Self Medication as a Response to Parasitoid Infection in the Tobacco Hornworm, Manduca sexta

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Self-Medication as a Response to Parasitoid Infection in the Tobacco Hornworm, *Manduca sexta*

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Abstract
Allelochemicals (plant chemicals not involved in growth/development) play an essential role in aiding insects that store these chemicals in their ability to thwart predators and parasitoids. Relatively little research has addressed the ability of polyphagous (feeding on several different food types) herbivorous insects to self-medicate by selecting diets that moderate infection intensity (self-medication). In the specific case of *Manduca sexta* [Tobacco Hornworm] (Sphingidae), ingestion of the alkaloid nicotine hinders the success of the parasitoid *Cotesia congregata* (Braconidae), yet whether tobacco hornworms switch to a diet of high nicotine concentration when infected is unclear. In this study, the ability of *M. sexta* to discern between diets and to choose a high-nicotine diet to battle infection, and thus self-medicate, was analyzed. The relationship between nicotine in the diet and the insect’s encapsulation response (immune response against invading bodies) was also studied. I predicted that *M. sexta* would exhibit behavioral plasticity and choose diets of higher nicotine concentration as a response to infection, and that higher nicotine concentrations would be correlated with a greater encapsulation response. 5th instar (the final molting stage before pupation) *M. sexta* were injected with glass silica beads and subjected to a series of dietary choice tests. They were subsequently dissected to retrieve beads and rate of melanization was determined for each caterpillar. While injected *M. sexta* differ in their dietary choices compared to healthy larvae, nicotine does not drive a trend in dietary choice. No relationship between nicotine concentration and melanization was found, although healthy caterpillars did consume significantly more food than those that were injected. *M. sexta* exhibit behavioral plasticity in diet choice, but no clear relationship between allelochemicals and encapsulation was found.
INTRODUCTION

Differences in diet breadth among insect herbivores are shaped by strong selective pressures created by the defenses of the plants they eat and by their need to defend themselves against predators and parasites (Bernays and Chapman 1994). Many factors thus influence what an insect can eat, one of these being the defensive components of plants (Duffey 1980). Mechanisms that have evolved to avoid herbivores include mechanical and chemical defenses, camouflage/mimicry, leaf shedding and indirect defense (ie, attracting enemies of the potential herbivore through symbiosis) (Freeman & Beattie 2008). These have evolved to improve the survival and reproduction of the plant species, while simultaneously diminishing herbivore performance and fitness. Honing in on a particular defensive attribute, chemical defenses generally involve secondary metabolites (also known as allelochemicals; these are organic compounds that are not involved in the natural ontogeny or reproduction of the plant to fend off enemies. Such compounds can be further broken down into several different types, such as alkaloids (which cause a bitter taste) and glycosides (which react with enzymes to cut off cellular respiration) (Rhoades et al 1979, Vetter et al 2000). These elaborate means of defense shape what an insect herbivore can eat, therefore imposing strong evolutionary selection.

Despite plants’ defensive adaptations, many herbivorous insects have evolved to overcome these noxious chemical defenses. Many species of Lepidoptera (moths and butterflies) are capable of digesting and metabolizing allelochemicals, or taking this a step further via sequestering plant metabolites (storing them as a defensive mechanism against predators and parasitoids) (Reddy & Guerrero 2004). Diet breadth can also be shaped by predators and parasites if these sequestered chemicals decrease the risk of the herbivore being attacked by its enemies. Pressures from both enemies and plants can thus lead to the evolution of insects’ ability
to digest allelochemicals, thereby increasing their dietary range. If herbivores specialize in highly toxic diets and they are capable of sequestering the toxins in their tissues, they can counter predators by being toxic themselves. However, the specific role of allelochemicals in herbivore-parasitoid (organism involved in a parasitic relationship with host that ultimately sterilizes and kills the host) interactions remains unclear (Duffey 1980, Goss 1993).

The primary physiological response against parasitoid infection is encapsulation. This is an immune response in which specialized cells known as hemocytes swarm invaders (in this case, parasitoid eggs). Hemocytes then undergo the process of melanization (process by which melanin is produced to swarm the intruder), and inevitably asphyxiate the foreign invader (Godfray et al 1994). The relationship between sequestered allelochemicals and the encapsulation response remains uncertain. In some cases, concentrations of secondary metabolites such as iridoid glycosides have been correlated with either enhanced or diminished rates of encapsulation, while in many other cases no clear correlations were found (Smilanich et al 2009, 2011). Chemicals may have differing effects against predators and parasitoids, and sometimes do not lower the fitness of parasitoids (Barbosa and Letourneu 1988). Accordingly, a secondary metabolite that frees an herbivore from predation may actually attract more parasitoids; the herbivore is more likely to survive, leading to the survival of parasitoid larvae (Charnov et al 1995). Parasitoids can in turn use chemical cues to find a suitable and safe host (Vinson 1976).

Given this dual role of diet breadth and plant allelochemical influence on immunocompetence (ability to develop an immune response), a generalist insect (feeding on multiple plants) should be able to alter foraging strategies to increase the effectiveness of encapsulation in response to infection. Although changes in diet choice have been demonstrated
in species such as *Grammia incoruupta* [woolly bear caterpillar] (Arctiidae) and *Platyprepia virginalis* [Ranchman’s tiger moth] (Arctiidae), other studies have shown no ability to use diet choice as a means of self-medication (Karban et al 1994, Singer et al 2003, 2004, 2009). The vast majority of studies have analyzed these tri-trophic interactions (a three-link food web relationship which in this case involves a plant, an herbivore, and a parasitoid) without considering diet choice by generalist herbivores. The physiological effects of secondary metabolites on parasitoids have been studied extensively in a wide range of specialist insects, as have the oviposition choices of parasitoids in relation to herbivore diet (Smilanich 2011, Lampert and Bowers 2011). The ability of insects to actually self-medicate, however, is a relatively new and emerging field that is poorly understood.

