even when they are not abundant in the habitat. Milkweed contains several types of secondary metabolites, including the cardenolides which the monarch caterpillars can sequester (Parsons, 1965; Nishida, 2002). Individual milkweed plants vary in their composition of cardenolides and can contain high levels of these toxic compounds.

Specialism allows caterpillars to not only manipulate and tolerate their host plants, but also to evolve strategies to reduce parasitism and predation (Krieger et al., 1971; Whittaker & Feeny, 1971). Differences in cardenolide content amongst species of milkweed can affect the growth and vulnerability to parasites and diseases in caterpillars (de Roode et al., 2011). Many specialist insect herbivores tolerate a toxic host plant for the use of a potent arsenal of secondary metabolites for their own self-defense (Baldwin, 1989; Brower, 1984; Brown, 1981; Nishida, 2002; Naumann et al., 2002). For example, larvae of the European cinnabar moth (*Tyria jacobaeae*) feed on a pyrrolizidine alkaloid-containing plant tansy ragwort (*Senecio jacobaea*) that is toxic to insects and vertebrates

(Naumann et al., 2002). These moths are able to efficiently *N*-oxidize the pyrrolizidine alkaloids (PAs) via the gene senecionine *N*-oxygenase, which is highly specific for these PAs. Thus, *T. jacobaea* larvae are able to accumulate predation-deterrent PAs in their hemolymph, increasing their chances of survival (Naumann et al., 2002).

In addition to using these chemicals for defense, many of these specialists have been able to repurpose plant secondary metabolites for their own development and mating (Rothschild & Reichstein, 1976; Peterson et al., 1987; Boppre, 1984; Conner et al., 1990). For example, Pliske et al. (1976) found that *Heliotropium indicum* L. (Boraginaceae) contains pyrrolizidine alkaloids, which are a powerful attractant for male ithomiine and danaine butterflies. These butterflies congregate on *Heliotropium*, feeding on the dead shoots and using the consumed alkaloids for the formation of male courtship pheromones. By influencing mating success, host plant chemistry has a lifelong effect on an individual's fitness.

Despite the benefits of plant secondary metabolites for defense and mating, use of plant secondary metabolites can have a cost. A consequence of feeding on plants with defensive chemicals can result in reduced fitness (e.g. longer development time, survival, etc.) for the herbivore. Thaler (1999) found that when beet armyworm (*Spodoptera exigua*) caterpillars feed on tomato plants (*Lycopersicon esculentum* var. Ace), the plants release volatiles that attract *Hyposoter exiguae*, an endoparasitic wasp. Thus feeding resulted in increased parasitism of the *S. exigua* caterpillars, reducing their individual fitness.

Many species of tiger moths exemplify adaptive herbivory because of their close interactions with specific host plants during larval stages. Incorporation of toxic plant

secondary metabolites allows tiger moth larvae to deter predation and parasitism (Azambuja et al., 2005; Mason et al., 2014). Tiger moths are also able to repurpose those same defensive chemicals for reproductive purposes as well by incorporating them into courtship pheromones (Wink & Schneider, 1990; Hartmann, 2004). Despite the known benefits of sequestering plant secondary chemicals for courtship and predator deterrence, there is little information on potential costs of these chemicals on the development and survival for several tiger moths. Here I examined potential costs between incorporation of toxic plant secondary metabolites on, larval growth and development time in three poorly studied tiger moths, *Grammia figurata*, *Grammia virgo*, and *Grammia virguncula*, to address the following questions and test the associated hypotheses:

- 1) Will there be significant variation in development time of *Grammia* spp. caterpillars on one of the four diet types?

*Hypothesis A:* Caterpillars that are feeding on plant species will have a longer development time than those fed on an artificial diet.

*Hypothesis B:* Caterpillars feeding on an artificial diet will have a longer development time than those fed on plant-based diets.

*Hypothesis C:* Caterpillars will have similar development time regardless of diet.

- 2) What are the effects of food sources on the weight gain of tiger moths caterpillars through the course of their development?

*Hypothesis A:* Caterpillars feeding on either *Plantago* species will have higher weight gain than those feeding on the other diet treatments.

*Hypothesis B:* Caterpillars feeding on the artificial diet will have higher weight gain than those feeding on the plant diets.

*Hypothesis C*: There will not be significant differences in weight gain across any of the diet treatments.

## METHODS & MATERIALS

### Rearing Protocol

I performed a no choice feeding test, using three species of *Grammia* and four food sources. Once the eggs hatched, I individually weighed the caterpillars and placed one caterpillar in a 60-mL plastic cup with one of the following food source: broadleaf plantain *Plantago major* (Plantaginaceae) (contains aucubin), narrowleaf plantain *P. lanceolata* (Plantaginaceae) (contains aucubin and catalpol), white clover *Trifolium repens* (Fabaceae) (= control), and a wheat-germ based diet (Yamamoto, 1961) (=control).

I used an insect pinning needle to puncture 10 holes in each plastic lid to control for air circulation and decrease humidity. The caterpillars were raised individually in 60 mL plastic cups for the first five instars, and were then placed in 160 mL plastic cups until pupation or mortality. The caterpillars were reared in an environmental growth chamber set at 27 °C and 12:12 L: D in the preparation room in the greenhouse in Starcher Hall. The experimental design consisted of five replicates with a total of one hundred caterpillars from each species of *Grammia* (N = 300 caterpillars/3 species/5 replicates = 20 caterpillars/species/replicate; Figure 2.1). The caterpillars were fed mostly young leaves from their designated plant species or a small mound (~1.0 g) of the

artificial diet (control). I weighed caterpillars, checked food quality, checked for mortality, and made behavioral observations every 24 to 48 hrs throughout the experiment.

## Data Analysis

### *Question 1: Development Time*

Development time was assessed in five replicates of no-choice feeding trials by tracking the development of each individual caterpillar. To look at development time across the different treatments and between the three species, I performed a two-way ANOVA in JMP 12 (SAS Institute Inc., Cary, NC, USA) on log transformed total number of days of development (=response variable). Diet type, moth species, and their interactions were included as the explanatory variables. The data collected on larvae feeding on *T. repens* were discarded from the analyses due to high mortality. A post hoc Tukey test was used to assess significance between moth species-diet type interactions on development time.

### *Question 2: Weight Gain*

Weight gain was calculated by subtracting the weight from the first instar from the weight of the final instar for each individual. Two-way ANOVA analyses were performed using log-transformed data. Weight gain was included as the response variable. Diet type, moth species, and diet-moth species interactions were included as explanatory variables. ANOVAs were performed for all moth species fed on either *P. major*, *P. lanceolata* or the artificial diet. The data collected on larvae feeding on *T.*

*repens* were discarded from the analyses due to high mortality. A post hoc Tukey test was used to assess significance between species versus diet type interactions on weight gains.

## RESULTS

Caterpillar development time and weight gain varied significantly across the three species of *Grammia*. Overall, larvae were successful on all treatments, with the exception of those feeding on *T. repens*. It is also important to note that the caterpillars used in the experiments circumvented diapause, which is part of their development in natural conditions (see Chapter 1).

### Development Time

The larvae of *G. virguncula* exhibited significantly prolonged development time while those of *G. virgo* had the shortest development time (Table 2.1; Figure 2.2). Larvae that fed on *P. major* had the longest development time compared to larvae that fed on the artificial diet, which had the shortest development time (Table 2.1; Figure 2.2).

### Weight Gain

All three tiger moth species that fed on the different diet types were significantly different in their weight gain (Table 2.1). These results indicate that the caterpillars feeding on artificial diet gain the most weight than versus those feeding on the plants. There appears to be no difference in weight gain for *G. figurata* or *G. virgo* feeding on

either species of *Plantago* (Figure 2.3). *Grammia virguncula* fed on a diet of *P. lanceolata* gained more weight than those that fed on *P. major*.

## DISCUSSION

These experiments were able to conclusively answer the questions I pose in the introduction:

1. Will there be significant variation in development time of *Grammia* spp. caterpillars on one of the four diet types?
2. What are the effects of food sources on the weight gain of tiger moth caterpillars through the course of their development?

The null hypotheses are rejected for each of the two questions. Caterpillars fed on plants took longer to develop, in general, than those fed on an artificial diet. Further, caterpillars fed on artificial diet gained more weight than those fed on plants. Here I discuss the causes and implications for these observations.

Rapid growth is advantageous for caterpillars, decreasing exposure to predation and parasitism (Benrey & Denno, 1997). Reduction in development time, as observed here (Figure 2.2), would limit exposure to environmental dangers. In addition, rapid growth can result in larger overall body sizes in developing caterpillars. Larger caterpillars pupate at a larger size, resulting in larger adults that can be more fecund than smaller adults (Iyengar & Eisner, 1999). These results of this study show that food quality is centrally important to growth and development in caterpillars. Because of the

importance of nutrient dense food, polyphagous caterpillars should consume the highest quality diet available to take advantage of rapid development and larger size.

### **Development Time**

In these experiments, larvae did not undergo diapause, which reduced overall development time by half compared with caterpillars developing in natural conditions. Experimental caterpillars developed under warm conditions and had constant access to food; thus, these larvae continued growing without interruption. The increased pace of development decreases the chance of larval exposure to parasitism, and increases chances of survival for these larvae. Access to quality food is central to successful development and survival.

In order to grow and reproduce as adults, caterpillars need to consume a diet that provides a suite of nutrients including: carbohydrates, amino acids, and nitrogen (Chapman, 1982; Allen et al., 2007; Slansky, 1992). The increased pace of development and growth on artificial diet observed here (Figure 2.2) suggests that this food source provides a complete source of necessary nutrients for *Grammia*.

Caterpillars fed on *P. major* took the longest to develop, indicating that *P. major* is suboptimal for *Grammia*. The unsuitability of *P. major* as a host may be due to lack of available nutrients, low water content and/or the presence of plant secondary metabolites. The polyphagous herbivore *Trichoplusia ni* took longer to develop on *P. major*, compared with the other two host plants (*P. lanceolata*, *T. officinale*) (Lampert & Bowers, 2010). Further investigation is required to understand why *P. major* negatively impacted development times of these species of *Grammia* caterpillars. Despite this



disadvantage, species of *Grammia* are still able to complete development on suboptimal hosts, indicating that the selection pressure imposed by a host plant can be overcome.

Complete caterpillar mortality observed on a diet of *T. repens* suggests that white clover is not a viable source of forage; however, other lepidopteran species successfully complete development on this host (Schittko et al., 1999; Blanco et al., 2008). Based on this information, it seems likely that mortality of *Grammia* caterpillars feeding on *T. repens* was not due to a lack of nutrients, but due to differing host plant chemistry containing cyanogenic glycosides.

### **Weight Gain**

As stated earlier, the artificial diet was optimal for an increased weight gain for *Grammia*. Differences in weight gain may have been due to an increased energy cost the caterpillars expended to chew through and digest fibrous materials in plant diets (Choong, 1996). Despite a significant difference in weight gain between the artificial and plant diets across moth species, those differences did not affect successful development of these caterpillars. These caterpillars did not need to reach a critical weight in order to continue molting; critical weight does not seem to be an important consideration during lepidopteran development (Davidowitz et al., 2003; Callier & Nijhout, 2011). Starved *Manduca sexta* caterpillars never reached their critical weight but molted regardless (Callier & Nijhout, 2011). Although the critical weight may be an important factor in determining body size it is not as influential in completion of larval development. Understanding the interplay of all cues leading to larval molts is a multifaceted

complicated topic. Further study is required to tease apart the roles of diet, weight, environmental cues, and physiology in larval development.

