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LETTER

Immunological cost of chemical defence and the evolution of herbivore diet breadth

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Abstract

Selective pressures from host plant chemistry and natural enemies may contribute independently to driving insect herbivores towards narrow diet breadths. We used the specialist caterpillar, *Junonia coenia* (Nymphalidae), which sequesters defensive compounds, iridoid glycosides, from its host plants to assess the effects of plant chemistry and sequestration on the larval immune response. A series of experiments using implanted glass beads to challenge immune function showed that larvae feeding on diets with high concentrations of iridoid glycosides are more likely to have their immune response compromised than those feeding on diets low in these compounds. These results indicate that larvae feeding on plants with high concentrations of toxins might be more poorly defended against parasitoids, while at the same time being better defended against predators, suggesting that predators and parasitoids can exert different selective pressures on the evolution of herbivore diet breadth.

Keywords

Diet breadth, encapsulation, herbivory, immune response, iridoid glycosides, natural enemies, parasitoids, plant chemistry, secondary metabolites, tritrophic interactions.

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INTRODUCTION

Two prominent questions in ecology and evolutionary biology are: (i) how are herbivore populations regulated and (ii) how does herbivore diet breadth evolve? Studies of tritrophic interactions have provided partial answers to these questions, revealing strong effects of both plant secondary chemistry and natural enemies on the ecology and evolution of insect herbivores (Dyer 1995; Lill *et al.* 2002). While a diverse array of plant compounds are critical in deterring herbivorous insects from feeding on plant tissues, many insects have evolved physiological mechanisms to detoxify secondary compounds (Wittstock *et al.* 2004; Zangerl *et al.* 2008). A more complex evolutionary response to plant chemical defence is found in those herbivores that have co-opted these toxins for their own benefit by sequestering them and using them as a defence against natural enemies (Bowers 1990; Nishida 2002; Hartmann 2004). While several studies have demonstrated that sequestration is an effective defence against many vertebrate and invertebrate predators (reviewed by Nishida 2002; Hartmann 2004), here we show that sequestration of plant secondary metabolites is more complex and may lead to higher mortality from more specialized natural enemies such as parasitoids. Thus, insect herbivores may experience an evolutionary trade-off between specializing to sequester secondary metabolites for protection against certain natural enemies, while becoming more vulnerable to parasitism.

To examine this potential mechanism for trade-offs in sequestration, we tested the hypothesis that sequestered secondary metabolites, iridoid glycosides (IGs), may interfere with the immune response of the buckeye caterpillar (*Junonia coenia*). Costs associated with detoxification or sequestration of plant compounds, linked with trade-offs in performance on alternative hosts, are assumed to be central to the evolution of insect specialization, and more generally for contributing to the high diversity of insects and flowering plants. Both sequestration and detoxification can be energetically costly, because these physiological processes involve the production of a number of different enzymes needed to transport toxins or to convert compounds into a non-toxic state for storage or metabolism (Bowers 1992; Hartmann 2004).

Once an insect has ingested a secondary metabolite destined for sequestration, the compound goes through the following sequence of events: (i) resorption through the gut wall, (ii) transportation to the haemolymph and (iii) deposition in a particular site of the body (Nishida 2002).

The mechanism by which insects achieve this path is likely to differ for each sequestered compound and can also vary between insect species for the same compound (Nishida 2002; Hartmann 2004), but all mechanisms are potentially costly.

One way to assess the cost of sequestering secondary metabolites is by measuring the insect's immune response. The insect immune response targets foreign objects inside the haemocoel and functions to defend against parasitoids, parasites and pathogens (Beckage 2008). The immune response consists of three main components: phagocytosis, nodule formation, and encapsulation (reviewed by Carton *et al.* 2008; Strand 2008). While phagocytosis and nodule formation protect against small pathogens such as bacteria, encapsulation targets larger invading objects such as parasites and parasitoid eggs. Encapsulation is generally composed of both a humoral and cellular response, although they do not always occur together. The humoral response is composed of recognition proteins that function to identify non-self objects and activate specialized cells (e.g. haemocytes) that are part of the cellular response. The cellular response continues with haemocytes attacking the foreign object and beginning the process of encapsulation, in which cells adhere to the foreign object and begin to build layers of cells, which eventually die and harden onto the surface. When the cells die, they undergo the chemical process of melanization, which includes the production of cytotoxic molecules. The combination of encasement in cells (encapsulation) and the oxidative reactions involved with melanization act to asphyxiate and poison the foreign body (Strand 2008). The encapsulation and melanization response is immediate and provides insects an effective defence mechanism against parasitic wasps and flies, pathogens, and other parasites that live in the insect haemocoel (Godfray 1994; Beckage 2008).