The goals of the present study were to assess whether or not a generalist caterpillar (*Manduca sexta*) can make dietary choices as a means of combating infection, as well as to analyze whether a correlation exists between a particular allelochemical (nicotine) and the encapsulation response. As *M. sexta* are generalists and nicotine diminishes parasitoid survival in infected larvae (Thorpe and Barbosa 1986, Barbosa 1986, 1988, 1991), it was predicted that the insect would be able to self-medicate by switching to diets of higher toxicity. Additionally, nicotine treatment level was expected to be correlated with the rate of encapsulation.
BACKGROUND

Parasitoids

In many studies, parasitoids represent the third link of a tri-trophic interaction, as is the case in my thesis. A parasitoid by definition is an organism that spends a significant portion of its life within or attached to a host, in a relationship that is essentially parasitic. In contrast to parasites, parasitoids are insects which always sterilize and kill their hosts (Eggleton and Belshaw 1992). Many parasitoids lay their eggs within insects, where the eggs develop and utilize their host’s internal resources for growth. Parasitoids are mostly Diptera (flies) or Hymenoptera (wasps), and because they kill their hosts (often herbivores), they are a crucial driver of selection for defenses in herbivorous species. Parasitoids can be further classified into one of two groups-ectoparasitoids and endoparasitoids (Eggleton and Belshaw 1992). Endoparasitoids, or those whose development occurs within the host, are considered one of the most important causes of mortality for many caterpillars, and accordingly present a significant threat towards larval survival (Kenis et al 2004).

Herbivore Immune Defenses Against Parasitoids: The Role of Host Plant Allelochemicals

Due to the lethality associated with parasitoids, insects have developed effective defenses against them (Goss 1993). Sequestration (the ability to digest and store allelochemicals) is in many cases a defensive response to parasitism, albeit with different levels of success among different species (Duffey et al 1986). Through sequestration, allelochemicals may serve as toxins against foreign parasitoid eggs, thereby diminishing parasitoid fitness through the influence of survival, development, size, and morphology (Goss 1993, Duffey et al 1986). The allelochemical α-terthienyl has an adverse effect on the parasitoid Diadegma terebrans, hindering the
development and rate of emergence in *D. terebrans* larvae in infected *Ostrinia nubilalis* [European corn borer] (Pyralidae) (McDougall et al 1988). Campbell and Duffey (1979, 1981) found that α-tomatine had adverse effects on the survival of *Hyposoter exiguae* (Viereck), a parasitoid of the tomato fruitworm, *Heliothis zea* (Boddie). *Drosophilia melanogaster* larvae can feed on particular species of yeast to enhance their encapsulation defense against parasitoid eggs. In this case, microbial agents in the yeast may help to fight off infection (Anagnostou et al 2010). Additionally, wood ants have been shown to utilize resin as a method of quelling infection intensity (Chapuisat 2007). The nicotine alkaloid is shown to reduce the number of emerging parasitoids in fall armyworms (Thorpe and Barbosa 1986). These examples emphasize the role of diet in regard to countering infection, thus showing that not everything an herbivore feeds upon may contribute towards growth and nutrition. In many instances, herbivores can take advantage of food chemicals to improve their immunocompetance against pathogens and parasites, thereby self-medicating and warding off infection.

Plant metabolites do not always serve as a foolproof defense against parasitoids. Sequestration may provide enemy-free space that favors parasitoid development, in which allelochemicals repel predators. Assuming there are no negative effects on parasitoid larvae development, allelochemicals can make hosts more appealing to parasitoids in that the host is less likely to be eaten (Gentry and Dyer 2002). Immune responses may be diminished as a result of an increased chemical load, as well (Gentry and Dyer 2002, Smilanich et al 2009). One can therefore not generalize that all sequestered secondary metabolites act as a defensive component against parasitoids. While specialist insects (those feeding on a single host species) may be able to feed upon such allelochemicals without experiencing problems, generalists tend to encounter more complications. In some cases, allelochemicals can hinder growth and, in turn, negatively
influence encapsulation rate (Bernays and Chapman 1994). For this reason, the immunologic response of encapsulation and the toxicity of allelochemicals, in regard to parasitoid defense, may not necessarily work in conjunction with one another. Secondary chemicals simply do not aid the insect in fighting off infection in many systems. It is therefore inconclusive whether or not secondary chemicals help in insect defenses against parasitoids.

Similarly, allelochemicals have various effects on the host encapsulation response depending on the species of caterpillar. In recent studies by Smilanich (2009, 2011), the influence of iridoid glycosides (IGs; a secondary compound) on melanization was analyzed in two species with differing responses. The IG-sequestering specialist species Junonia coenia (Nymphalidae) showed diminished encapsulation responses associated with higher levels of iridoid glycosides. Interestingly enough, while this was true when the caterpillar was fed plant diets, no relationship was established when it was fed an artificial diet containing iridoid glycosides (Smilanich 2008). In a follow up study, Smilanich studied the generalist species Grammia incorrpta (Arctiidae) in relation to how encapsulation was affected by diets of either low or high iridoid glycoside concentration. No variation in encapsulation was found in relation to the level of iridoid glycosides, yet lower levels of encapsulation were exhibited when the caterpillar was reared on an artificial diet (Smilanich 2009).

These studies suggest that sequestered allelochemicals may not play a universal role in herbivores. As shown above, a clear dichotomy can be established based on how IGs influence a specialist versus a generalist. Although caterpillars of both species are capable of sequestration as a defensive mechanism, sequestration may not contribute to an immune response against infection. Tradeoffs between sequestration and encapsulation may be involved, depending on the value of each tactic against a particular threat. While sequestration can ward off certain enemies,
it may diminish the encapsulation response and influence the defensive capability of an herbivore against other species (i.e. predators vs. parasitoids). Herbivores may utilize a different means of defense depending on the type of foe, and adjust their diet accordingly. Allelochemical intake may act independently from the encapsulation response, making these two separate lines of defense. No definite conclusion can be made about how secondary metabolites influence encapsulation. Simply put, the role sequestered allelochemicals play in encapsulation varies among different herbivores.

