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Effects of Ingested Secondary Metabolites on the Immune Response of a Polyphagous Caterpillar *Grammia incorrupta*

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Abstract We considered the effects of plant secondary metabolites on the immune response, a key physiological defense of herbivores against pathogens and parasitoids. We tested the effect of host plant species and ingested iridoid glycosides on the immune response of the grazing, polyphagous caterpillar, *Grammia incorrupta* (Arctiidae). Individuals of *G. incorrupta* were fed either one of three plant diets with varying secondary metabolites, or an artificial diet with high or low concentrations of iridoid glycosides. An immune challenge was presented, followed by measurement of the encapsulation response. We failed to detect a significant difference in the immune response of *G. incorrupta* feeding on diets with varying concentrations of iridoid glycosides, or feeding on different host plants. However, the immune response was lower in caterpillars consuming the artificial diet compared to those consuming the plant diets. When caterpillar performance was measured, pupal weights were lower when caterpillars ingested high concentrations of iridoid glycosides due to a decrease in feeding efficiency. Overall, individuals of *G. incorrupta* that consumed different plant diets exhibited a high immune response with low variation. We conclude that the immune response of *G. incorrupta* is adapted to feeding on a variety of plants, which may contribute to the maintenance of this caterpillar's polyphagous habit.

Key Words Immune system · Encapsulation · Diet breadth · Iridoid glycosides · Sequestration · Secondary chemistry · Parasitism · Insect · Arctiidae

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Introduction

Insect herbivores rely on host plants to meet daily nutritional requirements needed for proper metabolism and growth. Beyond these basic physiological functions, host plant secondary metabolites may help structure herbivore interactions with natural enemies (Turlings et al., 1990; Dyer, 1995; Smilanich et al., 2009a). Detoxification or accumulation of these metabolites may incur physiological costs (Camara, 1997; Despres et al., 2007), leading to ecological and evolutionary consequences (Bowers, 1992; Nishida, 2002). A variety of studies have demonstrated that ingestion and sequestration of secondary metabolites renders herbivores unpalatable to predators such as birds, wasps, and ants (e.g., Bowers, 1992; Dyer, 1997; Vencl et al., 2005), thus providing protection from predators. However, ingestion of secondary metabolites also can increase susceptibility to natural enemies such as parasitoids because (1) chemically-defended herbivore hosts provide enemy-free space for parasitoid development (Gentry and Dyer, 2002), or (2) sequestration and/or detoxification of secondary metabolites alters herbivore immune responses (Smilanich et al., 2009a).

The insect immune response is an effective defense against parasitoids and pathogens (Beckage, 2008; Smilanich et al., 2009b). In this paper, we investigated

whether diet causes variation in the immune response, which could lead to vulnerability to parasitism. In particular, we presented an immune challenge to polyphagous, sequestering caterpillars that were consuming different levels of secondary metabolites.

The immune response consists of three main components: phagocytosis, nodule formation, and encapsulation (Carton et al., 2008). Encapsulation is the concerted action in which specialized immune cells (hemocytes) adhere to a large foreign body, such as a parasitoid or parasitoid egg, and build layers of cells that eventually are melanized. The cytotoxic effect of melanization and asphyxiation from encapsulation both contribute to killing the pathogen (Carton et al., 2008). Host plant chemistry can have negative, positive, or neutral effects on the immune response (Klemola et al., 2007, 2008; Bukovinszky et al., 2009; Smilanich et al., 2009a). Plant nutrients (e.g., protein) can enhance encapsulation and lysozyme-like antibacterial activity, but may have little effect on other immune system components like phenoloxidase activity (Lee et al., 2008; Povey et al., 2009). Ingestion and sequestration of some plant secondary metabolites (e.g., iridoid glycosides) can weaken the immune response by directly interfering with the melanization component of immunity (Smilanich et al., 2009a), whereas other metabolites (e.g., hydrolyzable tannins) may act indirectly on immunity via reductions in herbivore performance (Haviola et al., 2007; Yang et al., 2008). In contrast, carotenoids, flavonoids, and synthetic chemicals may enhance immunity, perhaps by quenching harmful oxygen species (Babin et al., 2010).