### **Chemicals and Caterpillars**

In addition to the tradeoffs generalist caterpillars face during development on particular plants, there can also be constraints that limit success on particular host plants. In this study, these generalist caterpillars were forced to adopt a specialist diet. In a similar study, Ellis and Bowers (1998) found that while the specialist butterfly (*Junonia coenia*) had improved growth and survival on a diet of *Plantago* spp, and the generalist butterfly (*Vanessa cardui*), did best on an artificial diet. Similarities between the Ellis and Bowers (1998) results and these results suggest that a generalist suffers costs when constrained to one host plant, and that specialists are more efficient at feeding and digesting their host plants than generalists (Feeny, 1976; Wiklund, 1982). Without the 'buffet' afforded to generalist caterpillars while grazing, there is the pressure of adapting to one food source for the entirety of development, with exposure to associated toxic secondary chemicals.

The ingestion of defensive compounds for all three species of caterpillars feeding on *T. repens*, resulted in high mortality. *Trifolium repens* produces cyanogenic glycosides (Conn, 1979); non-toxic forms of linamarin and lotaustralin are stored in vacuoles of *T. repens* cells. When released via tissue damage, linamarin and lotaustralin interact with enzymes resulting in the production of hydrogen cyanide, a respiratory poison. High mortality rates suggest that *Grammia* caterpillars could not detoxify the cyanogenic glycosides. Without the ability to switch to other plants devoid of these chemicals,

*Grammia* caterpillars were unable to complete development, indicating that the ability to generalize is not universal across plant lineages.

*Grammia figurata*, *G. virgo* and *G. virguncula* were successful on both species of *Plantago*, suggesting they can detoxify and/or exploit various defensive chemicals (e.g. iridoid glycosides). A related species, *G. incorrupta* (Bernays et al., 2000; Hartmann et al., 2002; Singer et al., 2009), performed similar in an analogous study, indicating that the ability to sequester iridoid glycosides evolved early in the lineage. Of course, just because *Grammia* spp. can tolerate these chemicals during ingestion does not mean that they are actually sequestered into their tissues; this issue will be examined in further detail in the following chapter.

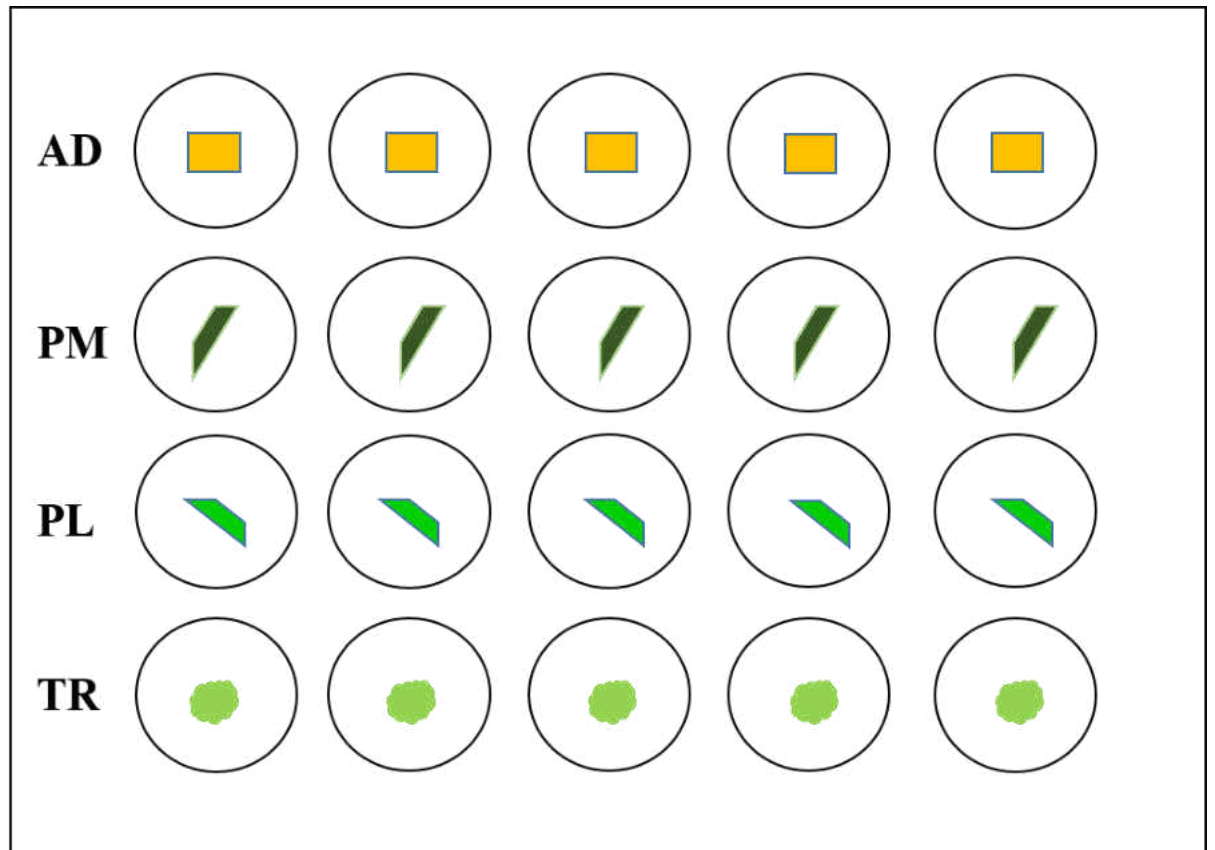


Figure 2.1. No-choice feeding design set-up. Set-up of one replicate for a species of *Grammia* caterpillars on the following diets: artificial diet (AD), *P. major* (PM), *P. lanceolata* (PL), and *T. repens* (TR). This was repeated for each tiger moth species, for a total of five replicates for each species (N = 300).

Table 2.1. Mean development time and weight gain across moth species and diet type  
(N=214; \* = P <0.0001).

<b>Variable</b>	<b>df</b>	<b>F Development time (days)</b>	<b>F Weight Gain (g)</b>
<b>Moth species</b>	2	2329*	1588*
<b>Diet</b>	2	1015*	374*
<b>Moth species*Diet</b>	4	17.18*	47.29*
<b>Species</b>		LS Mean	LS Mean
<b><i>G. figurata</i></b>		156 <sup>b</sup>	2.3 <sup>a</sup>
<b><i>G. virgo</i></b>		151 <sup>c</sup>	1.9 <sup>b</sup>
<b><i>G. virguncula</i></b>		165 <sup>a</sup>	1.7 <sup>c</sup>
<b>Diet</b>			
<b>Artificial Diet</b>		153 <sup>c</sup>	2.1 <sup>a</sup>
<b><i>P. lanceolata</i></b>		157 <sup>b</sup>	1.8 <sup>c</sup>
<b><i>P. major</i></b>		162 <sup>a</sup>	1.9 <sup>b</sup>

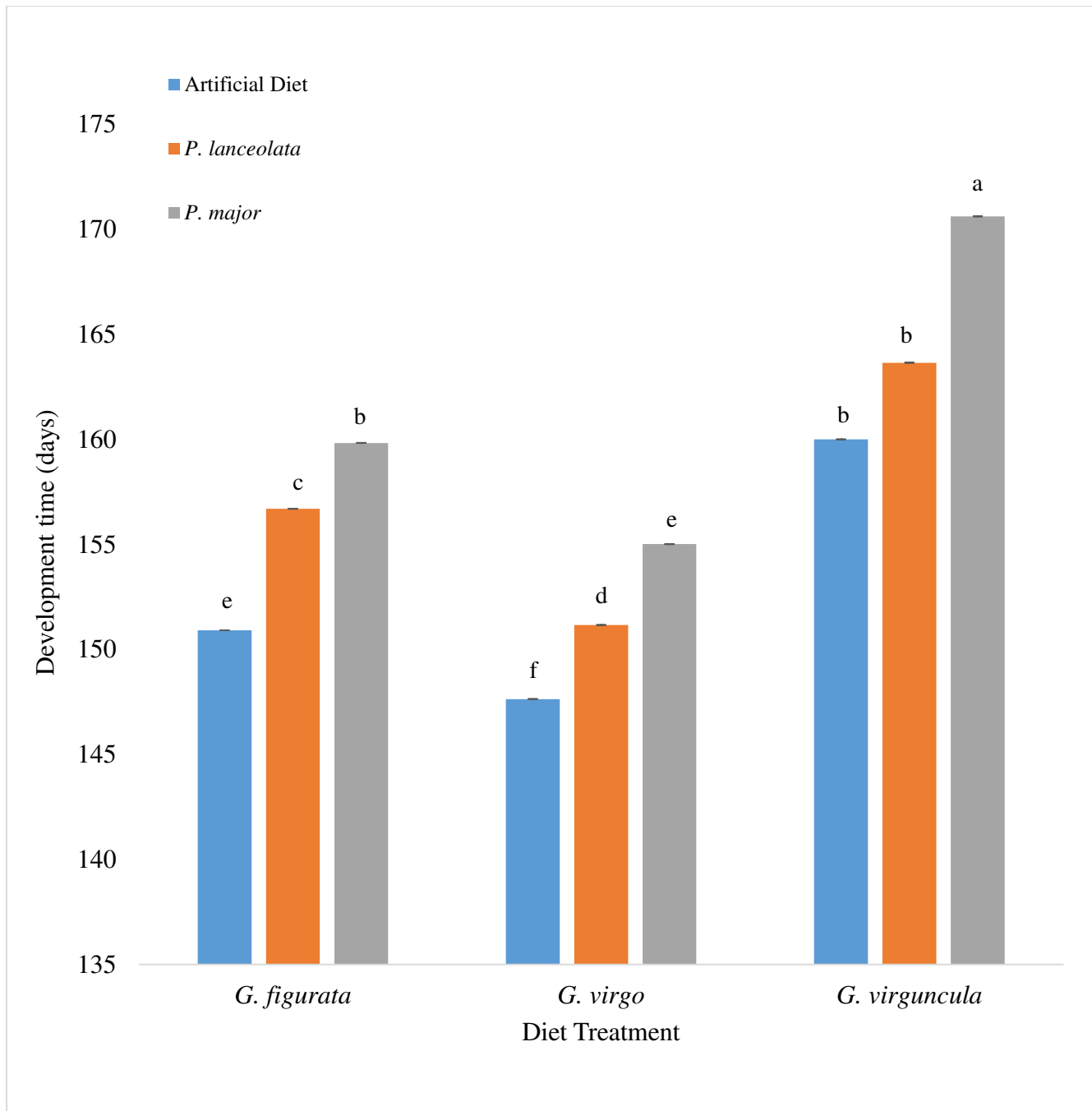


Figure 2.2. Mean development time of species and diet type (N=214, df =205). Error bars calculated by using standard error of the mean error bars as calculated by the Tukey's HSD post hoc tests.