**Herbivore Immune Defenses Against Parasitoids: The Role of Herbivore Diet Choice or Self-medication**

Research regarding self-medication (the ability to choose a metabolite rich diet when infected) has been conducted principally by Dr. Singer and his collaborators (Singer et al 2002, 2003, 2004, 2009, Smilanich et al 2009, 2011). Much of the research regarding behavioral plasticity through self-medication has focused on bi-trophic systems without considering how a third link in the food chain could influence outcomes. Predators and parasitoids may both play a critical role in the dietary choices of their hosts (Bernays and Bright 1993, Bernays and Minkenberg 1997, Hagele and Rowell-Rahier 1999, Tikkanen et al 2000, Singer 2001). Work done involving a polyphagous (feeding on multiple diet types) woolly bear caterpillar, *Grammia geneura* (Arctiidae), has shown that this species sacrifices a more nutrient rich diet for one higher in toxicity when infected by a parasitoid (Singer 2002, 2004).

Singer (2004) has speculated that the evolution of herbivore diet has been driven by the enemy free space hypothesis and the physiological efficiency hypothesis. The enemy free space hypothesis states that the chief driver of an herbivore’s host plant choice is maximizing defense
against enemies. The physiological efficiency hypothesis, on the other hand, stresses natural selection of food nutritious quality driving the evolution of herbivore diet (Singer et al 2004). While there is no concrete evidence to support either of these hypotheses as the sole driver in diet choice evolution, Singer et al (2003) presented a fair amount of evidence supporting the enemy-free space hypothesis from studies of *Estigmene acrea* (Arctiidae); these caterpillars preferred to feed on plants which yielded superior defense rather than those which provided nutrients for optimal growth.

Further work on parasitized and non-parasitized *Platyprepia virginalis* (Arctiidae) caterpillars also emphasized a degree of self-medication through diet choice. Infected individuals increase their rate of survival by feeding on poison hemlock plants, and feed on this plant to a greater extent than their healthy counterparts (Karban and English-Loeb 1997). Unfortunately, the results of this study are ambiguous, as it remains unclear whether the change in host behavior is a behavioral adaptation by the host against parasitoids, or a result of direct behavioral manipulation by the parasitoid.

More direct and clear-cut evidence of behavior plasticity through self-medication has been demonstrated with the polyphagous caterpillar *Grammia incorrupta* (Arctiidae) (Singer et al 2009, Smilanich et al 2011). This work pinpoints the adaptive behavioral plasticity found within the caterpillar’s diet choice, as parasitized larvae given a choice of various artificial diets showed a higher ingestion of pyrrolizidine alkaloids relative to uninfected larvae (Singer et al 2009). Infected larvae sacrifice nutritionally rich artificial diets and consume more pyrrolizidine artificial diets than their healthy counterparts, clearly as a result of self-medication.

In subsequent studies (Smilanich et al 2011), *G. incorrupta* larvae were given a choice among three host plant species—two containing different allelochemicals (pyrrolizidine and
iridoid glycosides), and the other a nutritious plant with antioxidants (flavinoids and phenolics; nontoxic molecules that inhibit oxidation of other molecules and can be associated with immunity). Infection with early-stage wasp parasitoids resulted in the caterpillar consuming more of the plant species containing antioxidants in comparison to the other diets available. Interestingly, the dietary choice shifted in response to infection with a fly parasitoid; the caterpillars directed their feeding towards a plant of higher iridoid glycoside content (Smilanich et al 2011). Such a study demonstrates the plasticity generalist species of caterpillars possess in their diet selection, and stresses the necessity of considering multiple trophic links prior to understanding how a polyphagous species may feed. In this manner, different parasitoids may impose varying effects on the host immune system, which in turn call for different types of secondary chemicals to ward off infection.

**Manduca sexta**

Tri-trophic systems have been studied to a great extent in relation to how diet breadth and subsequent chemical composition influence insect herbivores’ parasitoids. A particular system of interest, and the one the present thesis experiment uses, is *Manduca sexta* (Sphingidae), more commonly known as the Tobacco Hornworm. Despite being a generalist, *M. sexta* is capable of feeding on tobacco plants containing the alkaloid nicotine (Thorpe and Barbosa 1986). Nicotine generally serves as a defensive toxin to ward off herbivores, yet *M. sexta* is capable of metabolizing and digesting it (larvae do not sequester the compound, rather it causes the hemolymph and internal organs to be toxic) (Barbosa et al 1986, 1988, 1991). *M. sexta* has a unique tolerance for high levels of nicotine; larvae are capable of consuming diets with up to 1-1.5% nicotine concentrations (on a wet weight basis) without experiencing any inhibition of
growth and development, and can ingest up to 20 mg of nicotine within 24 hours without experiencing toxicity-associated symptoms (Self et al 1964, Parr and Thurston 1972). Absorbing tissues such as the gut take in the nicotine, which is slowly metabolized (Snyder et al 1993). Nicotine therefore expresses no negative influence on the insect, but has a toxic effect on the hornworms’ parasitoid, *Cotesia congregata* (Thorpe & Barbosa 1986). When *M. sexta* infected with *C. congregata* were raised on diets with moderate amounts of nicotine, parasitoid larvae showed a significant decline in survival rate and levels of fitness (Thurston & Fox 1972, Thorpe & Barbosa 1986, Krischick et al 1988, Barbosa et al 1991). Trace amounts of nicotine in the 0.1% of wet weight range resulted in diminished larval survival rates of *C. congregata* raised in the *M. sexta* host compared to infected hosts raised on diets void of nicotine. Fewer larvae emerged from the caterpillar, and those that did emerge were less successful in pupation in hosts raised on nicotine diets (Thorpe & Barbosa 1986). Increasing nicotine concentrations above this 0.1% range have also been shown to correlate with a decline in parasitoid survival, although those above 2% of wet weight typically have an adverse effect on the caterpillar’s own growth and development (Thorpe & Barbosa 1986).

Multiple studies show the same results in that nicotine is deleterious towards *M. sexta’s* parasitoid. Further research confirms that higher concentrations of nicotine result in diminished survival for *C. congregata* (Barbosa 1986, 1991). While tri-trophic studies deal with many aspects of plant-insect-parasitoid systems, little research has been done on the ability of herbivore insects to express self-medication. Behaviorally, oviposition choice of parasitoids has been assessed in relation to egg-laying preference based on host diet, although the plasticity of the herbivorous caterpillar (the middle level in this tri-trophic system) in altering diet has been little studied (Charnov et al 1985, Nieminen et al 2004, Lampert and Bowers 2010, Singer et al
2009). There is in turn a gap in the research regarding tri-trophic interactions. The direct influence of allelochemicals on parasitoid infection has been investigated in a number of systems, yet diet choice and self-medication (i.e., behavioral plasticity) associated with middle-trophic (herbivorous) generalists is relatively unexplored (Barbosa and Letourneau 1988, Singer 2009).