Insect herbivores may be immunocompromised by diverting energy away from immune function to detoxify or store toxins (Moret & Schmid-Hempel 2000). Alternatively, plant toxins may have a direct negative effect on specialized immune cells in the insect's haemolymph. When herbivores are immunocompromised, they become particularly vulnerable to attack from pathogens, parasites and parasitoids (Schmid-Hempel & Ebert 2003). Previously, we demonstrated that caterpillars feeding on a diet with high concentrations of secondary metabolites have greater parasitism rates than those feeding on diets with low concentrations of secondary metabolites (Dyer *et al.* 2004). Here, we report experimental evidence that provides unique support for the hypothesis that lepidopteran larvae feeding on plants with high concentrations of secondary metabolites become vulnerable to attack by natural enemies (Hunter 2003) via direct cellular toxicity and metabolic interference. The resulting effect may be that selection for host plant specialization, as imposed by predators, which are deterred by high levels of toxins, is quite different than that by parasitoids and pathogens.

This hypothesis, which we term the 'vulnerable host hypothesis', contradicts the 'nasty host hypothesis' first proposed by Gauld *et al.* (1992). According to the 'nasty host hypothesis', parasitoids have not evolved tolerance to secondary metabolites encountered in their host and, therefore, are less successful developing within herbivores feeding on plants containing toxic secondary metabolites. In contrast, we propose that secondary metabolites sequestered by herbivore hosts compromise the immune response, making herbivores more vulnerable to successful parasitism. Recent studies support this hypothesis, showing that consumption of plants with certain secondary metabolites (e.g. phenolics and salicyl glucosides) can decrease the strength of the immune response (Haviola *et al.* 2007), and also lead to higher parasitism rates (Zvereva & Rank 2003).

To examine how sequestered plant toxins affect the insect immune response, we performed a set of three experiments using the specialist caterpillar, *Junonia coenia* Hubner (Nymphalidae). Two of these experiments used leaves of host plant species and a third used an artificial diet. Immune response was determined by measuring the encapsulation and melanization of glass beads injected into caterpillars. Respiration rate and feeding efficiency variables were also measured as additional indicators of sequestration costs.

METHODS AND MATERIALS

Study organisms

Junonia coenia, the common buckeye caterpillar, consumes plants in the families Acanthaceae, Plantaginaceae, Scrophulariaceae and Verbenaceae (Robinson 2002). These families all contain IGs, which are a class of monoterpenoid secondary metabolites that are present in about 57 plant families (Boros & Stermitz 1990). *Plantago lanceolata* (Plantaginaceae) is a common host plant of *J. coenia* and contains the IGs, catalpol and aucubin. Individuals of *J. coenia* use IGs as both feeding stimulants and oviposition stimulants (Pereyra & Bowers 1988). *Junonia coenia* caterpillars sequester IGs from these host plants in concentrations as high as 25% dry weight (Theodoratus & Bowers 1999). Larvae concentrate IGs in their haemolymph, but begin to metabolize them before pupation, near the end of fifth instar and eventually eliminate them in the meconium just after eclosion (Bowers & Collinge 1992). Another host plant that is utilized by *J. coenia* is *Plantago major* (Plantaginaceae), which has lower concentrations of aucubin and no catalpol, and *J. coenia* larvae reared on this species sequester 1–5% dry weight IGs (Theodoratus & Bowers 1999). Both of these host plants were used in this study. The quantity of IGs that caterpillars

sequester varies depending on the concentration in their diet, which in turn determines the degree of protection the individual has against predators (e.g. Dyer & Bowers 1996). In addition, *J. coenia* sequestering high quantities of IGs experience lower feeding efficiency but suffer minimal physiological costs (Camara 1997a). *Junonia coenia* caterpillars were obtained from lab colonies maintained at University of Colorado, Boulder, and augmented from natural populations found in Southern Mississippi and Alabama.

Overview of experiments

In a series of three laboratory experiments, individual *J. coenia* caterpillars were randomly assigned to diets containing different levels of IGs. For the first experiment ('plant-species' experiment), caterpillars fed on either *P. lanceolata*, which contains primarily aucubin and catalpol, at high concentrations of 5–12% dry weight (Bowers & Stamp 1993), or *Plantago major*, which contains only aucubin at low concentrations (0.2–1% dry weight) (Barton & Bowers 2006). For the second experiment ('artificial diet' experiment), we used an artificial diet with *P. lanceolata* extract added, so that the only difference between diets was the concentration of IGs. *Junonia coenia* larvae were assigned to either an artificial diet containing 5% IGs, or an artificial diet containing 1% IGs. For the third experiment ('surface application' experiment), caterpillars were assigned to a leaf diet of *P. major* with isolated IGs added to the leaf surface at 10% or 2% concentrations, or a control diet in which caterpillars were fed *P. major* leaves with no added IGs. Sequestered IGs in larvae were quantified for the 'artificial diet' experiment. Each of these three experiments represents a unique mode for delivering IGs to *J. coenia* caterpillars.