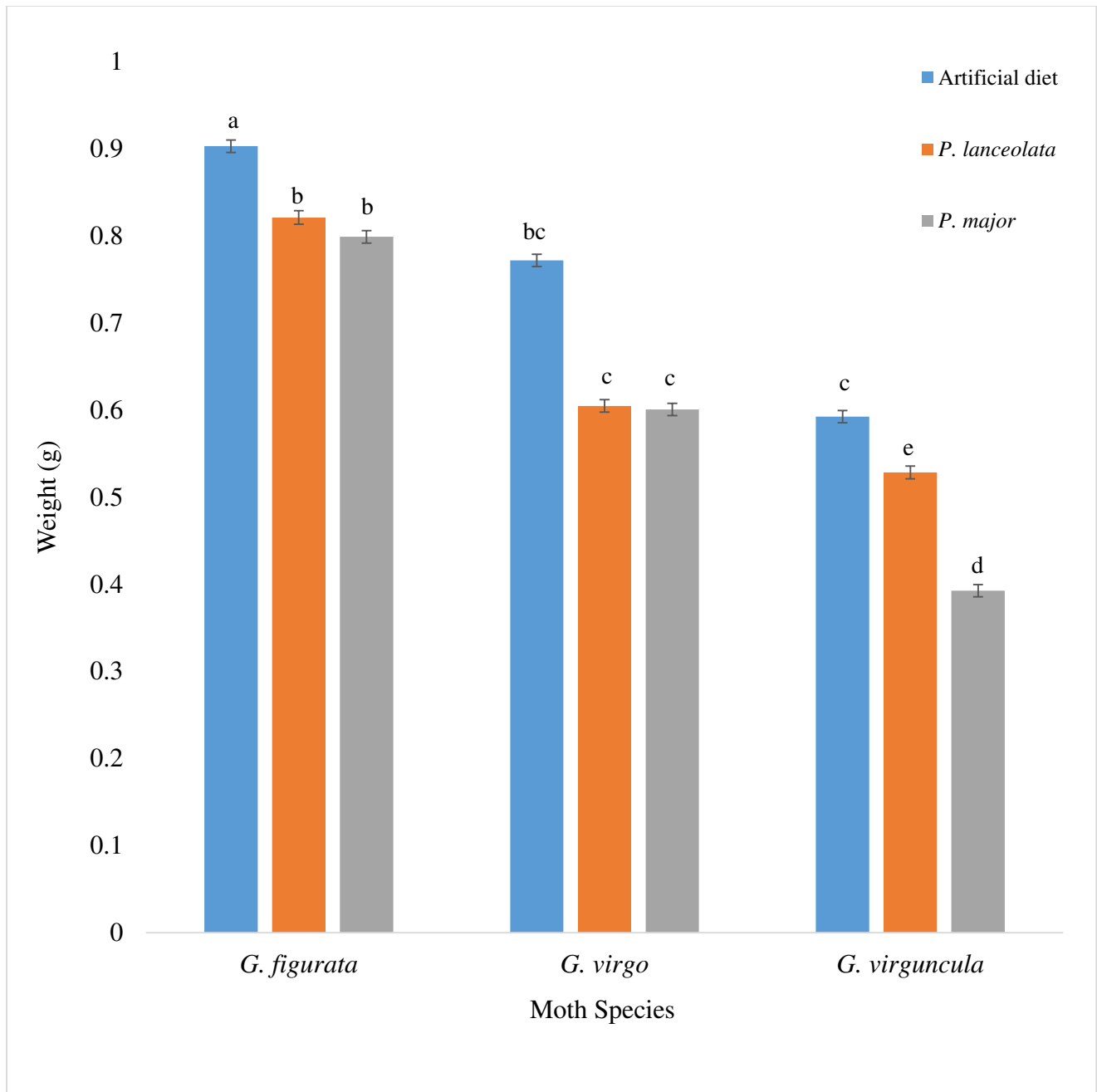


Figure 2.3. Mean weight gain of species and diet type (N=214, df =205). Error bars are calculated by using the standard error of the mean error bars, calculated by a Tukey's HSD post hoc test.

CHAPTER III  
INTERACTIONS BETWEEN PLANT SECONDARY METABOLITES AND SPECIES  
OF PRAIRIE TIGER MOTHS

(Lepidoptera: Erebidae: Arctiinae: *Grammia* spp.)

INTRODUCTION

Insects are among the most diverse group of animals on the earth, representing more than half of all known living organisms. Most insects rely on the primary metabolites of plants to complete their development (e.g. carbohydrates, lipids and proteins); this herbivory harms host plants by reducing fitness or causing mortality. In response, plants have evolved a high diversity of secondary metabolites (e.g. alkaloids, terpenoids and phenolics) to deter herbivory (Nishida, 2014). Insects are continuously challenged to develop mechanisms to detoxify or circumvent plants' defense systems while gaining adequate nutrition. In such a complex ecological network, insects have developed highly sensitive and specific biochemical processes to detect secondary metabolites.

The ability to detect plant secondary metabolites enables insects to locate host plants. Most insects utilize volatile organic compounds for host plant recognition. The tobacco hornworm (*Manduca sexta*) has a strong preference for plants containing the compound indioside D compared to plants without this compound (del Campo et al., 2001). Some insects can detect specific secondary metabolites, which they sequester for



their own defense mechanisms against natural enemies. Vencl et al. (1999) studied larvae of tortoise beetles (*Plagiometriona clavata*) that carry fecal shields on their bodies. These shields function not only as a protective barrier but also contain excreted plant secondary metabolites that deter predators (Vencl et al., 1999). The ability to detect volatiles from host plants and to utilize plant secondary chemicals in deterring predators indicates that these insect-plant relationships are the result of long co-evolutionary associations.

Butterflies and moths are known to use chemicals produced by plants facilitate the interactions they have with predators, other herbivores, host plants and with each other (Conner, 2009). The use of secondary metabolites is key for larvae to protect against predators, parasites and pathogens during development (see review in Chapter 2). Chemical defenses in caterpillars may be produced *de novo*, meaning these chemicals are manufactured by the insects, most often in specialized glands (Bernays, 2002). The more common approach of acquiring secondary metabolites is by feeding on their host plants (Conner, 2009).

Sequestration of plant secondary metabolites typically occurs during the larval stage; the acquired compounds may or may not be retained through to the adult stage (Bowers, 1992). Although sequestration of plant secondary metabolites has been documented in adults of several species of Arctiinae (Weller et al., 1999; Conner, 2009), the presence and amount of sequestered compounds within each larval instar remains poorly known. The focus of this study will be to quantify sequestration patterns of secondary metabolites acquired from selected host plants for different larval species within the genus *Grammia*.

*Grammia incorrupta* (Hy. Edwards) has been of particular interest to researchers (Bernays et al., 2002; Hartmann et al., 2005; Smilanich et al., 2011) because of its ability to sequester specific compounds (pyrrolizidine alkaloids and iridoid glycosides) while still exhibiting polyphagous feeding behavior. Pankoke et al. (2012) has argued that these same chemicals can reduce larval body size and overall fitness. Though this is an intriguing tradeoff, there is still much to learn about the life history of members of *Grammia* and how sequestered chemicals affect these species' life history. Gathering this information will help discern fitness costs associated with host plant choice. This study will focus on the following three species: the virgin tiger moth (*Grammia virgo* (L.)), the little virgin tiger moth (*Grammia virguncula* (L.)) and the figured tiger moth (*Grammia figurata* (L.)). These species have not been studied for their sequestering of secondary metabolites at any life stage (egg, larval, pupa or adult). Rearing species that share host plants species allows for broad comparisons and conclusions about sequestration patterns within this lineage of moths.

Iridoid glycosides are terpenoid compounds that are sequestered by insects in four orders: Lepidoptera, Hymenoptera, Hemiptera and Coleoptera (Nishida & Fukami, 1989; Bowers, 1991). Humans find these compounds very bitter tasting. Feeding experiments have shown that insects that sequester iridoids are unpalatable to both vertebrate and invertebrate predators (Bowers, 1980; Dyer & Bowers, 1996; Theodoratus & Bowers, 1999). The specific secondary metabolites that were surveyed in this study are aucubin and catalpol. Both are iridoid glycosides found in several host plants of tiger moths including *Grammia* spp., and in other butterfly and moth species (Nishida & Fukami, 1989; Bowers, 2003).

In most cases, the butterflies and moths that have been studied are specialists on iridoid-containing plants (Nishida, 2002). Moths that are polyphagous during larval development are thought to be able to select plants based on the secondary metabolites produced (Reisenman & Riffell, 2015). In a comparative study, Bowers and Stamp (1997) tested the ability of four generalist arctiines to sequester iridoid glycosides when fed on *Plantago lanceolata* (Plantaginaceae). Three species (*Spilosoma congrua*, *S. virginica* and *S. latipennis*) sequestered the compounds while one (*Pyrrharctia isabella*) did not. Their study demonstrated that not all polyphagous caterpillars are capable of sequestering plant secondary metabolites. Further, the ability to sequester these chemicals may be restricted to lineage, as those that are able to sequester iridoid glycosides were in the same genus.

Though it is clear that some lineages can sequester iridoid glycosides, little is known about the pattern of sequestration throughout larval development. Here I examined the sequestration of iridoid glycosides for each instar of three *Grammia* species. These observations demonstrate how and when generalist caterpillars sequester and retain compounds from host plants. Specifically, I will address the following questions and associated hypotheses:

Q1. Can *Grammia* spp. larvae sequester aucubin and/or catalpol from the *Plantago* spp.?

*Hypothesis A: Grammia* spp. larvae feeding on either *Plantago* spp. will sequester aucubin and catalpol.

*Hypothesis B: Grammia* spp. larvae feeding on either *Plantago* spp. will sequester aucubin, but not sequester catalpol.

*Hypothesis C: Grammia* spp. larvae feeding on either *Plantago* spp. will sequester aucubin, but not sequester catalpol.

*Hypothesis D:* *Grammia* spp. larvae feeding on either *Plantago* spp. will not sequester either aucubin or catalpol.

Q2. When are larvae of *Grammia* spp. able to sequester aucubin and/or catalpol during development?

*Hypothesis A:* Larvae of *Grammia* spp. are able to sequester aucubin and/or catalpol in the first half of their larval development (1-5 instars).

*Hypothesis B:* Larvae of *Grammia* spp. are able to sequester aucubin and/or catalpol during the last half of their larval development (5-10 instars).

*Hypothesis C:* Larvae of *Grammia* spp. are able to sequester aucubin and/or catalpol at any stage of their larval development

*Hypothesis D:* Larvae of *Grammia* spp. are not able to sequester aucubin and/or catalpol at any stage in their larval development.

## METHODS & MATERIALS

Information on the study sites and field collections can be found in the generalized methods section in the first chapter.

### **Rearing and Experimental Methods**

#### *Insects*

Eggs collected from the females were placed in 160-mL plastic cup lined with filter paper to collect frass. Caterpillars were reared in a growth chamber set at 27 °C and 12:12 L: D, and checked daily to record molting and mortality. Larvae selected for analysis were weighed before being placed in a -80 °C until sample preparation.

### *Insect feeding experiments*

A no-choice feeding study was conducted with the following food sources: broadleaf plantain *Plantago major* (Plantaginaceae) (contains aucubin), narrowleaf plantain *Plantago lanceolata* (Plantaginaceae) (contains aucubin and catalpol), white clover *Trifolium repens* (Fabaceae) (=control) and a wheat-germ based artificial diet (=control). The experimental design consisted of four replicates with a total of four hundred caterpillars for each species of *Grammia* (N = 1200 caterpillars/3 species/4 replicates = 100 caterpillars/species/replicate). The larvae were fed young leaves from their designated plant species or a cube of the artificial diet (approximately 10 mm x 10 mm). Larvae were raised individually in 60-mL plastic cups for the first five instars, then placed in 160-mL plastic cups for the rest of their development.

### *Chemicals*

Analytical standards of aucubin and catalpol, and internal standard (I.S.): phenyl  $\beta$ -D-glucopyranoside (PBG) of >97% purity, were purchased from Sigma-Aldrich (St. Louis, MO, USA). All of these compounds with analytical parameters are listed in Table 3.1. Pyridine, methanol (MeOH), dichloromethane (DCM), and acetone (LCMS grade) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Also, N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% TMCS was purchased from Sigma-Aldrich (St. Louis, MO, USA). Calibration standards for aucubin in the concentration range of 0.11-110  $\mu\text{g mL}^{-1}$  and catalpol in the concentration range of 0.13-137  $\mu\text{g mL}^{-1}$  were prepared in DCM using serial dilutions.

### *Sample Preparation*

Caterpillar and frass samples of first, fifth and tenth instars for all species were prepared in triplicates (N=162). All other caterpillar samples for instars two through four and instars six through nine were prepared in duplicates or triplicates (N = 288). The sample preparation protocol developed by Bowers and Stamp (1992) for secondary metabolites analysis in moths was modified as described below.