Given the possibility that plant chemistry may have a negative effect on the immune response, herbivores face the dual challenge of digesting and assimilating plants of variable quality and at the same time maintaining the immune response in order to evade parasitoids. Here, we used experiments with the grazing generalist caterpillar, *Grammia incorrupta* Hy. Edwards (Arctiidae), to address the following questions: (1) what are the effects of three host plant species with different nutrient and secondary chemical profiles on the immune response, and (2) what are the effects of iridoid glycosides (IGs) added at different levels to a standard diet on the immune response? *Grammia incorrupta* larvae sequester pyrrolizidine alkaloids (0.3 to 2.7 mg/g dry wgt, Hartmann et al., 2005) and low levels of IGs (Bowers, 2009).

To address these questions, larvae were reared on three different host plants: *Plantago major* (Plantaginaceae), which has low concentrations of one IG, aucubin (Barton and Bowers, 2006); *P. lanceolata*, which has high concentrations of two IGs, aucubin and catalpol (Bowers and Stamp, 1992), and *Taraxacum officinale* (Asteraceae), which has no IGs, but does contain flavonoids, phenolics, and sesquiterpenes (Schutz et al., 2006). Larvae were presented with an immune challenge and the immune response was analyzed. To more specifically examine the effects of IGs, larvae were fed artificial diets that contained extracted IGs (aucubin and catalpol) and were then presented with an immune challenge. For the artificial diet experiment, we also measured feeding efficiency in order to test for a relationship between food utilization and the immune response. Based upon previous work (Smilanich et al., 2009a), we predicted that ingestion of IGs would negatively affect the immune response, and that host plants with the highest content of IGs would be detrimental to the immune response.

Methods and Materials

Experimental Overview Experiments with *G. incorrupta* were performed at Tulane University, New Orleans, LA, USA during fall 2006, and at University of Colorado, Boulder, CO, USA during fall 2008. Egg masses were obtained from an established lab colony at Wesleyan University, Middletown, CT, USA (courtesy of M.S. Singer). The first experiment tested the effects of host plant species on the immune response. The second experiment tested the effects of high and low concentrations of IGs on the immune response and feeding efficiency by using an artificial diet with added IG extract.

Immune Assay To measure the immune response, *G. incorrupta* caterpillars were injected with Sephadex beads as a proxy for parasitization (Lavine and Beckage, 1996; Rantala and Roff, 2007; Smilanich et al., 2009a,b). The beads (Sigma-Aldrich, Sephadex A25, 40–120 μm) were dyed red using 0.1% Congo red and were suspended in Ringer's solution so that 5–10 beads could be injected into the base of the third proleg (Lavine and Beckage, 1996). Caterpillars then were returned to their test diets and after 24 hr were freeze-killed. Caterpillars were dissected in 95% methanol, and beads were photographed with a camera mounted on a dissection microscope focused at 80× magnification (Carl Zeiss Discovery V.8, AxioVision software). Since the beads were dyed red before injecting them into the caterpillars, we quantitated melanization by measuring the red value (r-value, Adobe Photoshop ver. 6.0), a scale ranging from 0–255, where 0 = pure gray, and 255 = pure red, for each bead. The mean r-value for all the beads from each caterpillar was statistically compared between treatments, using ANOVA with r-value as the dependent variable and treatment as the independent variable. The r-value was transformed into percent melanization [1−(r-value/maximum r-value)] where the maximum r-value is 255 for a non-injected, unmelanized bead.