Injections

The immune response was measured by injecting silica beads (40–120 μm diameter) into caterpillar haemolymph and allowing 24 h for encapsulation and melanization (Lavine & Beckage 1996) (Fig. 1). Beads used for injections were DEAE Sephadex-A25 silica chromatography beads obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Beads were dyed with a 0.1% solution Congo Red Dye and allowed to dry completely. Beads were injected into caterpillars using the Hamilton 7000 series syringe (Sigma-Aldrich) or hand-made fine glass needles fashioned from Pasteur pipettes. Solutions of 5 μL Ringer's solution, containing 5–15 beads were injected per individual at the base of the third proleg. Once injected, the injection site was sealed using New Skin Liquid Bandage (Medtech Products, Jackson, Wyoming, USA). After 24 h, caterpillars were freeze killed and later dissected to retrieve beads. Beads were stored in Ringer's solution until photographed. Beads

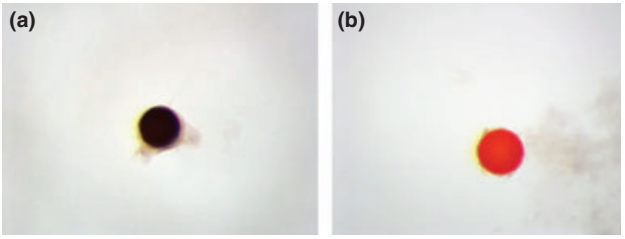


Figure 1 Bead encapsulation and melanization: (a) Left image shows an encapsulated and melanized bead from a caterpillar feeding on a low iridoid glycoside diet. (b) Right image shows a bead without encapsulation retrieved from a caterpillar feeding on a high iridoid glycoside diet.

were photographed using a dissecting microscope mounted with a digital camera (Carl Zeiss Discovery V.8, AXIOCAM Software, Oberkochen, Baden-Wurttenburg, Germany). All photographs were taken at 80× magnification. As beads were dyed red before injecting them into the caterpillars, we were able to obtain a measure of encapsulation and melanization strength by measuring the red value (*r*-value) of each bead. The *r*-value is a numerical measure of the red saturation of an image on a scale ranging from 0 to 255, where 0 = pure grey, and 255 = pure red. Using ADOBE PHOTOSHOP (version 6.0) Adobe Systems Inc., San Jose, California, USA, the *r*-value was obtained for each bead within a caterpillar and these values averaged to provide an *r*-value score for each individual caterpillar. The mean *r*-value was transformed into a percentage of melanization [1 − (*r*-value/maximum *r*-value)] for ease of interpretation.

This technique of presenting caterpillars with a foreign object to assess the immune response has been shown to be significantly correlated with the immune response against real parasites and pathogens (Rantala & Roff 2007). In addition, in a separate study using this method, we measured the immune response of 16 species of caterpillars with known frequencies of parasitism. Parasitism frequencies were determined from 15 years of rearing data (Dyer *et al.* 2007). In that study, we found a significant negative correlation between per cent melanization and per cent parasitism (Pearson's correlation coefficient $R = -0.58$, $n = 15$, $P = 0.02$; Smilanich *et al.* 2009), indicating that individuals with a weak immune response (as measured by bead melanization) have higher parasitism rates. These results show that the injection technique accurately reflects the caterpillar immune defence against parasitoids.

Iridoid glycosides used in the experiments were extracted from bulk collections of *P. lanceolata* collected in Alabama and Mississippi in 2005. Leaves were dried and extracted in methanol. The plant material was filtered out and the extract dried under vacuum. The extract was partitioned between water and diethyl ether, the ether layer removed and the water layer partitioned with butanol. The water layer

(containing the IGs) was evaporated to dryness and the IG content of the extract quantified by gas chromatography (Bowers & Stamp 1993). Appropriate amounts of the extract were added to artificial diets or leaves to provide the concentrations of IGs desired.

Iridoid glycosides in caterpillars were also quantified by gas chromatography using methods previously described (Bowers & Stamp 1993; Barton & Bowers 2006).