### Method Optimization

The larvae collected throughout the experiments were weighed and placed in 4 mL or 8 mL closed glass vials and stored in a -80 °C freezer. The larvae were removed

individually from the freezer, weighed to collect a frozen weight, and placed in a ceramic mortar that was frozen beforehand to keep temperatures consistent between the specimen and equipment. Liquid nitrogen was poured over the larva before being ground down for 10 seconds with a pestle.

The homogenized larva was transferred into an 8 mL glass vial by repeatedly adding MeOH and transferring suspension into the vial for a total volume of 5 mL of MeOH for each larva. The samples were capped, sealed with parafilm, and sonicated for one hour at 55-65 °C using Branson 2510 ultrasound bath at 42 kHz (Branson Ultrasonics, Danbury, CT, USA). Samples were then placed in a -20 °C refrigerator for 72 hours to continue the extraction. The resulting solution for each larva was filtered

through 17 mm Teflon syringe filters (0.2 $\mu$ m pore size) from Thermo Scientific (Rockwood, TN) into a 7 mL glass tube. Each filtered sample was spiked with 10  $\mu$ L of the internal standard PBG (0.840  $\mu$ g/mL) and evaporated under a gentle stream of nitrogen to approximately 200  $\mu$ L. Contents were then transferred using Pasteur pipets into 1 mL autosampler vials and evaporated to dryness. Once the vials were completely dry, 20  $\mu$ L of pyridine and 50  $\mu$ L of BSTFA were added. Samples were then capped, vortexed, and derivatized in a GC oven overnight at 70 °C. Samples were cooled down and spiked with 200  $\mu$ L of DCM. The resulting solution was transferred into inserts placed in autosampler vials, injected and analyzed one to three times (= analytical replicate) using gas chromatography coupled with mass spectrometry (GC-MS).

### **Instrumentation**

Gas chromatographic analyses were performed using a 6890 Series II Plus GC coupled to a 5972 MS (Hewlett-Packard, Santa Clara, CA). Separations were carried out using a 20 m-long DB-5MS column with 0.25 mm internal diameter and a stationary phase 0.25  $\mu$ m film thickness (J&W Scientific, Rancho Cordova, CA, USA) at a constant helium flow rate of 0.8 mL min<sup>-1</sup>. Samples (1.0  $\mu$ L) were injected in a pulsed splitless (splitless time 0.75 min, 25.0 psi for 0.75 min) mode at 250 °C. The temperature program started at 160 °C, held for 1 min, then heated to 300 °C with 15 °C min<sup>-1</sup> temperature gradient, and held for 8.5 min. A total run time was ~ 19 min. The transfer line was set to 280 °C and a solvent

delay for 5 min. The MS data were acquired in the full scan mass range of 50-1000  $m/z$  using the electron ionization (70 eV).

### **Data Processing**

The analytes were identified based on their retention times and mass spectra that match the analytical standards. The internal standard method of calibration was used to quantify the analytes. Limits of detection (LODs) were calculated in Microsoft Excel 2010 from the calibration curves generated using a least square linear regression. The following equations were used:  $LOD = 3.33*s_y/k$ ,  $LOQ = 10*s_y/k$  and  $LLOQ = 5*s_y/k$  (Harris, 2010), where the  $s_y$  is a standard error of the predicted  $y$ -value for each  $x$ -value and  $k$  is a slope of a calibration curve.

### **Data Analysis**

I performed a three way mixed effects model in JMP 12 (SAS Institute Inc., Cary, NC, USA) with moth species, diet, and instar (and all two and three way interactions between variables) as the fixed effects and the technical replicates and analytical replicates as the random effects. Moth species, diet, and instar were fixed effects because replicate number was the same for each variable. The technical and analytical replicates were imbalanced due to variance in the number of replicates sampled across the fixed effects. The response variables were log transformed catalpol and aucubin concentrations ( $\mu\text{g/g}$ ). The first two instars for each moth species were discarded because they were too small to record accurate weights. I performed a three way mixed effects model using the same parameters to analyze the frass data. The response variables were log transformed catalpol and aucubin concentrations ( $\mu\text{g/g}$ ) found in the frass. Interactions that were significant



from the three way mixed effects model were examined using two-way analysis of variance (ANOVA) with a 95% confidence interval. A post hoc Tukey test was performed when appropriate.

## RESULTS

### Sequestration of Analytes

The three species of *Grammia* caterpillars are able to sequester both catalpol and aucubin from both plant diets and the artificial diet (see examples in Figure 3.1 & 3.2). Caterpillars fed on *T. repens* did not survive development and were not analyzed for presence or absence of iridoid glycosides. The earliest instar that catalpol and aucubin were detected on the GC-MS was first instar of *G. virgo* fed on *P. lanceolata* and *P. major*, and first instar of *G. virguncula* fed on *P. major*. Table 3.1 lists the quantifying (with the highest signal to noise ratio) and qualifying ions for each analyte BSTFA derivative as well as for the I.S.

### Caterpillar and Analyte Interactions

Diet type did not influence the amount of catalpol and aucubin sequestered by the *Grammia* spp. caterpillars (N = 232:  $F = 0.731$ ,  $P = 0.765$ ;  $F = 1.39$ ,  $P = 0.179$ , respectively). However, moth species-instar interactions did influence the amount of catalpol sequestered by *Grammia* spp. caterpillars (two way ANOVA:  $F_{16, 170} = 3.18$ ,  $P < 0.0001$ ). The amount of catalpol sequestered differed between species during development (Figure 3.3 & 3.4). *Grammia figurata* caterpillars sequestered the highest amounts relative to body weight of aucubin and catalpol early in development. The interaction between moth species-instar did not influence the amount of aucubin sequestered by the *Grammia* spp. caterpillars (two way ANOVA:  $F_{16, 170} = 1.64$ ,  $P =$

0.6154). The same analysis did find the interaction between instar-aucubin to be influencing the amount sequestered ( $F_{9, 170} = 10.1, P < 0.0001$ ) (Figure 3.5 & 3.6). *Grammia* spp. caterpillars sequestered smaller amounts of aucubin during development versus the amount of catalpol sequestered from the same caterpillars (Figure 3.3 & 3.5). The amount of catalpol sequestered was over ten times higher than the amount of aucubin sequestered in the *Grammia* spp. caterpillars. *Grammia virgo* and *G. virguncula* sequestered less of the iridoid glycosides than *G. figurata*. Both *G. virgo* and *G. virguncula* had negligible amounts of aucubin into their late instars from both plant diets (Figure 3.5). Differences in catalpol and aucubin concentrations may be the result of larvae converting aucubin to catalpol during development; catalpol would increase as the precursor, aucubin is converted in this process.

### Frass and Target Analyte Interactions

Diet did not influence the amount of catalpol and aucubin found in the frass of *Grammia* spp. caterpillars (N = 125:  $F = 1.26, P = 0.2103$ ; N = 122:  $F = 1.98, P = 0.173$ , respectively). Species-instar interactions did not influence the amount of catalpol found in the frass of *Grammia* spp. caterpillars (two way ANOVA:  $F_{14, 101} = 1.27, P = 0.2399$ ) (Figure 3.7). *Grammia virguncula* caterpillars, had the highest amounts of aucubin and catalpol in their frass early in development (third and fourth instar) (Figure 3.7 & 3.8). The interaction between species-instar did influence the amount of aucubin found in the frass of *Grammia* spp. caterpillars (two way ANOVA:  $F_{14, 98} = 3.82, P < 0.0001$ ). *Grammia* spp. caterpillars expelled smaller amounts of aucubin in their frass during development versus the amount of (Figure 3.7 & 3.8). The amount of catalpol found in frass was almost five times higher in *G. figurata* and *G. virguncula* third instar

caterpillars than the amount of aucubin found in frass of these caterpillars. *Grammia virguncula* had the highest amounts of catalpol in the frass of 6-9 instar caterpillars than *G. figurata* and *G. virgo* (Figure 3.7). All three species of *Grammia* caterpillars had very low amounts of aucubin in their frass with the exception of fourth instar *G. virguncula* caterpillars (Figure 3.8 & 3.9).

## DISCUSSION

In summary, this study provides the first identification and quantification of the sequestration of iridoid glycosides by *Grammia* larvae. Aucubin and catalpol were sequestered in detectable amounts across instars. Second, these species of *Grammia* are also capable of converting aucubin into catalpol from *P. major* plants. Third, the concentration of catalpol in these species of *Grammia* larvae was highest overall during the early to middle instars and decreased in their final instars. Overall, my results show that although these compounds may be sequestered by these species, the sequestration efficiency and amounts of those compounds can vary considerably. Specifically, these results best support the idea that *Grammia* spp. larvae feeding on either *Plantago* spp. will sequester aucubin and catalpol (Q1. Hypothesis A), or that catalpol can be synthesized from sequestered aucubin during development. Further, it appears that these larvae can sequester/manufacture aucubin and catalpol at any stage in their development (Q2. Hypothesis C). I will discuss the support and implications for the findings below.

### **Conversion of Aucubin to Catalpol**

The presence of catalpol in *Grammia* caterpillars is significant. All three species of *Grammia* fed both plant diets contained catalpol; however *P. major* does not possess catalpol as a secondary metabolite (see appendix Figure 3.10; Long et al., 1995; Samuelsen, 2000; Ronsted et al., 2000). Instead caterpillars appear to sequester aucubin from *P. major* and later convert it to catalpol. Catalpol is more toxic to insects than aucubin (Puttick and Bowers, 1988); it is not clear why *Grammia* caterpillars would synthesize a more toxic compound after sequestering its precursor. Nonetheless, the ability to sequester catalpol from a host plant can be advantageous for *Grammia* caterpillars. Stromeyer et al. (1998) reports that predators preying on iridoid glycoside-containing insects had difficulty gaining body weight. Thus, sequestering more toxic iridoids can be useful for caterpillar survival as predators may try to avoid eating too many iridoid-containing caterpillars.

### **Patterns of Sequestration across Species and Instars**

Though it was not possible to quantify iridoid glycosides in the first and second instars for these *Grammia*, these chemicals were detected in both instars. Surprisingly, iridoid glycosides were found in all first instars, including those fed on diets not containing these chemicals. The amounts of iridoid glycosides found in some samples fed on artificial diet could be from parental investment of secondary chemicals provisioned in the egg, or contamination in the GC column. Other species of tiger moths have been found to provision their eggs with sequestered secondary chemicals (Dussourd et al.,

1988). In order to determine if parental investment is occurring, a separate study focusing on caterpillars feeding on an artificial diet should be performed.

The amounts of iridoid glycosides sequestered across all *Grammia* spp. caterpillars were low to moderate relative to body weight. The exception to this trend was the fourth instar of *G. figurata*. It is unclear why this particular instar in *G. figurata* was significantly higher than the rest of the samples. This difference could be due to the time these caterpillars were sampled; excess amounts of the iridoid glycosides may not have been eliminated at that time. With the exception of *G. figurata*, it appears that *Grammia* are not effective at sequestering iridoid glycosides.

These observations reveal a pattern in sequestration of iridoid glycosides for these species that is similar to results found in late instar stage *G. incorrupta* caterpillars (Smilanich et al., 2010). *Grammia incorrupta* caterpillars sequester low amounts of catalpol and aucubin, which indicates that they are inefficient at sequestering these compounds (Smilanich et al., 2010). Furthermore, other tiger moth species sequester iridoid glycosides poorly during larval development (Bowers & Stamp, 1997; Lampert & Bowers, 2010). The inability to effectively sequester these secondary chemicals may stem from a relatively recent association with these host plants. Both species of *Plantago* are not native to North America (Samuelson, 2000), while the lineage *Grammia* evolved in the Great Plains (Schmidt, 2008). Co-evolutionary relationships between tiger moths and their hosts require long periods of time, allowing for the evolution of chemical pathways that detoxify and alter secondary metabolites. It may be that *Grammia*'s association with *Plantago* has not been occurring for a sufficient period of time to determine the outcome of this evolutionary arms race.