There is a gap in tri-trophic research regarding the ability of *M. sexta* to self-medicate, as well. Work on parasitized *M. sexta* diet choice focused primarily on nutrient intake rather than on nicotine. When given choices of protein and carbohydrate diets, uninfected *M. sexta* consumed the nutrients in a rough 1:1 ratio (of protein to carbohydrates) for optimal growth. In contrast, larvae parasitized with *C. congregata* exhibited no discrete 1:1 protein/carbohydrate intake distinction and fed randomly (Thompson & Redak 2005, 2008). Thompson and Redak’s work thus shows a difference in consumption choices made by infected *M. sexta*, and altered consumption choice should continue with nicotine offered diets given the alkaloid’s role in parasitoid defense.

Increased levels of sequestered nicotine can be utilized for defensive purposes, but is there any real evidence for self-medication in the species, as discussed in the above scenarios? Knowing that the species is polyphagous and that nicotine diminishes the survival of its parasitoid *C. congregata*, can infected *M. sexta* discern between a basic nutritious diet and one that is toxic? Thompson & Redak (2005) established that the ability to intake nutrients in a 1:1 ratio is hindered in parasitized larvae, indicating behavioral plasticity in diet choice between healthy and parasitized caterpillars. In addition, Barbosa (et al 1986, 1991, Thorpe and Barbosa 1986) highlighted the role of sequestered nicotine in decreasing parasitoid fitness. Based on this gap in our knowledge, I made the decision to analyze the behavioral plasticity of *M. sexta* in
response to parasitism; can this species engage in self-medication through means of dietary alterations when its parasitoid imposes a threat?

As other generalist species showed the ability to alter their diet in response to infection, I predicted that *M. sexta* may do the same (Karban et al. 1997, Singer et al. 2003, 2004, 2009). In my experiments, fifth instar *M. sexta* caterpillars were offered a choice among various nicotine-rich diets and a standard artificial diet and analyzed for evidence of self-medication. After being injected with glass silica beads to simulate an infection, the caterpillars were given the choice between these varying diets, and later they were dissected to determine whether or not the nicotine diets were in fact enhancing the immunologic defense of the caterpillar by increasing the rate of encapsulation. I predicted that injected *M. sexta* would choose diets of higher toxicity and subsequently select diets of the greatest toxicity concentration. Previous research showed that caterpillars fed with higher nicotine concentrations tend to have greater survival against parasitism (Thorpe and Barbosa 1986, Barbosa 1988). Increasing nicotine concentration in turn diminishes parasitoid fitness. Because the encapsulation response combats foreign invaders (or in this case, the silica beads as parasitoid eggs), as outlined above, I also hypothesized that higher concentrations of nicotine would be positively correlated with the rate of encapsulation.
MATERIALS & METHODS

Insect Rearing

*Manduca sexta* eggs were obtained from Carolina Biological. They were reared in growth chambers with a photoperiod of 14hr day: 10 night, and day-night temperatures of 27°C/22°C. A colony was established to be used in the experimental procedure. *Manduca sexta* typically feed on a host of dietary plants, generally tobacco, tomato, and several *Datura* species (Barbosa et al 1986). The artificial diet is wheat germ based and specified for *Manduca sexta*, and thus contains all essential nutrients required for successful growth. The caterpillars grow rapidly through five molting stages (instars). Immediately following a molt, *M. sexta* will cease eating and become temporarily lethargic for several hours. Upon reaching their final instar, the caterpillars enter a “wandering stage” just prior to pupation, in which they move around frantically, stampeding over their food. In the pupation process, the caterpillars tend to shrink drastically in size, and last in this stage roughly 18 days under laboratory conditions. Following pupation, emerged moths will mate and lay their eggs on host plants (in this case, a tomato plant was used).

Colony maintenance

Groups of caterpillars (generally ~30) were raised until pupation for the sole purpose of establishing a colony. Groups of 15-20 caterpillars were placed in a petri dish and fed artificial diet (Bioserv *Manduca sexta* diet). Upon reaching fifth instar, groups were further split into subgroups of five individuals and placed in crispers to prevent overcrowding as well as overfeeding. Once pupated, caterpillars were moved into a butterfly net within the same growth chamber. After hatching, a tomato plant (purchased from Home Depot) was placed within the
cage, as well as sponges containing honey water for a source of food. Honey water was prepared by first boiling water, then adding Kroger Brand honey. This was stirred and allowed to simmer, then placed in the refrigerator to cool down. Eggs laid by the sphinx moths on the tomato plants were collected and placed in petri dishes until they hatched.

*Treatment caterpillar rearing prior to diet choice experiments*

Caterpillars to be used for experimentation were placed in petri dishes and raised on an artificial diet (Bioserv *Manduca sexta* diet) void of nicotine up until their third instar. Upon reaching this stage, caterpillars were fed varying nicotine diets (i.e. standard artificial diet with either 0.1-0.5%, 0.1-1%, or 0.5-1% wet weight nicotine) daily pending their future choice test diet (see below). This early exposure to variable nicotine concentration diets was done to ensure no dietary preference prior to the choice test stage.

*Nicotine Diet Preparation*

Artificial diets prepared for experimental *M. sexta* caterpillars were obtained from Bioserv. Liquid nicotine 0.5% solution was obtained from the same company. In order to add nicotine to the artificial diet, the wet weight of the diet mixture was first taken. Nicotine would then be added as a percentage of this artificial diet wet weight, in quantities of 0.1%, 0.5%, and 1%. This percentage was calculated into mL of the nicotine solution, and added into the diet with a pipette prior to letting the diet cool down. In wild tobacco plant populations, nicotine concentrations generally range between 0.3% and 2% of the wet weight, however, percentages on the higher end of the spectrum (1.5-2%) have been shown to hinder growth and development of the caterpillar (Barbosa et al 1991, Snyder et al 1994). Therefore, three contrasting nicotine
concentrations were used in the diets: 0.1% = low, 0.5% = moderate and 1% = high. In this way, chosen nicotine treatment diets represent the range observed in natural populations adequately, but excluded the higher concentrations levels detrimental for *M. sexta* performance.