Respiration experiments

Junonia coenia respiration rates were measured using a CO_2 gas analyser (LI-COR 820) connected to a 400-mL closed dynamic chamber (Chambers *et al.* 2001). Actively feeding caterpillars were placed in the chamber (one caterpillar per trial), and the increase in CO_2 concentration in the closed chamber was recorded every second over the time span of 3 min. Respiration was measured as micromoles of CO_2 per millilitre per second ($\mu mol\ mL^{-1}\ s^{-1}$), which was then standardized to micromoles of CO_2 per gram of caterpillar ($\mu mol\ g^{-1}\ s^{-1}$) (dry weight). The mean respiration rate was used for all analyses. Respiration data were collected from the 'surface application' experiment only. We predicted that respiration rates would increase with high IGs because individuals sequestering more IGs may have an increase in metabolic activity due to increased production of detoxification proteins as well as transport proteins required to move the IGs across the gut wall and into the haemolymph.

Feeding efficiency experiments

For the 'surface application' experiment, the feeding efficiency of caterpillars was calculated. In addition, we collected pupal weights and growth rates (GR) from all individuals in this experiment. Feeding efficiency can give an approximation of the metabolic costs of feeding on diets with high concentrations of secondary metabolites. Feeding efficiency was calculated using the standard gravimetric method (Waldbauer 1968). For each caterpillar, we collected data for the following three nutritional indices, as well as GR:

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/average larval dry weight during interval

The ECI measures the overall efficiency at which the caterpillar assimilates food into biomass. This measurement is composed of the AD and ECD, where AD measures the proportion that is assimilated from the ingested food (pre-digestion), and ECD measures the proportion of assimilated food that is turned into caterpillar biomass. All measurements were gathered over a fixed time interval starting at newly moulted third instar larvae and ending at pre-pupal fifth instar. Data gathered included food mass, body mass and faecal mass each day. In addition, a subset of larvae and diet was dried and weighed at the beginning and the end of the experiment to obtain dry weight calculations.

Statistical analyses

For all analyses, individual caterpillars were the unit of replication, transformations were applied to insure normality of residuals, and SAS statistical software (SAS Institute Inc., Cary, N.C., USA) was utilized. Analyses of Variance (ANOVA) were used to compare mean transformed *r*-value (per caterpillar) between treatments for each of the different experiments. Pearson's correlation coefficients were used to examine associations between sequestered iridoids (aucubin, catalpol and total iridoids) and *r*-values. Effect sizes (Hedges D; Gurevitch & Hedges 2001) were calculated for all experiments.

We used the SAS Calis (Covariance Analysis of Linear Structural Equations) Procedure to test goodness of fit of specific *a priori* causal hypotheses, examining direct and indirect relationships between diet, nutritional indices, GR, respiration, and melanization. Data for path analyses were from the 'surface application' experiment, which included all of the response variables and in which IG concentrations were 10% (high). All models were specified based on our predictions; specification, estimation and tests of model fit followed recommendations of Ullman (1996) and Shipley (2000 and references therein). Normal theory maximum likelihood (ML) methods were used for estimation, and parameter vectors were estimated iteratively by a nonlinear optimization algorithm to optimize a goodness of fit function. Chi-squares were calculated for the ML goodness of fit to assess the fit of the models to the data; *P*-values of greater than 0.05 are considered to indicate a good fit (Ullman 1996). When appropriate, models were statistically compared with one another by subtracting the chi-squares and degrees of freedom for the two models being compared ($P < 0.05$ indicates a significantly better fit for the model with lower chi-squared value; Ullman 1996).

RESULTS

Immune response

In the 'plant-species' experiment, individuals reared on the high IG plant, *P. lanceolata*, had a significantly lower melanization response compared with individuals reared on the low IG plant, *P. major* (Fig. 2, $F_{1,7} = 30$, $P = 0.0015$), indicating that *J. coenia* feeding on the high IG plant were less efficient at melanization. This represents a considerably costly trade-off (Bowers 1992): an 18% decrease in an important immune function caused by consumption of plants with high concentrations of IGs.

For the 'artificial diet' experiment, we found that individuals sequestering higher concentrations of catalpol (the more toxic IG, Puttick & Bowers 1988), had a significantly lower melanization response (Fig. 3, $R = -0.28$, $P = 0.032$, $n = 59$), suggesting that high concentrations of this compound interferes with the immune response. There were marginally significant, yet relatively high negative correlations between melanization and sequestration for aucubin and total IG (aucubin: $R = -0.24$, $P = 0.063$, $n = 59$; total IG: $R = -0.25$, $P = 0.058$, $n = 59$). Interestingly, the overall melanization response for the 'artificial diet' experiment was lower compared with the experiments using plant diets (artificial diet average per cent melanization = 48.56 ± 12.13; plant diet average per cent melanization = 76.38 ± 2.69). This low degree of melanization on artificial diets vs. plant diets may be a result of a lack of certain plant components necessary for melanization that are missing from artificial diets (Lee *et al.* 2006).