### **Iridoid Glycosides Found in Frass of *Grammia* Caterpillars**

Amounts of aucubin and catalpol found in the frass were lower than the amounts of aucubin and catalpol found within all *Grammia* spp. caterpillars. The lower concentration of these chemicals in the frass indicates that *Grammia* caterpillars are sequestering more iridoid glycosides as they develop. These results are supported by another study that reports low amounts of iridoid glycosides in frass of other tiger moth species (Bowers & Stamp, 1997). In all of these cases, caterpillars in the final instar sequester and expel low amounts of aucubin and catalpol, suggesting that the amount sequestered per instar does not change as caterpillars gain weight. Thus, as caterpillars gain weight, the concentration of these toxins may decrease as they approach pupation and adulthood. *Grammia* undergo a diapause at mid larval development (instar 5-7) presumably beneath soil and leaf litter; pupation also occurs in soil and litter (Schmidt, 2009). Late instar *Grammia* are found in leaf litter and loose soil, lessening their exposure to parasitism. With less pressure from parasitoids, larvae may store lower concentrations of chemicals per body weight, allowing them to optimize the use of these chemicals and to reduce the risk of auto-toxicity during pupation.

Table 3.1. List of analytes used to determine retention times and identify MS qualifying and quantifying ions for their BSTFA derivatives.

Analyte	Molecular formula	MW (g mol <sup>-1</sup> )	Retention (t <sub>R</sub> )	Quantification ion m/z	Confirmation ions m/z	Purpose	Manufacturer
Aucubin	C <sub>15</sub> H <sub>22</sub> O <sub>9</sub>	346.33	10.29	361 (100)	217 (70), 221 (5)	Analyte	Sigma-Aldrich
Catalpol	C <sub>15</sub> H <sub>22</sub> O <sub>10</sub>	362.33	9.86	361 (100)	217 (40), 221 (40)	Analyte	Sigma-Aldrich
<i>Internal Standard</i>							
phenyl β-D-glucopyranoside	C <sub>12</sub> H <sub>16</sub> O <sub>6</sub>	256.25	7.45	361 (100)	217 (40), 221 (1)	I.S.	Sigma-Aldrich

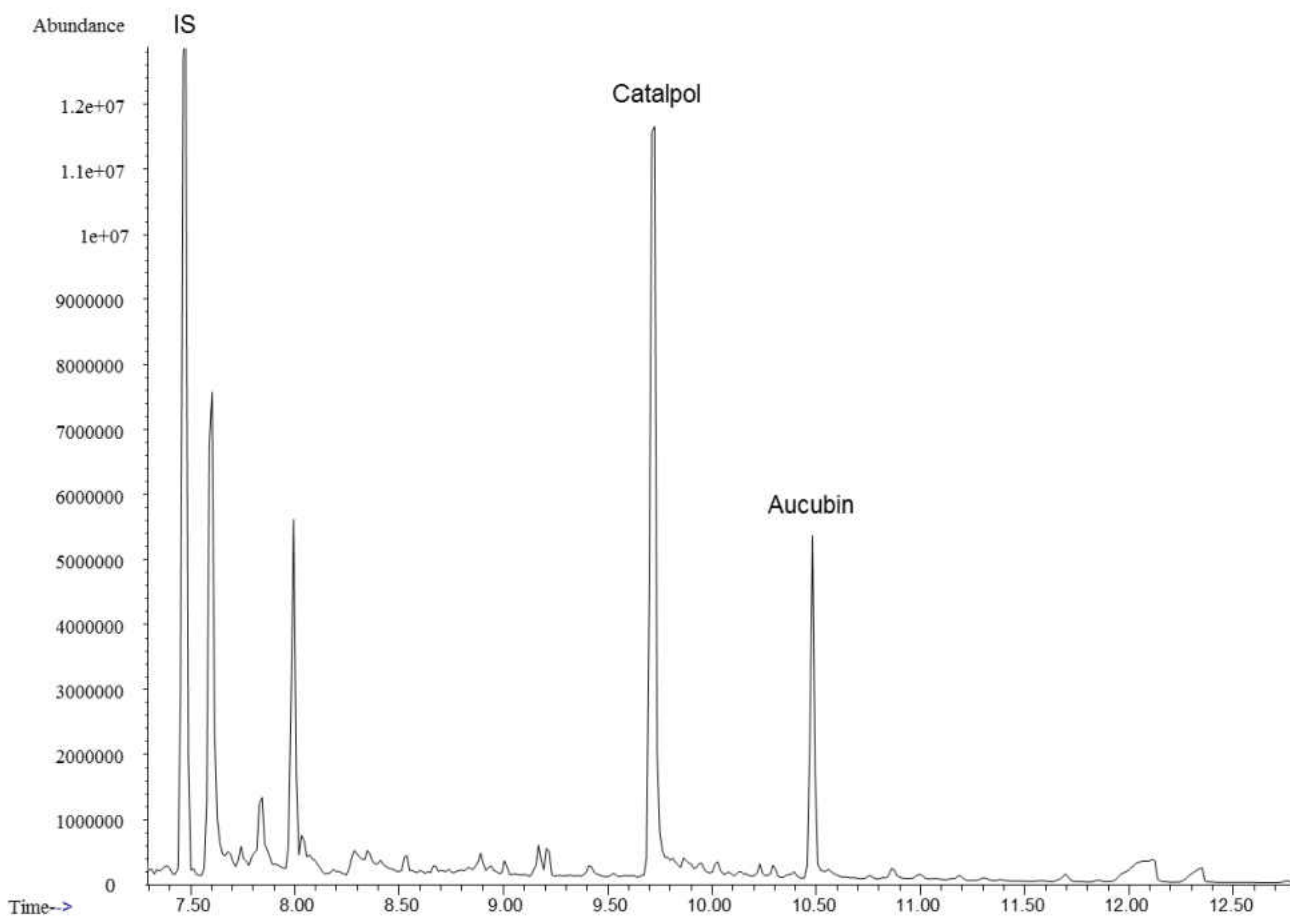


Figure 3.1. GC-MS TIC chromatogram showing the identification of aucubin and catalpol of a *G. figurata* larval sample that fed on *P. lanceolata*.



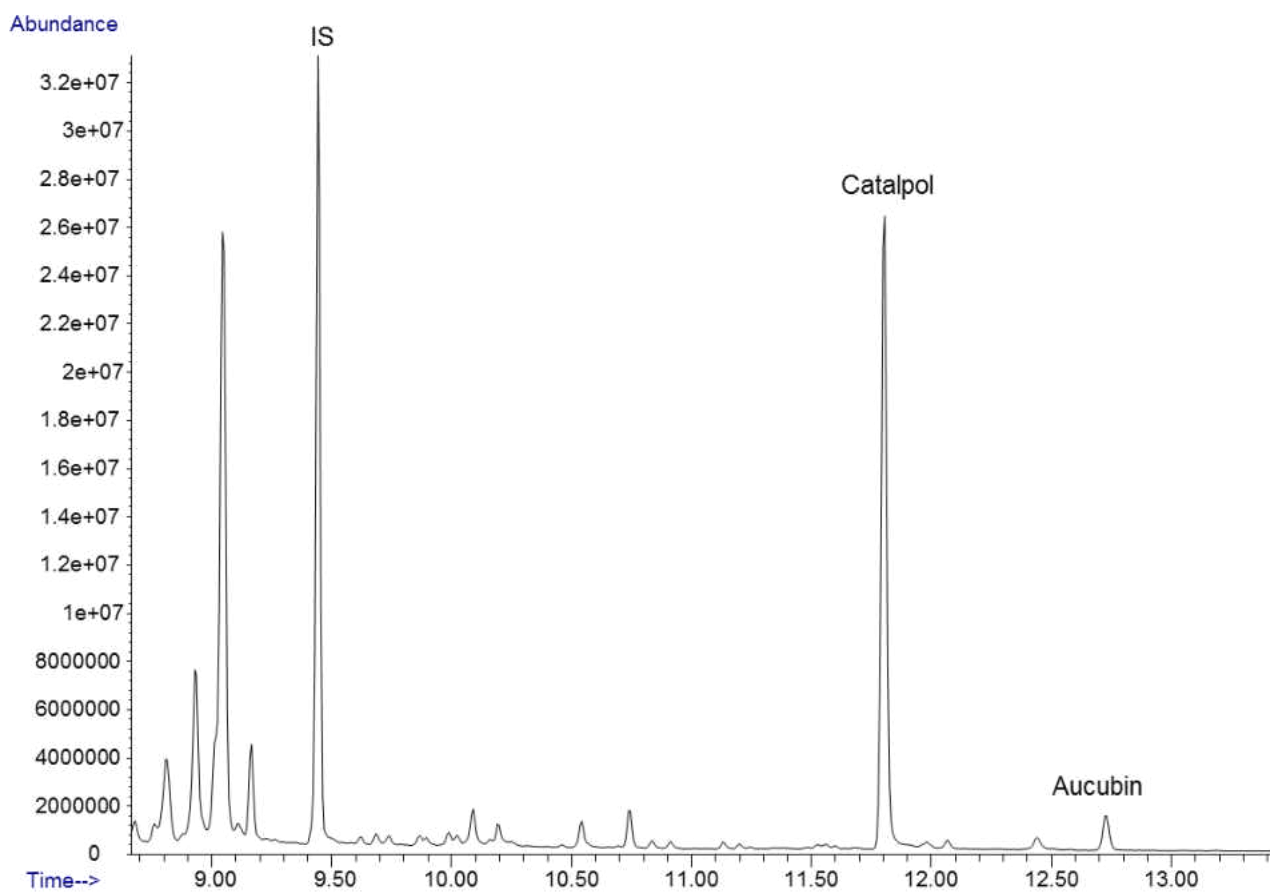


Figure 3.2. GC-MS TIC chromatogram showing the identification of aucubin and catalpol of a *G. virgo* larval sample that fed on *P. major*.

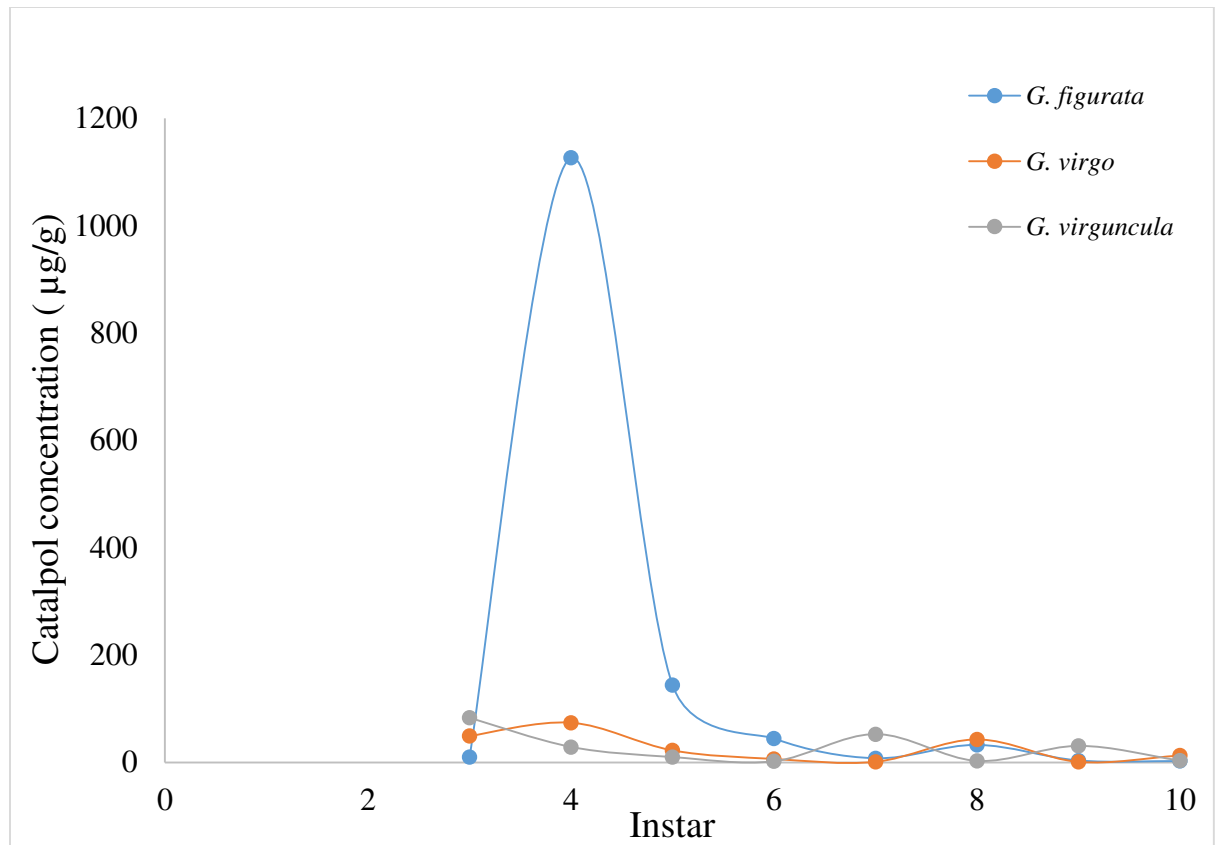


Figure 3.3. Comparison of sequestration patterns of catalpol in *Grammia* spp. caterpillars during larval development (N = 230, df = 16).