Experiments with Three Host Plants Caterpillars were randomly assigned to one of three diets: *T. officinale*, *P. lanceolata,* or *P. major*. *Plantago major* contains only one iridoid glycoside, aucubin, at relatively low levels (0.2–1.0% dry weight) (Barton and Bowers, 2006), while *P. lanceolata* contains both aucubin and its derivative catalpol at much higher concentrations, ranging from 5–12% dry weight (Bowers and Stamp, 1992). *Taraxacum officinale* does not contain iridoid glycosides, but does contain flavonoids generally considered less toxic to herbivores (Harborne, 1991; Schutz et al., 2006). Newly hatched larvae were reared in groups of five in plastic rearing cups in growth chambers with 16:8 h L:D control, and 25°C daytime, 20°C nighttime temperatures. Plants were collected from Boulder County, CO, USA, soaked in dilute bleach solution for approximately 10 min, rinsed three times, and spun dry in a salad spinner before they were offered to caterpillars *ad libitum*. After molting to the 6th instar, 25 caterpillars feeding on each of the three host plants were removed in order to test the immune response. An additional 20 *G. incorrupta* caterpillars from each of the *Plantago* treatments were removed, injected, and immediately frozen for assessment of IG sequestration. Ten individuals from each host plant were dissected in methanol, and 10 were left intact in order to determine whether the dissection procedure affected IG sequestration. Five subsets of leaves of *P. major* and *P. lanceolata* from the material fed to caterpillars were dried at 50°C to a constant weight, ground to a fine powder, and subsampled to quantify IGs.

Chemical Analysis Iridoid glycosides in plant material and caterpillars reared on *P. major* and *P. lanceolata* were quantified by gas chromatography. Frozen caterpillars were ground in 5 ml of 95% methanol and extracted overnight. Those caterpillars that had been previously dissected were extracted overnight in the methanol solution utilized for the dissection. A subsample (25 mg) of dried plant material was extracted in 5 ml of 95% methanol overnight. Caterpillar and leaf samples were filtered, and the methanol was removed by evaporation. An internal standard (phenyl-β-D-glucose, Sigma-Aldrich, St. Louis, MO, USA) was added, and the sample was partitioned between water and ether in order to remove hydrophobic compounds. The water phase was collected, evaporated to dryness, and the residue was dissolved in 1 ml methanol. An aliquot of 100 μl was dried overnight, derivitized with 100 μl Tri-Sil-Z (Pierce Chemical Company, Rockford, IL, USA) and injected (1 μl) into a Hewlett Packard 5890A gas chromatograph equipped with a FID detector for separation as described by Bowers and Stamp (1992). The two iridoid glycosides, aucubin and catalpol, were identified by comparison with standards of the pure compounds that were extracted and isolated from plant material (Camara, 1996) and quantified using Agilent Chem Station software.

Artificial Diet Experiment This experiment was designed to measure the effects of IGs on the immune response while holding all other diet variables equal. Thus, we used an artificial diet (Lei and Camara, 1999) to which we added known concentrations of IGs. Iridoid glycosides were extracted and purified from *P. lanceolata* collected from Southern Alabama (Camara, 1996). The crude extract comprised 8% IG making it impossible to adjust the artificial diet to 12% IG, which is the upper limit found in natural plant populations. Crude extract was dissolved in water and added to the artificial diet mix to yield a 1% (low concentration) and a 5% (high concentration) IG diet. Newly hatched caterpillars were assigned randomly to high or low diets and placed in individual plastic rearing cups (30 per treatment) with food. Rearing cups were kept in a walk-in growth chamber with environmental conditions set at 12:12 h L:D at 23°C. The immune response was tested at the beginning of 6th instar.