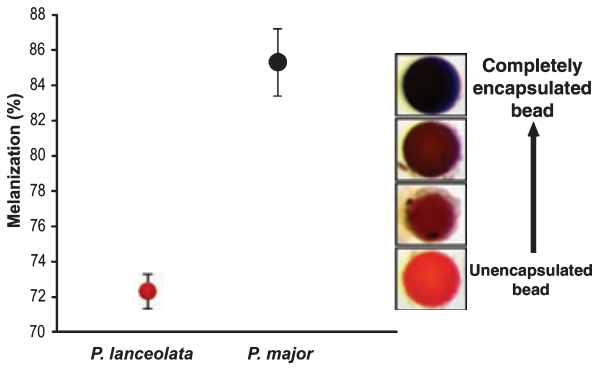


Figure 2 Percent melanization between treatments for plant-species' experiment. Buckeyes (*Junonia coenia*) reared on *Plantago lanceolata* (high iridoid glycoside plant) had significantly lower levels of melanization ($n = 4$ caterpillars, 5–15 beads per caterpillar, mean ± SEM, per cent melanization = 72.34 ± 0.9775) as measured by red hue saturation than *J. coenia* reared on *Plantago major* (low iridoid glycoside plant) ($n = 4$ caterpillars, 5–15 beads per caterpillar, mean ± SEM, per cent melanization = 85.29 ± 1.8936).

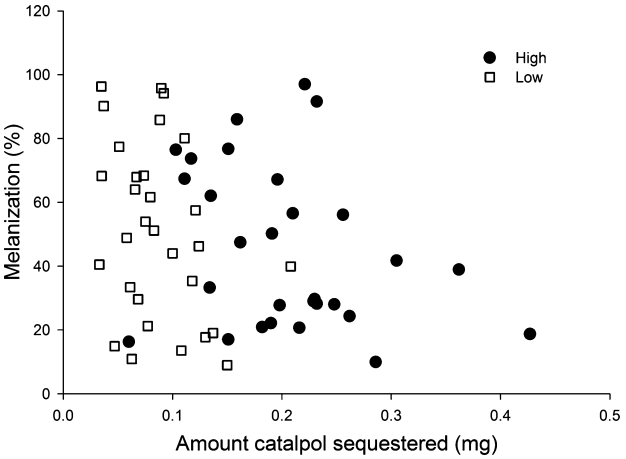


Figure 3 Relationship between amount of catalpol IGs sequestered and the per cent melanization of injected silica beads. These results indicate that as catalpol sequestration increases the per cent melanization decreases. As *Junonia coenia* will sequester more catalpol when it is in high concentrations in their host plants, they may be more vulnerable to parasitism and pathogens when feeding on high catalpol plants.

For the 'surface application' experiment, in which isolated IGs were pipetted onto the leaf surface of *P. major*, caterpillars feeding on the 10% IG-treated leaves had significantly lower melanization than the caterpillars in the control group (Fig. 4, ANOVA, $F_{2,43} = 4.09$, $P = 0.024$), while those fed on the 2% IG diet were intermediate. Among all of these experiments, the 'plant-species' experiment yielded the greatest effect size (effect size for 'plant-species' = 2.26, 'surface application' = 0.351, 'artificial diet' = 0.123).

Metabolic activity and feeding efficiency

When the difference between larval CO_2 output was analysed for the 'surface application' experiment, we found that individuals feeding on the high 10% IG diet had significantly lower respiration rates compared with individuals feeding on the low 2% IG diet ($n = 94$ caterpillars, $F_{1,93} = 20.62$, $P < 0.0001$). Caterpillars feeding on the low IG diet had significantly higher GR than caterpillars feeding on the high IG diet (Table 1). Pupal weights were not significantly different on the low IG diet compared with the high IG diet (Table 1).

Feeding efficiency data (Table 1) showed that caterpillars feeding on the low 2% IG diet had a significantly higher index for efficiency of conversion of ingested food (ECI) compared with the high 10% IG diet. The ECI can be partitioned into approximate digestibility (AD) and the index for efficiency of digested food (ECD). The results of these two indices differed, with AD significantly higher on