**Caterpillar Injections**

Upon reaching their fifth instar, caterpillars were injected with silica beads (40-120 um diameter) in order to measure their immune response. The beads serve as a simulation for a parasitoid infection, and take the role of foreign eggs being injected into the hemolymph. Beads used for injection were obtained from Sigma-Aldrich (St. Louis, Missouri, USA), also known as DEAE Sephadex-A25 silica chromatography beads. These were dyed with a 0.1% solution Congo Red Dye and allowed to completely dry. They were stored in insect Ringer’s solution prior to use (see Smilanich et al 2009). Prior to injection, the weight of the insect was taken as an initial measurement of health, and to be compared to the insect’s weight after the choice test was concluded. Weighed larvae were exposed to CO₂ for temporary paralysis. Under a dissecting microscope, beads were injected using a combination of a Hamilton 7000 syringe, a microliter pipette with disposable tips, and modified glass needles handmade from Pasteur pipettes. An average of 5 µl ringer solution containing 20-50 beads were injected into the base of the third proleg of the 5th instar larvae.

**Choice Tests**

After injections, caterpillars were given a small amount of food for 24 hours to ensure a feeding response. Following these first 24hrs post-injection, injected and non-injected caterpillars were given a choice of two artificial diets with and without different nicotine
concentrations. Discs of food were then used from the two diets being compared. These discs were weighed to the nearest 0.001g, and then placed within 4 cm of each other in a circular petri dish. The following dietary choices were compared, with ten injected and non-injected caterpillars being used in each diet test comparison (total of 60 caterpillars):

- low nicotine vs. control (0.1% vs. control)
- moderate nicotine vs. control (0.5% vs. control)
- high nicotine vs. control (1% vs. control)
- high vs. low nicotine (1% vs. 0.1%)
- high vs. moderate nicotine (1% vs. 0.5%)
- low vs. moderate nicotine (0.1% vs. 0.5%)

After the first six hours, the diet discs were removed from the petri dishes and weighed, as to obtain the total amount consumed. New diet discs were then weighed to replace the old. After a second six hour interval, these were again collected and weighed. Thus, the overall mass of artificial diet eaten was assessed twice over a 12 hour period, in six hour intervals. This small window of time helped to ensure that the final weight of the diet was primarily due to consumption rather than weight loss from water evaporation. After the choice tests, the caterpillar was weighed for a final time, then placed in a freezer for further analysis. This same process was done for an additional 60 control caterpillars that were not injected (control group), to ensure any preference in nicotine was due to injection.

*Caterpillar Dissection & Bead Analysis*

Once the choice tests were completed, frozen caterpillars needed to be dissected in order to retrieve the beads for additional analysis (see Smilanich et al 2009 for detailed methodology).
Briefly using a dissecting microscope, recovered beads from dissected caterpillar tissues were transferred via pipette into small Eppendorf vials and stored in ringer’s solution. Subsequently, beads were photographed with a dissecting microscope mounted with a digital camera (Carl Zeiss Discovery V.8, AXIOCAM software, Oberkochen, Germany). Pictures were taken for control beads (non-injected beads) and for all retrieved beads per caterpillar (only a small subset of caterpillars lack retrieved beads, less than or equal to 2 individuals per treatment). All photographs were taken with maximum lighting and at 80X magnification. A red value (hereafter r-value) per bead could then be obtained through the use of Adobe Photoshop (CS5, Adobe Systems Inc., San Jose, CA, USA). This r-value is a measure of the degree of red saturation in the bead, ranging from 0 (pure gray) to 255 (pure red). This in turn reflects the rate of encapsulation on each bead and is furthermore a proportionate measure of the caterpillar’s immune response—the more red the bead, the lower the level of melanization (see Figure 1).

Reference photographs were first taken for unused beads to establish a maximum r-value. Said value could then be used as a reference against the melanized beads to determine the effectiveness of the encapsulation response. An average r-value was then taken for each caterpillar using all beads found within each individual larvae. This could finally be converted into a percentage rate of melanization, \([1-(r\text{-value}/\text{maximum } r\text{-value})]\), done in order to simplify interpretation. Caterpillars that showed a significant preference for nicotine diets were analyzed more extensively, to determine whether or not the consumption of nicotine did in fact correlate with the degree of melanization found in the beads.

**Statistical Analyses**
Chi square analyses were performed to determine a difference in choice among injected and non-injected caterpillars. First, a chi square test was performed based on the treatment (injected) and control (non-injected groups). In this manner, a proportion was determined based on the number of caterpillars that chose the more nicotine-enriched diet over the entire 12 hour feeding period (a number out of ten for each of the six groups). Individual chi square analyses were then performed for each of the six pair-choice tests (i.e., a single 2x2 chi square comparing diet selection for treatment & control caterpillars). To further analyze whether or not caterpillars selected a higher toxic diet, a two way ANOVA was performed to analyze the proportion of the higher nicotine diet consumed in relation to treatment (infected or control caterpillars) and by choice test. Independently of their choice and to determine if feeding was hindered by any means due to infection, an additional two way ANOVA was performed to assess how total consumption (i.e., total mg consumed in 12hr) varied as a function of diet (six paired-choices) and caterpillar (injected vs. non-injected) treatment.