Feeding Efficiency Experiments For the artificial diet experiment, feeding efficiency was calculated using the standard gravimetric method (Waldbauer, 1968). All measurements were obtained over a standardized time interval starting at third instar and ending when caterpillars molted into 6th instar. Measurements included food mass, body mass, and fecal mass each day. In addition, samples of larvae and diet were dried and weighed at the beginning and the end of the experiment. Growth rate and feeding efficiency measures were analyzed using MANOVA where measurements at each instar were considered separate response variables (i.e., multivariate repeated measures). The following indices were calculated for each caterpillar:

Efficiency of conversion of ingested food (**ECI**) = larval dry weight gain/dry weight of food consumed
Approximate digestibility (**AD**) = (dry weight of food consumed − dry weight of frass)/dry weight of food consumed
Efficiency of conversion of digested food (**ECD**) = larval dry weight gain/(dry weight of food consumed − dry weight of frass)
Growth Rate (**GR**) = larval dry weight gain/(duration of feeding period*mean weight during growth interval).

(1)

Results

Three Host Plants Individuals of *G. incorrupta* that fed on the three different host plants did not significantly differ in their immune response ($F_{2,4}$=2.69, N=42, P=0.081) (Fig. 1a). Within each species, the iridoid glycoside content was quite variable but overall *P. lanceolata* had a higher IG content than *P. major* (*t*-test, P=0.015) (Fig. 2). *Grammia incorrupta* larvae sequestered very low levels of IGs (Fig. 2) and fewer than 40% of the larvae tested contained detectable amounts. To determine whether dissection of larvae affected IG content by promoting breakdown of the IGs, we performed a two-way ANOVA on the arcsine square root transformed proportion of total IGs in larvae, with host plant and dissection as the main effects. For this analysis, we used only those larvae in which IGs were detected (only aucubin for larvae reared on *P. major* and both aucubin and catalpol for larvae reared on *P. lanceolata*). The amount sequestered was not affected by host plant species ($F_{1,10}$=2.52, P=0.143) or by dissection ($F_{1,10}$=3.84, P=0.079), although there was a trend for dissected larvae to contain higher levels of IGs (Fig. 2), nor was there an interaction ($F_{1,10}$=0.220, P=0.649).

Artificial Diet When individuals of *G. incorrupta* were fed IGs in an artificial diet at 5% and 1% concentrations, there was no significant difference in the melanization response ($F_{1,70}$=0.01, N=71, P=0.907) (Fig. 1b). Pupal weights were higher on the 1% iridoid diet compared to the 5% iridoid diet ($F_{1,37}$=10.76, N=38, P=0.002). There were no significant differences in growth rates across all instars (Table 1), indicating that the higher pupal weights on the 1% diet were not a result of slower growth, which could allow more time to feed. The difference in pupal weights may be due to significant differences in the digestion indices. The ECI and ECD differed significantly between diets (Table 1). For both indices, the mean consumption (dry weight of food consumed / average larval dry weight during interval) for caterpillars feeding on the 1% IG diet were higher. However, AD did not significantly differ between caterpillars feeding on the 1% and the 5% diets (Table 1). These results indicate that while caterpillars may be assimilating nutrients from food (AD) efficiently on both diets, they are less efficient at converting the assimilated nutrients into caterpillar biomass on the 5% iridoid diet, thus resulting in lower pupal weights.

Discussion

Our initial hypothesis that ingestion and sequestration of iridoid glycosides would cause significant decreases in the immune response was not supported. Sequestration was a poor predictor of variation in the immune response, since overall sequestration was low, and many individuals had undetectable levels of IGs. Poor sequestration ability also was found in another generalist arctiid, *Estigmene acrea* (Lampert and Bowers, 2010). Individuals feeding on diets with high and low concentrations of IGs exhibited almost identical immune responses. The overall high levels of melanization on the plant diets, and the low amount of variation among all diets suggest that individuals of *G. incorrupta* have a reliably strong immune response.

There was, however, a 37% difference in melanization score between caterpillars feeding on the artificial diet and caterpillars feeding on the plant diets. The relatively lower degree of melanization in larvae fed on the artificial diets may be due to the lack of essential substrates necessary for the phenoloxidase cascade to occur. The phenoloxidase cascade promotes the melanization process that darkens the cells encapsulating a foreign object (Beckage, 2008). The artificial diet contains only essential nutrients so that caterpillars are not ingesting pigments or other plant components that may be required for melanization.