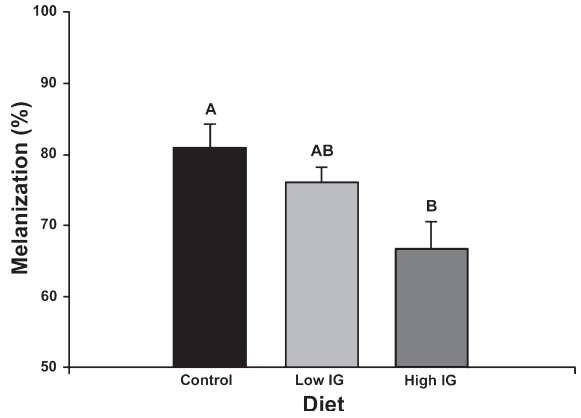


Figure 4 Per cent melanization between treatments for the 'surface application' experiment. Letters above bars indicate which treatments were significantly different from each other. As was observed in the 'plant-species' experiment, *Junonia coenia* feeding on plants with high IGs added had a decreased encapsulation response (n = 19 caterpillars, 5–15 beads per caterpillar, mean ± SEM, per cent melanization = 66.73 ± 3.8) compared with caterpillars feeding on the control diet (n = 6 caterpillars, 5–15 beads per caterpillar, mean ± SEM, per cent melanization = 81.50 ± 3.2). Low IG diet was not significantly different from the high IG diet (n = 21 caterpillars, 5–15 beads per caterpillar, mean ± SEM, per cent melanization = 76.04 ± 3.6).

the high IG diet and ECD significantly higher on the low IG diet.

The path analysis model with the best fit to the data included the pathway showing that high concentrations of IGs have a direct negative effect on melanization (Fig. 5, model 1; χ^2 = 0.90, d.f. = 1, P = 0.34). Of all the nutritional indices added to the models, AD (percentage of ingested food that is digested) consistently had the highest standardized path coefficient, thus explaining a greater proportion of the variation than the other indices. Several other models that included other feeding efficiency indices and melanization were also tested, but were rejected due to a statistically poor fit to the data (Table 2). Only when melanization was taken out of the model was there a significant effect of diet on other feeding efficiency indices. Model 2 is a simplified hypothesis that shows high IG diet has a negative effect on growth rate (Fig. 5, model 2; χ^2 = 0.0287, d.f. = 1, P = 0.8655).

DISCUSSION

We have provided clear empirical evidence that the immune response of *J. coenia* is negatively affected by the amount of IGs these caterpillars sequester from their diet. The largest effect sizes among experiments were found in the 'plant-species' experiment and the 'surface application' experiment, which had the greatest difference in IG concentration between diets. These results indicate that negative effects of secondary metabolites on the immune response of *J. coenia* are most likely to occur when larvae are feeding on plants with high concentrations of IGs. Thus, host plant selection is very important in resistance or susceptibility to parasitoids (Lill *et al.* 2002; Singer & Stireman 2003). The fact that the experiments utilizing normal plant diets yielded the greatest effect size also suggests that herbivore immune function is affected additionally by other characteristics that differ among host plant species, such as primary metabolites and nutritional composition of plants. There is recent evidence that parasitized herbivores have higher survivorship on nutritionally inferior host plants (Singer & Stireman 2003), and will modify their nutrient intake when faced with an immune challenge, such as a pathogen or parasitoid (Lee *et al.* 2006). We did not investigate other features of *P. lanceolata* and *P. major* besides IGs, but *P. major* does have lower IG concentrations and it likely varies in other nutritional features from *P. lanceolata*. Nevertheless, treatments in the 'artificial diet' experiment did not differ in nutritional content, and results still showed that high larval sequestration of IGs can significantly interfere with the immune response.

It appears that the immune cost of sequestering high concentrations of IGs is direct and is not reflected by metabolic load (i.e. respiration). If this is a general phenomenon, specialist herbivores that sequester will experience trade-offs between the benefit of chemical defence and the immunological cost of that defence. The

Table 1 Mean values (X), standard deviation (SD), sample size (n), F-statistic (F) and P-values (P) for feeding efficiency data from 'surface application' experiment

	High diet (10% IG)			Low diet (2% IG)				
	X	SD	n	X	SD	n	F	P
Pupal weight	296.07	54.10	20	331.86	51.44	27	2.80	0.071
Growth rate	0.038	0.021	50	0.049	0.023	54	3.70	0.028
ECI	1.38	0.96	51	1.95	1.27	54	3.51	0.040
AD	96.34	3.3	51	94.54	2.46	54	5.50	0.005
ECD	1.45	0.96	51	2.05	1.30	54	3.29	0.033

ECI, efficiency of conversion of ingested food; ECD, efficiency of conversion of digested food; AD, approximate digestibility.