To analyze changes in melanization rate as a function of diet treatment, a one-way ANCOVA was first run to analyze the influence of pair-choice tests on melanization, using the number of retrieved beads as a covariate. If treatment effect was significant, Bonferroni post-hoc test were used to detect differences among all paired-choice tests. Finally, linear regression was utilized for more concrete analysis to determine the relationship between nicotine consumption and melanization rate. To do so, the difference between the higher nicotine diet consumption and the lower nicotine diet consumption was first taken to scale the independent variable. In this way, positive values indicated a higher nicotine consumption, while negative values meant the opposite.
Figure 1-Top picture shows beads that have not undergone melanization and were used as reference (i.e. non-injected beads). Bottom picture shows retrieved beads from injected caterpillars that experienced a heavy amount of melanization, indicated by their lack of red color.
RESULTS

Diet Choice

There was a significant difference in dietary choice between injected and non-injected caterpillars ($\chi^2 = 47.9, \text{df}=5, P<0.0001$). Injected caterpillars did not make the same diet choices as their non-injected counterparts. Chi square analyses were performed for each choice group to determine whether a difference existed among the injected and control caterpillars for each of the six treatments. This revealed no overall trend, with rather different results for each treatment. In comparison to the control caterpillars, the injected caterpillars chose diets of greater nicotine concentration in the 0.1% vs. control and 0.5% vs. control tests at significantly greater percentages. There were no significant differences in diet choice between infected/control groups for the other diets with the exception of the 1% vs. 0.1% choice, in which the 1% diet was preferred by the control caterpillars and was significantly different than the preference of those injected (see Figure 2). It was expected that injected caterpillars would choose the higher nicotine diet at a significantly higher proportion than those that were healthy, but this was not always the case. However, the results of the 0.1% vs. control and 0.5% vs. control showed a significant difference in the proportion of caterpillars selecting the higher nicotine diet, supporting what was predicted.

To further elaborate on the degree of choice amongst treatment groups, a two-way ANOVA test assessed whether the proportion of the more toxic of the two diets consumed varied in relation to the treatment type (injected vs. non-injected) and the paired-choice test options. Injected and non-injected caterpillars did not exhibit a significant difference in the consumption of the diet with the higher nicotine content, expressed as a proportion of the total consumption of
the two diet choices ($F_{1,109}=0.249, P=0.619$). However, the proportion of the more highly toxic diet consumed varied significantly among the various choice tests ($F_{5,109}=5.605, P<0.0001$), indicating that there was no uniform preference amongst diets when caterpillars were presented with a choice. Rather, consumption in dietary selection differed depending on the choices that were offered. The interaction of these two independent variables, the proportion of the greater toxic diet consumed between groups and treatments, was significant ($F_{5,109}=2.342, P=0.046$) (see Table 1). Caterpillars make some degree of a choice when it comes to varying diets, although there is no distinct pattern separating those infected and their healthy counterparts. In general, although there are differences in the consumption patterns between groups, consumption preference is not always based on a highly toxic diet.

**Melanization**

A two-way ANOVA was performed to determine whether food consumption was hindered as a result of infection, via comparing the total amount of food eaten as a function of caterpillar treatment (i.e. injected vs. non-injected) and paired-choice test. There was a significant difference between injected and control caterpillars in the total amount of food consumed ($F_{1,109}=4.37, P=0.04$) as well as for the difference amongst the six paired-choice tests ($F_{5,109}=3.01, P=0.014$). In the interaction of these variables in relation to total consumption, on the other hand, there was only marginal significance ($F_{5,109}=2.21, P=0.06$) (see Table 1 & Figure 3).

For the relationship between caterpillar diet choice and melanization response, the one-way ANCOVA revealed no significant influence of the number of beads injected into the caterpillar on the rate of melanization ($F_{1,47}=2.57, P=0.116$). Melanization was significantly
different among the six different choice tests ($F_{5,47}=6.04, P<0.0001$) (see Figure 4). Finally, linear regression analysis was used to determine whether dietary choice was associated with the rate of melanization, which was predicted to show a positive linear relationship (i.e. higher nicotine concentrations would bolster the encapsulation response). The results did not support this prediction, as the regressions showed little relationship between caterpillar consumption of higher nicotine diets and encapsulation response. Of the six groups, only the 1% vs. 0.5% choice test showed a marginally significant positive correlation with the amount of the higher nicotine diet consumed (1% in this case) increasing the rate of melanization ($R^2=.496, P=.051$). Aside from this, there was no other significant correlation between nicotine consumption and melanization, essentially indicating no relationship between nicotine concentration and the efficacy of the encapsulation response ($P>0.05$) (see Figure 5).
Figure 2-Diet preference amongst choice tests based on injected vs. non-injected (control) caterpillars

*Chi square probability indicated with stars, with more stars indicating a lower probability that the results were due to chance (and thus higher significance). Proportions (dependent variable) were determined based on the number of caterpillars consuming the higher nicotine diet choice over the total 12 hour period, and is therefore a number out of 10. N=10 for each treatment.
Table 1-Statistical summary of two-way ANOVA for choice test analysis

<table>
<thead>
<tr>
<th>SOURCE OF VARIATION</th>
<th>TOTAL CONSUMPTION</th>
<th>PROPORTION OF HIGHER TOXIC DIET CONSUMED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>df</td>
</tr>
<tr>
<td>Treatment (Injected V control)</td>
<td>4.37</td>
<td>1,109</td>
</tr>
<tr>
<td>Choice Test</td>
<td>3.01</td>
<td>5,109</td>
</tr>
<tr>
<td>Interaction</td>
<td>2.21</td>
<td>5,109</td>
</tr>
</tbody>
</table>

*Statistical summary for two way ANOVA tests. The total amount of food consumed was analyzed between injected and noninjected caterpillars to determine if infection altered the amount of consumption. This was also done between the six different choice tests, and finally with an interaction amongst all variables. There was a significant difference between treatments (p=0.04) and amongst the six choice tests (p=0.14), but marginal significance with the interaction of the independent variables. This data was interpreted in regards to how melanization and food intake interact. As the caterpillars were given two dietary choices in the choice tests, the ANOVA also analyzed consumption via the proportion of the diet with the higher nicotine concentration in relation to the total amount consumed. Again, the ANOVA analyzed whether there were differences between injected and noninjected caterpillars in how much more of the (greater) toxic diet was eaten. There was no significant difference in the proportion of the more toxic diet being consumed between treatments (p=.619). This was done for the differences among the six choice tests and for the interaction among the variables, showing significant results (p=0, p=0.46, respectively).
Figure 3-Average total consumption amongst choice tests and groups, over 12 hour period
*Melanization is measured as a proportion, in which the r-values of the beads retrieved were averaged and compared to the reference bead’s r-value (beads subject to no melanization). This proportion was then averaged amongst the caterpillars in each choice test.
Figure 5—Linear Regression analysis between diet consumption and melanization rate