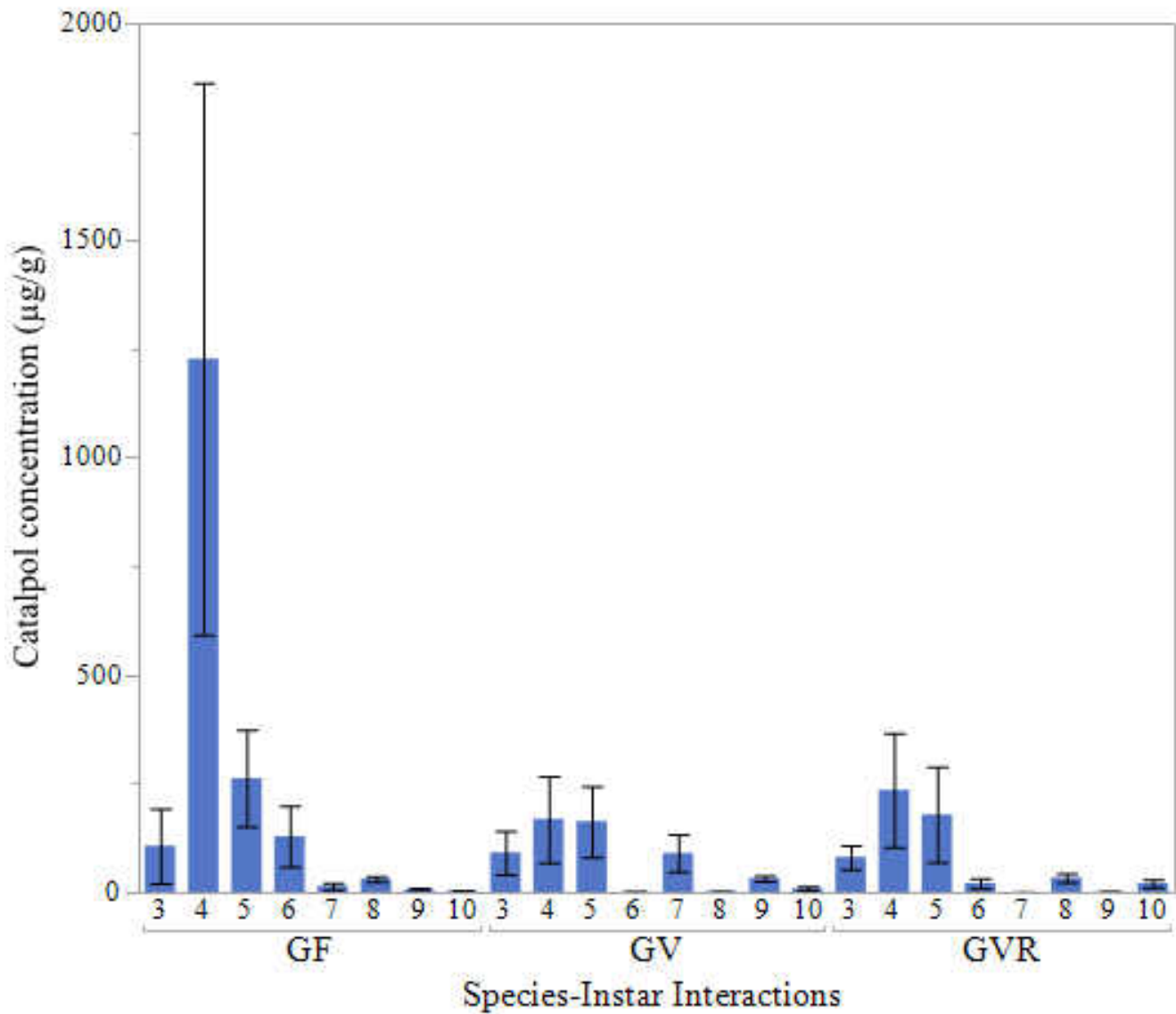


Figure 3.4. Species-instar interactions (GF = *G. figurata*, GV = *G. virguncula*, GVR = *G. virgo*) on sequestration of catalpol during larval development (N = 230, df = 16). Error bars constructed using standard error from the mean as determined by the Tukey's HSD post hoc test.

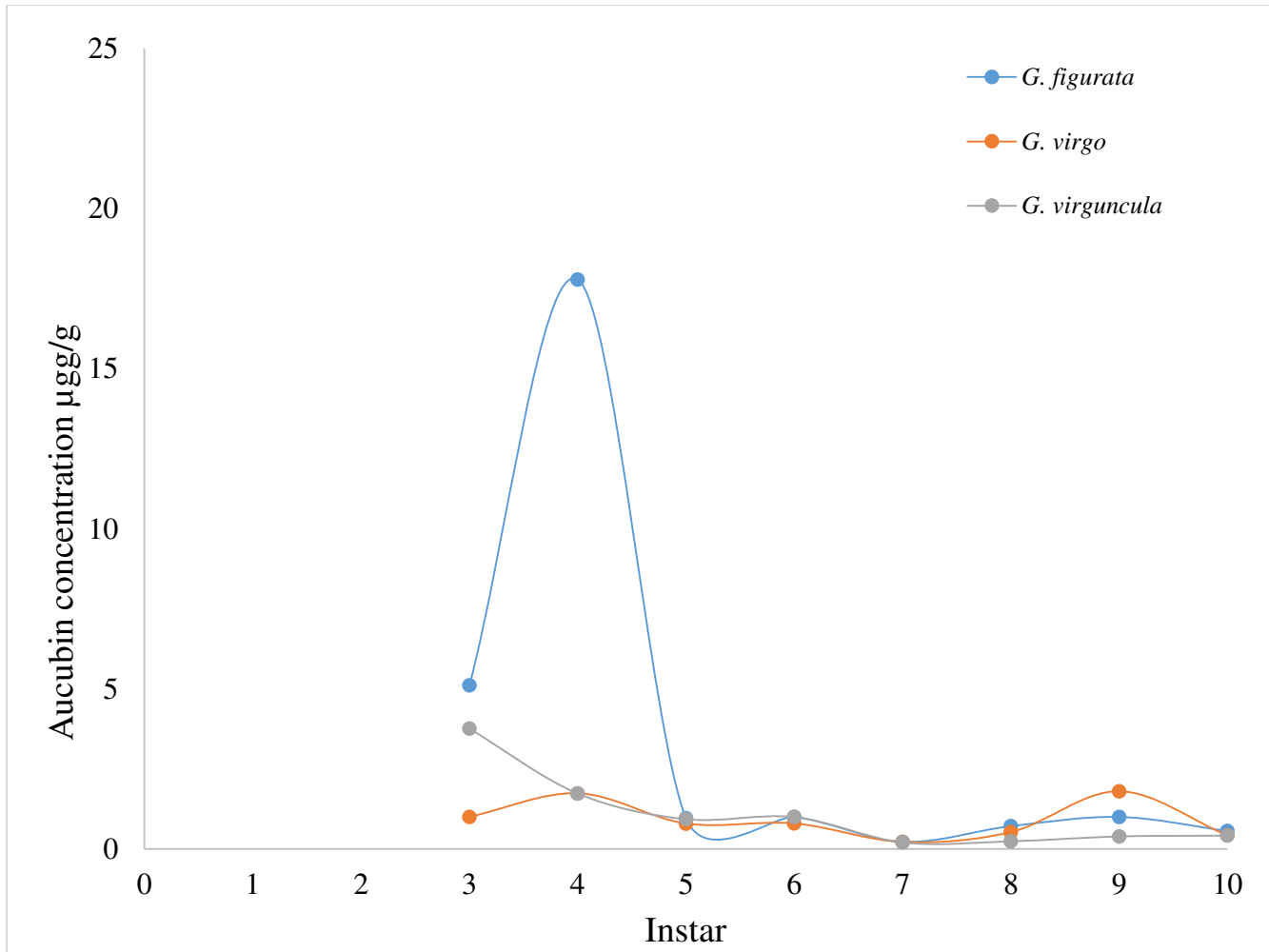


Figure 3.5. Comparison of sequestration patterns of aucubin in *Grammia* spp. caterpillars during larval development (N = 230, df = 16).