Immune responses can be highly variable in insects (Rolff and Siva-Jothy, 2003). This variation is attributed to environmental stress (van Ooik et al., 2008), diet (Smilanich et al., 2009a), genetic variation (Lazzaro et al., 2004), or predation pressure (Slos et al., 2009). In our study, the only variation in the melanization response was associated with artificial diets vs. leaf diets. The results with

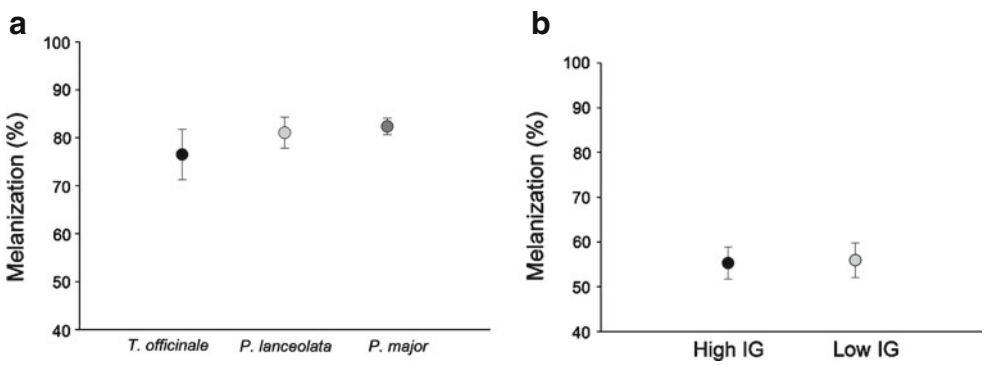

Fig. 1 Response to immune challenge for *Grammia incorrupta* consuming plant diets (*Taraxacum officinale, Plantago lanceolata*, or *Plantago major*) (**a**) or iridoid glycoside (IG)-containing artificial diets (**b**). The immune response was measured as % melanization of injected particles

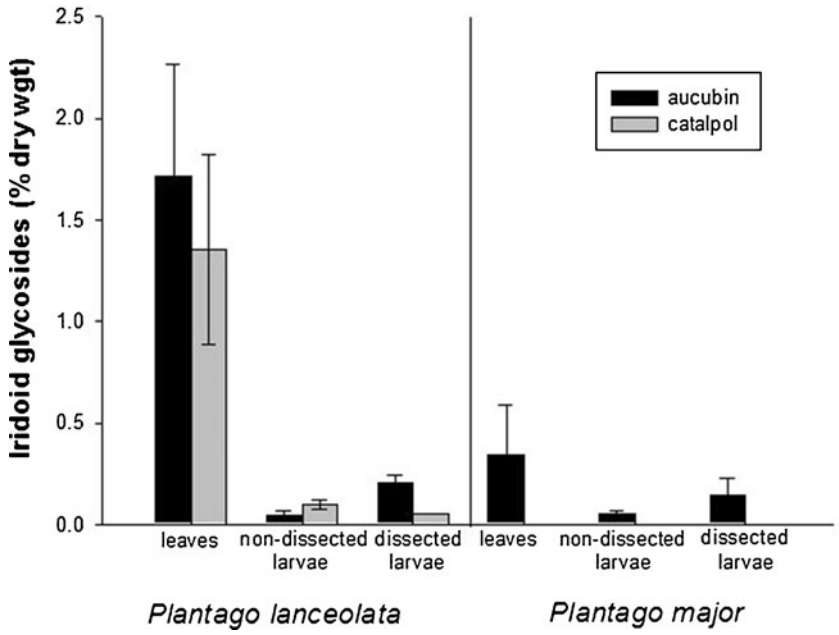

Fig. 2 Level of iridoid glycosides in *Plantago lanceolata* and *P. major*, and in intact or dissected *Grammia incorrupta* fed on either *P. lanceolata* or *P. major*