1% V 0.5%

\[ y = 0.186x + 0.3436 \]
\[ R^2 = 0.4962 \]
\[ p = .051 \]

1% V 0.1%

\[ y = 0.2921x + 0.4556 \]
\[ R^2 = 0.0459 \]
\[ p = .552 \]

1% V Control

\[ y = 0.0469x + 0.6391 \]
\[ R^2 = 0.1625 \]
\[ p = .248 \]

0.5% V Control

\[ y = 0.0194x + 0.5055 \]
\[ R^2 = 0.61 \]
\[ p = .446 \]

0.1% V Control

\[ y = -0.1863x + 0.6367 \]
\[ R^2 = 0.6339 \]
\[ p = .663 \]

0.5% V 0.1%

\[ y = 0.0684x + 0.0806 \]
\[ R^2 = 0.1794 \]
\[ p = .223 \]

Consumption Difference (High Nicotine–Low Nicotine)

*Consumption difference was determined by subtracting the total consumption of diet B (the diet of lower concentration or no nicotine) from diet A (diet of higher nicotine concentration). This produced a scale value for the independent variable (consumption). In this manner, a more positive value on the independent axis signifies a higher consumption, and in turn a higher preference for, a diet of greater toxicity. Each data point represents a caterpillar from within that specific choice test. Caterpillars whose beads could not be retrieved were excluded from the analysis. Melanization for each individual caterpillar was determined as a proportion of the r-value of the beads retrieved in relation to the r-value of the reference beads (who experienced no encapsulation). This was subsequently averaged to obtain one rate of melanization for each caterpillar.
DISCUSSION

The results obtained here demonstrate there is some behavioral plasticity in the dietary choices caterpillars make in response to a simulated parasitoid infection. Caterpillar treatment altered larval food choice behavior. Injected and control caterpillars also made different choices with respect to total food consumption.

Both these findings indicate that injected caterpillars are able to self-medicate to a certain extent. It appears that nicotine, at low concentrations, drives diet choice and self medication. However, this pattern does not continue at higher nicotine concentrations. One may speculate that these greater concentrations are toxic to the caterpillar, yet higher nicotine concentrations (i.e., 1%) have been shown to impede parasitoid survival without hindering caterpillar growth (parasitoid larvae fail to emerge from the host more in caterpillars fed these higher concentrations) (Thorpe & Barbosa 1986, Barbosa 1988). It is possible that the 0.1% and 0.5% concentrations are sufficient in warding off infection, and higher concentrations are above a threshold of palatability. More highly toxic diets may not actually provide as large an improvement in parasitoid defense as anticipated, and thus there could be a toxic range.

It is important to note limitations of the type of statistical analysis (chi square) in the present study. Regarding the proportion choosing the diet of higher toxicity (Figure 2), determining whether a caterpillar preferred the more toxic diet being offered was no more than a categorical “yes” or “no” based on which of the two offered diets was consumed more. The overall difference between the consumption of the two diets for each caterpillar may have been rather small, thereby producing misleading results. This problem, however, was ameliorated through further analysis of the two-way ANOVA regarding the proportion of the more toxic diet
consumed. These findings can then be taken together with the chi square findings to emphasize that injected and control caterpillars make different consumption choices and thus express behavioral plasticity in their diet choices. Nicotine may account for some amount of this choice to support self-medication in *M. sexta* (evidenced in the 0.1% vs. control and 0.5% vs. control tests), but it does not result in a trend of choosing higher concentrations, indicating a potential threshold above which concentration differences may not be detected.

A potential mechanism causing differences in diet choice between the two groups may be a deficiency in chemoreceptors (organs that pick up chemical cues from environment) in the caterpillars. This is highlighted in Thompson & Redak’s work (2005, 2008). Their research focused on protein/carbohydrate intake in diet in healthy and parasitized *M. sexta*. While healthy individuals regulated dietary intake to maintain a 1:1 ratio of protein to carbohydrates for optimal growth/development, infected caterpillars chose food randomly. Thompson & Redak (2005, 2008) established that regulation in feeding behavior, at least with respect to nutrient intake, involves a relationship amongst peripheral chemoreception, nutrient-allelochemical interactions, and feedback from blood-chemistry. Learning and memory plays a role in choice as well (Thompson & Redak 2008). The self medication process may not be as simple as choosing a diet of higher toxicity given the several variables that influence consumption choice.

One limitation of my study is the viability of the artificial diet. In a natural setting, *M. sexta* feeds on plants rather than synthetic diets. It is unknown how effective chemoreception is in detecting nicotine concentrations. In a natural setting, chemoreception may cue in on plant components absent in the artificial diet. Chemoreception is tied to multiple gustatory and olfactory organs in the larvae, which when surgically removed, have been shown to thwart the insect’s ability to choose nutritious food (de Boer & Hanson 2008). De Boer and Hanson (1987)
analyzed consumption preference of artificial diets containing various chemicals, such as tomatine, tomatidine, atropine and nicotine. Tomatine was found to slightly increase feeding, while the other chemicals (nicotine) did not induce a dietary preference (de Boer & Hanson 1987). It is possible that nicotine alone does not stimulate chemoreception adequately, but rather works in tandem with other chemicals specific to the tobacco plant. Alternatively, other plant chemicals may play a role in the immune response against parasitoids (such as antioxidants). In future studies, it would be interesting to control for several plant chemicals in diets offered to infected *M. sexta*.

Another interesting factor to note is the significant difference found in total food consumption between injected and healthy caterpillars. This difference in consumption may be related to the cost of feeding versus the energy requirements associated with the insect’s immune response. The immune system requires a substantial amount of energy to produce the encapsulation response, and may divert energy away from foraging and feeding. However, findings from other studies suggest that starvation can hinder immunity. *Bombus terrestris* (Apidae), when starved, show a diminished ability to encapsulate micro-latex beads, and starvation in 5th instar *Epirrita autumna* (Geometridae) elicits the same response (Schmid-Hempel et al 1998, Yang et al 2007). Other studies indicate trade-offs in energy use between behavior and encapsulation (Gustafsson et al 1994, Sheldon and Verhulst 1996, Moret and Schmid-Hempel 2000). Infection impairs reproductive effort in a number of insects, potentially due to encapsulation diverting reproduction’s energy demands (Hurd 1995, 2001).