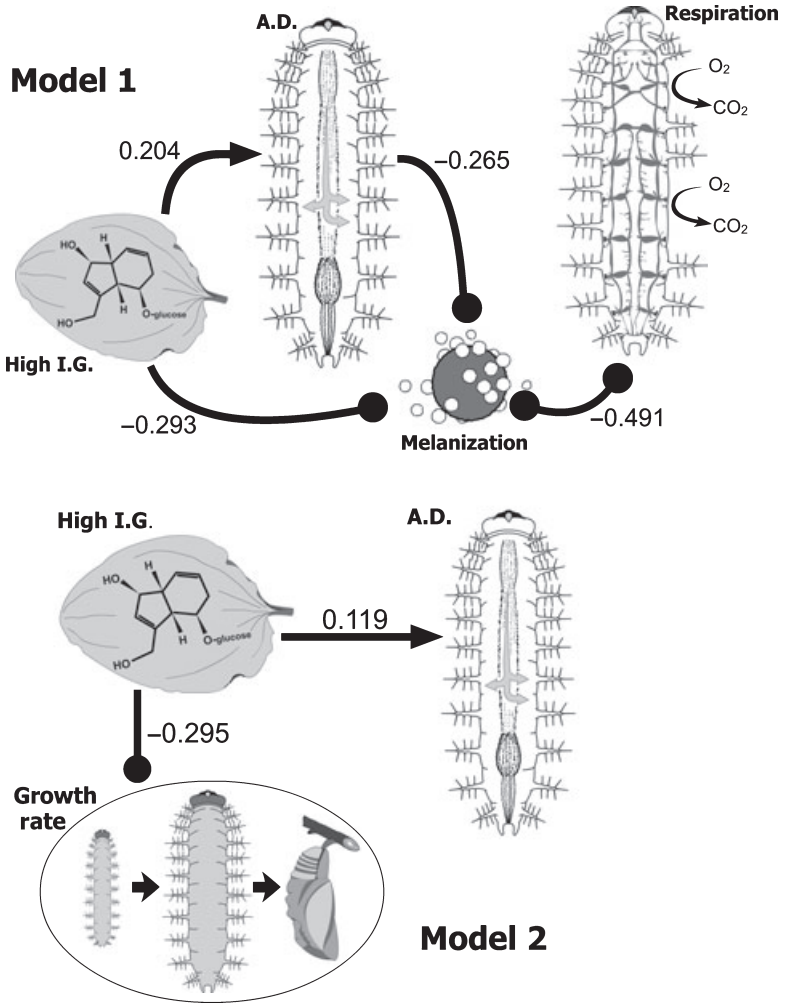


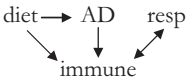
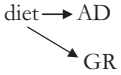
Figure 5 Path diagrams testing *a priori* hypotheses for relationships between diet, melanization and metabolism. Numbers above pathways are standardized path coefficients. Negative numbers and bulleted arrows indicate a negative effect of one variable on another. Model 1: of all AD path models tested (Table 2), model 1 had the best fit to the data, supporting our hypothesis that high concentration of IGs have a direct negative effect on the immune response. High concentrations of IGs also increased approximate digestibility (AD), which in turn had a negative effect on melanization. We hypothesize that with an increase in AD more IGs are crossing the gut wall and being sequestered in the haemolymph, which inhibits normal immune function. The negative association between melanization and respiration was an unanalysed correlation in the path analysis. Model 2: this model shows that high concentrations of IGs negatively affect caterpillar growth rate, which may partly explain the associated decrease in CO_2 output found in model 1.

ecological result is that effective anti-predator chemical defences of herbivores are an asset to parasitoids by compromising the immune response, in addition to creating enemy-free space. As parasitoids are considered the most important source of mortality for many caterpillars, they can exert considerable selective pressure (Godfray 1994; Hawkins *et al.* 1997), particularly when influencing diet breadth. Other studies investigating the effects of diet on the immune response have focused on plant nutritional quality (Ojala *et al.* 2005; Klemola *et al.* 2007). The results of these studies show that herbivores feeding on nutritionally low quality diets have an enhanced or equally effective immune response compared with herbivores feeding on high quality diets, while others have found high quality diets to confer the most resistance (Klemola *et al.* 2008). Thus, the effect of plant nutrition on the immune response is complex and can be quite different from that of plant secondary metabolites. The way that these two factors interact to affect the immune response has yet to be fully investigated.

Our results support the vulnerable host hypothesis, demonstrating that a high IG diet has a strong direct negative effect on melanization in addition to indirect negative effects via AD. The main effect of IGs on *J. coenia* feeding efficiency was a strong positive effect on AD, which in turn had a negative effect on melanization. As we found a positive correlation between high IG diet and AD, increasing the portion of the ingested food that is digested (high AD) could also increase the amount of IGs crossing the gut wall, so that caterpillars are sequestering more IGs in their tissue. The increased amount of IGs in the caterpillar's haemolymph, which could negatively affect immune cells as well as decrease resources available for mounting a strong immune response, may drive the observed negative effect of AD on melanization.