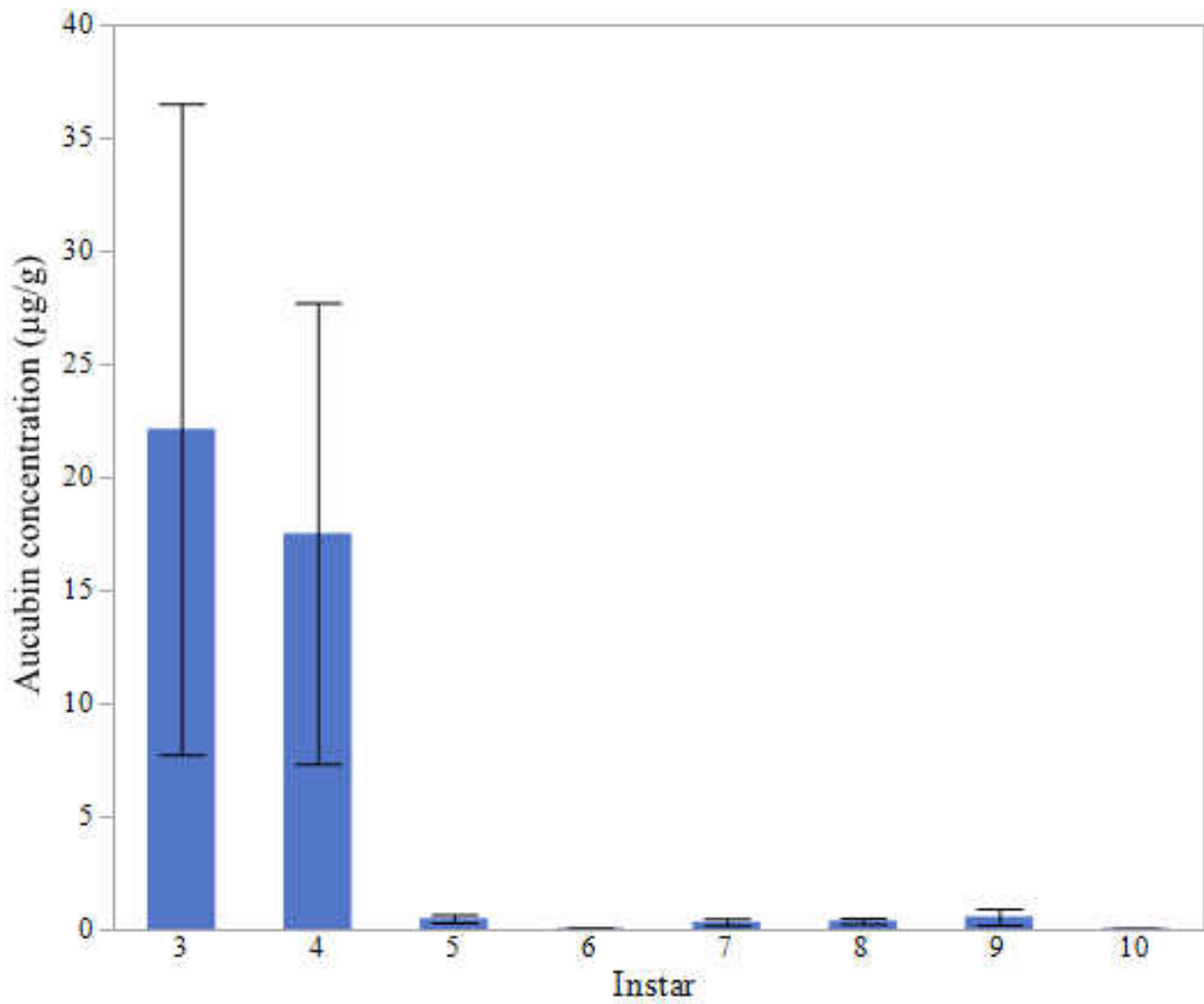


Figure 3.6. Sequestration of aucubin for all species combined for larval instars 3-10 (N = 230, df = 16). Error bars constructed using standard error from the mean as determined by Tukey's HSD post hoc test.

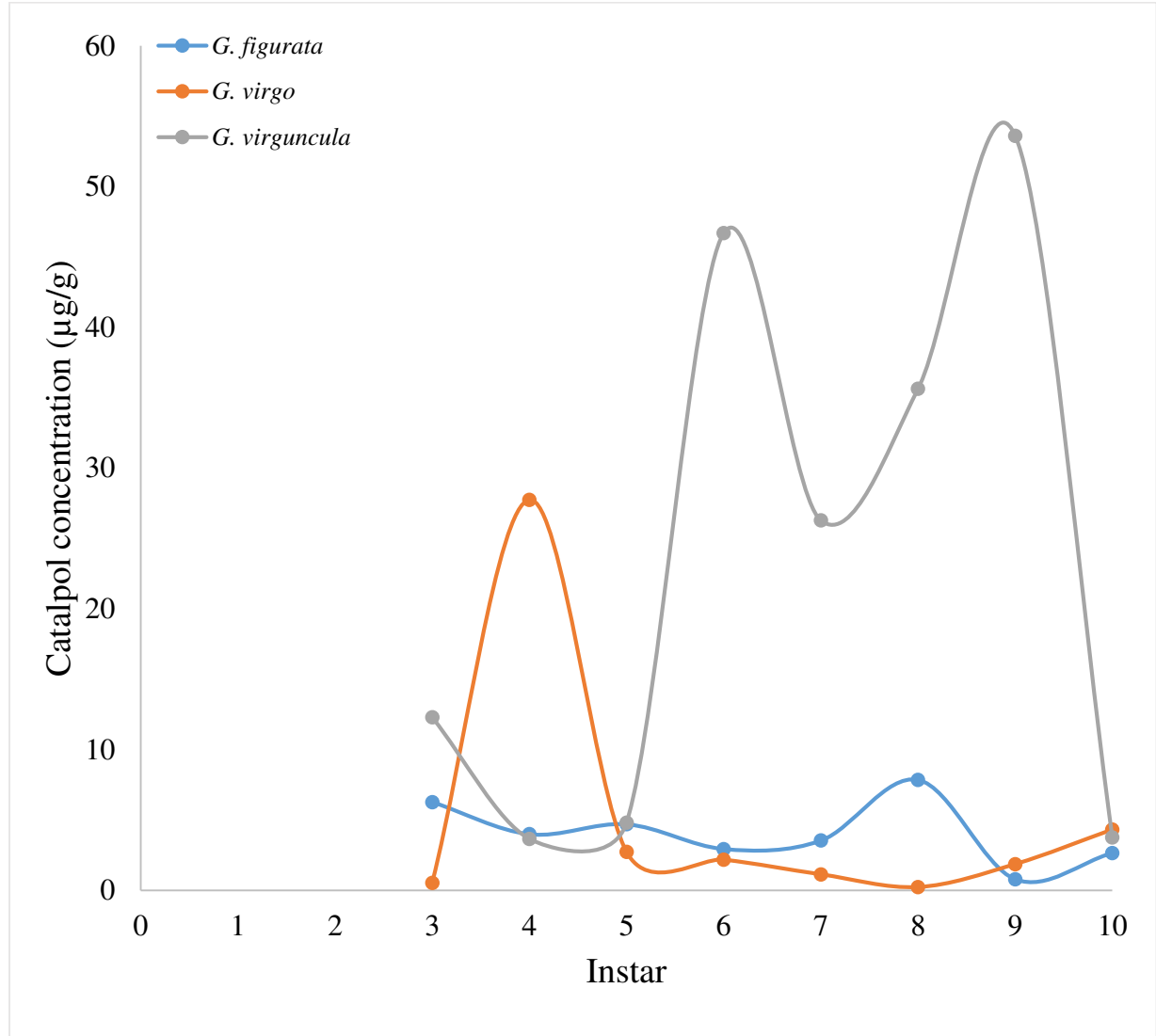


Figure 3.7. Comparison of catalpol found in frass in *Grammia* spp. caterpillars during larval development (N = 125, df = 14).

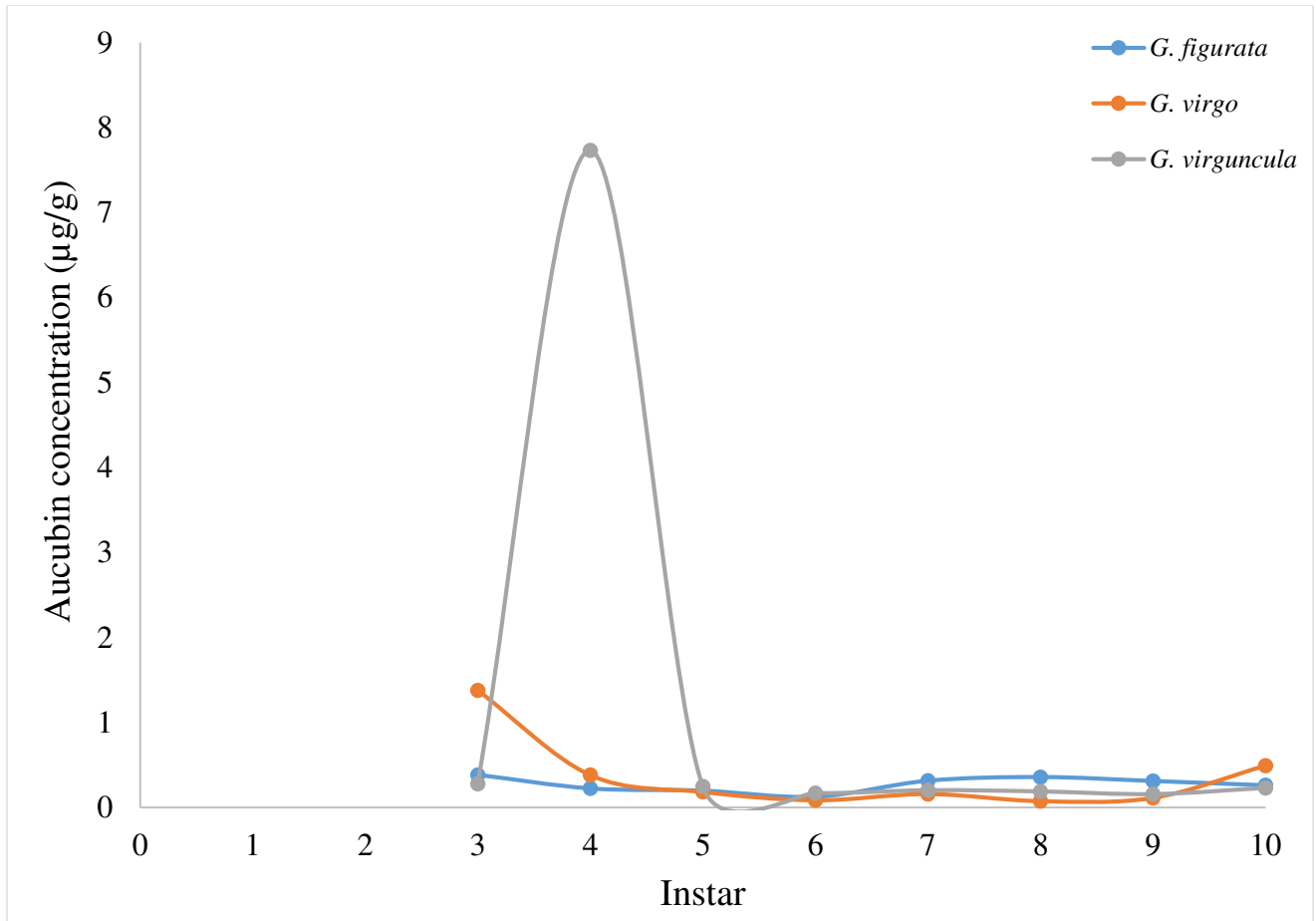


Figure 3.8. Comparison of aucubin found in frass in *Grammia* spp. caterpillars during larval development (N = 122, df = 14).