Intense infection may simply weaken caterpillars and decrease their desire to eat. The process of injecting the beads into the caterpillar resulted in hemolymph loss from caterpillars. Some larvae may have healed their wounds better than in others, and those suffering more from
the injection process may have lacked the strength to eat. Further experimentation is needed to test how much damage was caused to the caterpillars and whether this problem influenced their feeding preference.

The melanization results in this study revealed much about the role of allelochemicals in the encapsulation response. The number of beads did not correlate with the rate of melanization. After injection, only a portion of the beads originally injected were recovered. Beads that may have played a role in influencing the encapsulation response may not have been found in the dissection and thus affected the analysis.

Was encapsulation associated with the toxicity of the diet being consumed? The results differ from what was originally expected, i.e. that higher toxicity would be correlated with larger rates of melanization. The initial one-way ANCOVA provided promising results. Essentially, the test showed significant differences in melanization among the choice tests. However, post-hoc tests demonstrated that only one treatment (0.5% vs. 0.1%) showed a significantly lower melanization rate from all other choice tests (Figure 4). No patterns in melanization were demonstrated among the different choice test groups.

If nicotine concentration in diet is not associated with the extent of melanization, then nicotine does not have an influence in the caterpillar’s immune system, which may explain the lack of a pattern in consumption. There may be a two-way defense system in which melanization and nicotine toxicity act independently. Melanization could be the initial response to fighting infection, as it has been established as the basis of the immune response among all insects (Godfray et al 1994). Nicotine, on the other hand, makes the hemolymph and internal organs of the caterpillar toxic (Thorpe & Barbosa 1986). If a parasitoid were able to survive the encapsulation response, it would naturally utilize the caterpillar as a food source. This would
then prove deleterious given the toxicity associated with the host insect. Another possibility regarding the role of ingesting nicotine relates to the purpose by which the mechanism has evolved. While Barbosa’s (Thorpe & Barbosa 1986, 1986, 1988, 1991) work has emphasized how nicotine diminishes the burden of the parasitoid infection, the ability to metabolize nicotine may not have evolved for this specific purpose. In other words, *M. sexta* may have evolved the ability to digest nicotine as a method of increasing their diet breadth, and doing so later proved useful in fighting off infection due to the alkaloid’s toxicity. The defense may have also evolved principally for predators rather than parasitoids, thereby explaining why no relationship between encapsulation and nicotine concentration was found.

There were a number of other limitations in this study. The sample size was relatively low. Groups of 10 caterpillars were optimal given the time frame of this work, yet if more time were allocated, increasing the sample size to 15 or 20 caterpillars per choice test would certainly have strengthened the results. Another important aspect to consider is the nature of the diet offered. Artificial diets are not found in *M. sexta’s* natural setting, and there may be attributes of a natural plant diet that were not accounted for, as mentioned. Plants may influence caterpillar chemoreception differently, or other allelochemicals in plants may affect the diet preference of the larvae. Utilizing actual plants or leaves would have been ideal for this study, albeit impractical given the inability to control for nicotine concentrations within tobacco plants.

It may also be ideal to offer more distinct trade-offs in the artificial diets, as utilized in much of Singer’s work (2003, 2004, 2009). In many of his choice tests, Singer offers a diet of high toxicity that lacks nutrition, as well as a diet of high nutritious content with no toxicity (Singer 2003, 2004). Having choices like these would create a definite distinction between the choices being made—that is when infected, is the caterpillar choosing a diet to optimize growth
or defense? Such choices may also elicit a stronger behavioral choice, as each diet provides clear advantages and disadvantages that make precise diet choice more essential. My study had two diets of equal nutritional value (the only difference being that nicotine was added), which could have limited the results. Some of Singer’s work indicates that infected generalists choose the higher toxic diet offered, indicating a trade-off between defense and nutrition/growth (2004, 2009), and it would certainly be interesting to see how work with M. sexta choice compares when offering these same distinct diet options.

The analysis regarding melanization could also be refined. I originally planned to do an additional study using non-choice tests, in which injected caterpillars would be reared on a single diet. A comparison could then have been made among groups to verify if increasing nicotine concentration was associated with a stronger melanization response and a direct relationship between diet and melanization could be assessed. As it stands, the linear regression performed in this study has its flaws. As both diets were consumed, both then have some amount of influence, which can alter the rate of melanization to varying degrees (ie, certain caterpillars simply ate more than others). Non-choice tests would alleviate these concerns in making the relationship between variables (melanization and nicotine concentration) more precise.

This study ultimately provides unique insight into the role of behavioral plasticity within tri-trophic interactions. M. sexta use of nicotine to fight against a parasitoid injection was previously well documented, yet the ability of the larvae to make choices in diet pertaining to self-medication was unclear. This species is capable of discerning diets and selecting one of greater toxicity as a method of defense, to some degree. As the study illuminates, injected M. sexta make different consumption choices than healthy larvae. Nicotine may account for these differences in lower amounts (0.1%, 0.5%), but no pattern was established suggesting a potential
response threshold for higher nicotine concentrations. Nicotine may not be the sole component driving consumption plasticity in this caterpillar. Healthy larvae ate more than their injected counterparts as well, which may be associated with energy demands of the encapsulation process. As higher nicotine consumption did not correlate with melanization, no relationship was established between allelochemicals and encapsulation. Caterpillars fed from both diets being offered; a series of nonchoice tests would help to clarify the relationship between nicotine and melanization. While the original hypotheses derived were not completely supported, this study has highlighted the behavioral plasticity at play within *M. sexta* and their tri-trophic interactions that will certainly be analyzed further as behavioral entomology continues develop.
References


Freeman, B.C. and G.A. Beattie. 2008. An overview of plant defenses against pathogens and herbivores. The Plant Health Instructor. DOI: 10.10.94.


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