leaf diets suggest that high concentrations of IGs in the diet are not directly toxic to the immune system in this species. One reason for the low variation among diets in this study may be because this species is not efficient at sequestering IGs. In a previous study, we found that the specialist buckeye caterpillar, *Junonia coenia* (Nymphalidae), which can sequester IGs in concentrations as high as 25% dry weight (Bowers and Stamp, 1997), had a significantly lower melanization response when sequestering high concentrations of IGs (Smilanich et al., 2009a). Since *G. incorrupta* inefficiently sequesters IGs, the impact of the compounds on the immune response is probably minimal, thus leading to the small effect observed in our study.

Consumption of the 5% IG diet caused a significant decrease in pupal weights compared to the 1% IG diet. This effect most likely was driven by differences in the consumption indices, ECI and ECD, which were dramatically lower (36%) on the high IG diet. In our study of immune-challenged insects, feeding efficiency can give an approximation of the metabolic costs of feeding on diets with high concentrations of secondary metabolites coupled with mounting an immune response. The ECI measures the overall efficiency with which the caterpillar assimilates food into biomass. This measurement is broken down into the AD and ECD, where AD measures the proportion of nutrients that are assimilated from the ingested food (predigestion), and ECD measures the proportion of assimilated food that is turned into caterpillar biomass. If individuals on the high IG diet are consuming less and converting less of the digested food to biomass, then a decrease in pupal weights is expected. This difference in pupal weights was not correlated with growth rate or development time since there was no significant difference between diets for these variables. Instead, the increase of IG concentration in the caterpillar's diet negatively affected pupal weights by interference with consumption and post-digestive ability. Since pupal weights are correlated with adult fecundity (Gilbert et al., 1984), *G. incorrupta* fecundity may be lower when

Table 1 Feeding efficiency parameters from the artificial diet experiment

	F	*P*	1% Iridoid glycoside diet			5% Iridoid glycoside diet		
			Mean	SEM	*N*	Mean	SEM	*N*
GR	0.48	0.49	0.009	0.001	42	0.006	0.0007	47
ECI	8.34	0.006	3.10	0.49	42	1.96	0.19	47
AD	0.05	0.82	92.40	0.45	42	92.07	0.41	47
ECD	8.29	0.006	3.34	0.53	42	2.12	0.20	47

MANOVA was used to analyze GR, growth rate; ECI, efficiency of conversion of ingested food; AD, approximate digestibility; ECD efficiency of conversion of digested food. Mean values are reported (standard error of the mean, *SEM;* sample size, *n*). *F* is reported as Wilks' Lambda

feeding on plants with high concentrations of secondary metabolites. However, if individuals of *G. incorrupta* are able to switch hosts easily, then continued feeding on a nutritionally poor plant is unlikely unless individuals are parasitized (Singer et al., 2009).

The ability to switch host plants and feed on plants with suboptimal quality can be an asset for an insect challenged by a parasitoid (Karban and English-Loeb, 1997; Singer et al., 2009). Switching hosts as a defense against parasitoids may outweigh any developmental costs suffered by feeding on an inferior host. Our study provides further support that being a polyphagous feeder is advantageous by demonstrating that the immune response did not vary among three plant species, which are likely to have different nutritional qualities as well as differences in secondary metabolite contents.

The evolution of herbivore diet breadth is complex and influenced by many variables (Singer and Stireman, 2005). Here, we found that the immune response, which is one of the most effective defenses against parasitoids, did not vary among a select set of host plant species and was not affected by one group of secondary metabolites, iridioid glycosides. This lack of variability may allow individuals to feed on a variety of plants without detrimental effects on the immune response. Recent work has demonstrated that pyrrolizidine alkaloids, which are actively sequestered by *G. incorrupta*, also do not affect the immune response (Smilanich et al., 2011). In light of these results, we suggest that this polyphagous caterpillar is well adapted to feeding on host plants of variable quality with no detrimental effect on its immune response.

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