Unexpectedly, we found that metabolic rates were significantly higher on low IG diets compared with high IG diets, indicating that increased processing of IGs does not increase respiration. Instead, the observed increase in respiration on the low IG diet may be a result of the

Table 2 Summary of all the models that were selected *a priori* and tested using path analysis. Models 1 and 2 were statistically and biologically the best fits to our data. Statistical comparisons of nested models (via subtracting χ^2 and d.f.; Ullman 1996) indicate significantly better fit of Model 1 compared with Models 3–5 ($P < 0.05$ for all comparisons). Model 1 was also tested with ECI, ECD and GR in place of AD; these models were adequate fits ($P > 0.05$), but had lower or insignificant standardized path coefficients. Models 3–5 were tested with ECI, ECD and GR in place of AD and were inadequate fits to the data ($P < 0.05$)

Model	Structure	X^2	P	DF
1-costs of immune		**0.898**	**0.343**	**1**
2-AD + GR		**0.029**	**0.865**	**1**
3- no effect of resp. & AD on immune		6.72	0.082	3
4- no effect of diet on immune		5.27	0.153	3
5-direct diet effects		7.26	0.064	3

ECI, efficiency of conversion of ingested food; ECD, efficiency of conversion of digested food; GR, growth rate; AD, approximate digestibility; resp, respiration; immune, melanization.

increased growth rate and feeding efficiency indices (except AD) on the low IG diet. Consequently, the increase in CO_2 output in our study may reflect an increase in feeding metabolism, but not changes in IG processing costs. An alternative explanation for the unanalysed correlation (no specified direction of causation) between respiration and immune function is the possibility of a latent variable, such as changes in specific enzyme concentrations, that is correlated with both variables.

While some studies show that sequestered toxins are detrimental to developing parasitoid larvae by decreasing adult body mass, and generating smaller clutch size (Harvey *et al.* 2005; Lampert *et al.* 2008), here we demonstrate that they can actually benefit parasitoid larvae by suppressing the host's immune response. The same sequestered toxins that facilitate establishment of developing parasitoids within an immunocompromised host could also provide enemy-free space later in the parasitoid life cycle by reducing predation of the host and the vulnerable larvae inside it. These anti-predator and pro-parasitoid qualities of sequestration suggest a hypothesis that sequestration evolved in response to generalist predators, such as birds and a variety of invertebrate taxa, and the challenge facing insect herbivores from parasitoids arose more recently. For example, flies in the family Tachinidae are a dominant source of parasitoid-induced mortality among caterpillars, and this dipteran family arose relatively recently (*c.* 20 million years BP; Stireman *et al.* 2006), while specialized relationships between plant and insect genera may be much older (≤ 97 million years BP, Labandeira *et al.*1994). Among specialist caterpillars that sequester secondary metabolites, it is possible that parasitoids represent a stabilizing selective force that acts by decreasing extreme specialization and favouring a more general diet and lower levels of sequestration. The ability to host-switch for specialist caterpillars will be obviously more difficult than for generalist caterpillars. However, shifting host-plants within a particular genus of plants or between plants with similar chemistry may not be as difficult (Camara 1997b).

The vulnerable host hypothesis is most applicable to koinobiont parasitoids, which develop from egg to pre-pupal stage inside the host caterpillar, compared with idiobiont parasitoids, which can immobilize host caterpillars during development and thus avoid the host immune response altogether (Harvey 2005). Additionally, Harvey *et al.* (2005) showed that negative developmental effects of high host plant IGs were evident only for the generalist herbivore, *Spodoptera exigua* (which does not sequester IGs), and the generalist parasitoid, *Cotesia marginiventris*, while there were no detectable negative developmental effects on the specialist herbivore, *Melitaea cinxia* (which sequesters IGs), and its endoparasitoid, *Hyposoter horticola*. These results suggest that our hypothesis is most relevant to specialized tritrophic interactions, which involve host plant or chemical specialists, and specialist koinobiont parasitoids, because these taxa are most likely to have evolved with their host.

CONCLUSIONS

Our data are consistent with the hypothesis that plant chemistry and natural enemies can work in concert to influence the diet breadth of herbivores. Results from several experimental and statistical tests demonstrate that the relationship between sequestered plant secondary metabolites and the immune response is damaged by direct negative effects of sequestered toxins on melanization. Thus, chemical defences can lead to higher parasitism rates due to a diminished immune response. This discovery raises questions about the effectiveness of chemical sequestration

as a defence against all natural enemies and about its role in the evolution of diet breath in phytophagous insects (Dyer 1995). Research on the costs and benefits of insect chemical defences should further resolve these and other questions related to the regulation of herbivore populations and evolution of diet breadth. The interaction between chemical sequestration and the immune response against parasitoid eggs (rather than artificial eggs) is likely to be even more complex and variable, but research into this interaction will contribute substantially to understanding chemical defences and herbivore regulation.

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