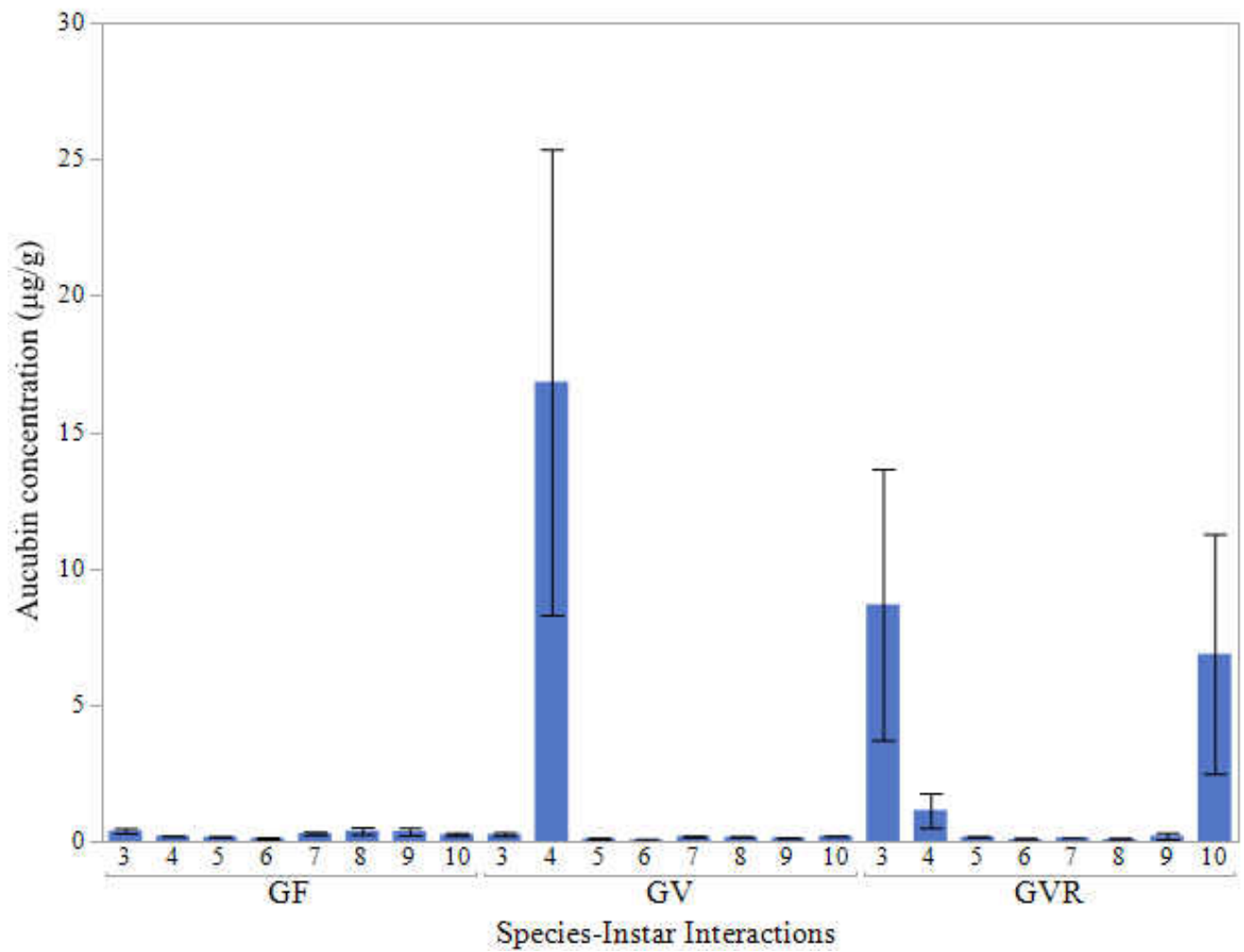


Figure 3.9. Species-instar interactions (GF = *G. figurata*, GV = *G. virguncula*, GVR = *G. virgo*) on concentration of aucubin found in frass during larval development (N = 122, df = 14). Error bars constructed using standard error from the mean as determined from Tukey's HSD post hoc.



## CHAPTER IV

### EPILOGUE

My journey into the field of chemical ecology was initially inspired by observations I made while working at an invasive research lab in south Florida. My research focused on studying the interactions between the invasive Brazilian peppertrees, *Schinus terebinthifolius* (Anacardiaceae, Raddi 1820) and insect pollinators that could serve as bio-controls. During the course of my research I came upon a bird and a raccoon that had ingested fruit from *S. terebinthifolius* plants; they were severely disoriented. The bird was trying to fly away and kept falling to its side. The raccoon was also trying to walk away and was stumbling along in a zig-zag pattern. I found their behavior fascinating and unexpected. I wanted to investigate this further and located the raccoon's scat (full of *Schinus* fruit) near a row of *S. terebinthifolius* plants. I started to look around and found a variety of insects moving on and around these plants. There were butterflies, moths, ants, bees and wasps feeding and taking pollen from these plants. But specifically, I was watching the wasps.

These wasps were hovering around the fruit of the *S. terebinthifolius* plants and I decided to take a cluster of fruit to analyze in-lab. I discovered that Brazilian peppertrees produced urushiol. This organic allergen is responsible for the itching sensation associated with plants like poison ivy (*Toxicodendron radicans*), also a member of the Anacardiaceae. Wasp larvae inhabited nearly every individual berry, feeding on the fruit

during development. It blew my mind that these tiny wasp larvae could feed on *S. terebinthifolius* fruit as their primary source of nutrition during development, and in contrast, two larger animals were severely affected by the same fruit. This observation gave rise to my fascination with the world of chemical ecology, and inspired me to search out research being done on tiger moths and butterflies, with an emphasis on their chemical ecology, evolution, and development. I was inspired by researchers such as Deane Bowers, Elizabeth Bernays, Thomas Hartmann, Suzanne Dobler, and Michael Boppré. The work of these scientists has played a major role in the development of my research and my interest with this field.

Below, I highlight some of the main conclusions from my thesis on the development and chemical ecology of these species of *Grammia* caterpillars. In doing so, I review the current state of knowledge and identify possible future directions for research that would improve our understanding of the larval development of *Grammia* spp.

***Grammia* spp. caterpillars take longer to develop and gain less weight on plant diets versus an artificial diet.**

Prior my research, there was no information available on the larval development of these *Grammia* species. The research reported in Chapter 2 demonstrated that these *Grammia* caterpillars developed fastest and gained the most weight on an artificial diet. However, in natural conditions these *Grammia* caterpillars develop on *Plantago* spp. that are non-native and abundant throughout North America. My results show that these polyphagous caterpillars are able to survive when forced into a monotypic diet, which may be increasingly observed as land conversion for human activities (e.g. exurban

expansion and agricultural intensification) leads to simplified landscapes containing these plants. These results hold promise for the survival of *Grammia*, as they can adapt to diet constraints within a single generation.

Though I forced these *Grammia* species to adopt a monotypic diet during development, multi-plant feeding trials would provide a more accurate platform to understanding larval feeding behaviors. In addition to this better understanding of larval feeding, there is also a need to document the complete life cycle of these *Grammia* moths, particularly in regards to reproductive success. To do this, it is necessary to follow these *Grammia* caterpillars through pupation and into adulthood to track body size and reproductive success. Further, multi-generation experiments are needed to determine if diet constraints lead to differential fitness and physiological and behavioral adaptations.

***Grammia* spp. caterpillars can store aucubin and catalpol from both *Plantago* species.**

Aucubin and catalpol are well known plant secondary metabolites that have been found in moths. This work was the first to investigate the sequestration patterns of caterpillars at this level of detail. By examining all instars of three species of *Grammia* (*G. figurata*, *G. virgo*, *G. virguncula*) for aucubin and catalpol sequestration, it is clear that these species have a life-time association with these chemicals that is not entirely positive for the caterpillars. Previous work examined late instar caterpillars, limiting the understanding of the chemical ecology for these species. Future studies will also need to encompass all instars to understand how caterpillars sequester plant secondary metabolites, and how they cope with negative aspect of this association.

These *Grammia* caterpillars have the ability to convert aucubin to catalpol; this is the first study to identify this occurrence in any lepidopteran species. Not only can these caterpillars sequester iridoid glycosides, but they also are able to store the catalpol in higher amounts compared to aucubin, even though catalpol is more toxic to insects than aucubin. I also found that *Grammia* caterpillars expel very small amounts of these iridoid glycosides in frass during development.

Despite these findings, it is not clear what the cost is for making/storing catalpol, or exactly how catalpol is expelled in frass. These results also demonstrated that *Grammia* caterpillars sequester higher amounts of aucubin and catalpol earlier in their development than in later instars. Low amounts of both iridoid glycosides sequestered in late instars could indicate that caterpillars allocate fewer resources to sequestration, shifting more resources into preparation for pupation.

As discussed in the previous section, designing multi-plant and multi-generation experiments would be important to gain further understanding of these species of *Grammia*. Multi-generation testing would provide a method to understand the affects plant secondary metabolites have on caterpillar and adult fitness. I would also be able to examine if the trends seen in the results from Chapter 3 in regards to iridoid glycoside sequestration patterns, are consistent in successive generations of these species of *Grammia*.

Here, I have attempted to broaden the scope of understanding the larval development and chemical ecology of these species of *Grammia*. My future investigations will examine interactions between caterpillars and predators/parasitoids and incorporate multi-plant and multi-generation experiments to further our

understanding of caterpillar interactions with their surrounding environment.

Understanding how parasitoids interact with the caterpillar at a physiological level is a new set of questions to address. Additionally, I am interested in observing the gut microbiome of these species; it is possible that some of these processes are mediated by endosymbionts that have a co-evolutionary relationship with tiger moth lineages.

Expanding on my completed research to include multi-trophic interactions will give a better understanding of how these fascinating species survive in a hostile, changing environment.

## APPENDIX

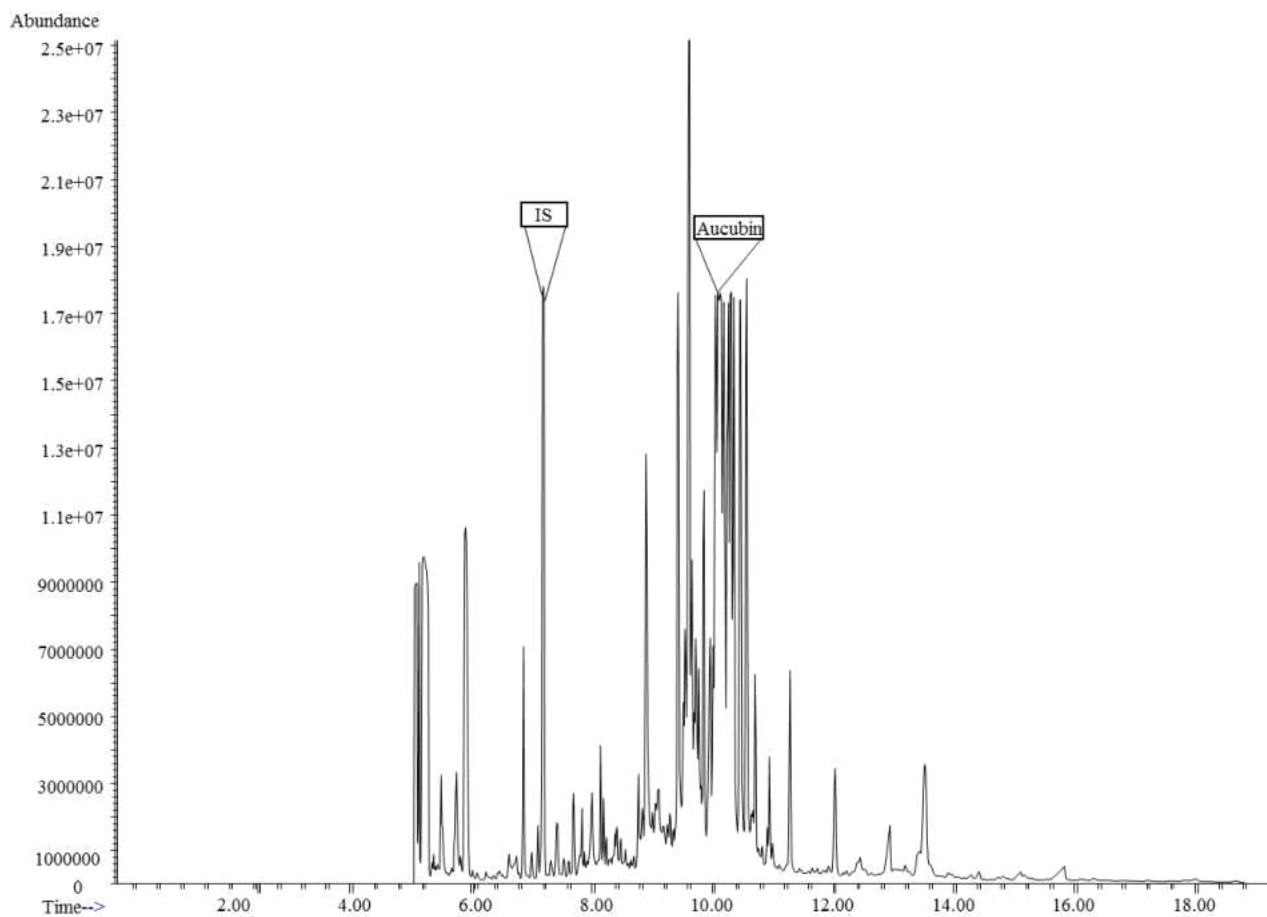


Figure 3.10. GC-MS TIC chromatogram showing the presence of aucubin and the I.S. in leaves of *P. major*.

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