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The insect immune response and other putative defenses as effective predictors of parasitism

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Abstract. Parasitic wasps and flies (parasitoids) exert high mortality on caterpillars, and previous studies have demonstrated that most primary and secondary defenses do not protect caterpillars against parasitoids. We investigated the efficacy of tertiary defenses (i.e., immune responses) against parasitoids. Using a bead injection technique to measure the immune response and a 15-year database to measure parasitism, we compared the immune response for 16 species of caterpillars in nine different families. We found that caterpillar species with a strong immune response had the lowest incidence of parasitism, and when statistically compared to other defensive traits, the immune response was the best predictor of parasitism. Parasitoids either avoid attacking caterpillar species with a capacity for high levels of melanization or are killed once they have parasitized. In either case, the immune response is clearly one of the most effective defenses that caterpillars have against parasitism, and elucidating consistent predictors of variation in encapsulation could improve understanding of parasitism patterns in time and space and could enhance biological control efforts.

Key words: caterpillar defense; Diptera; encapsulation; Hymenoptera; immune response; La Selva, Costa Rica; Lepidoptera; melanization; natural enemies; Nematoda; parasitism.

Introduction

A major goal of population and community ecology is to characterize the evolutionary responses of animals to selective pressure from natural enemies (Singer and Stireman 2005). Herbivorous insects have evolved an arsenal of defenses against natural enemies, including chemical, behavioral, morphological, and physiological characteristics, or a combination of each (Gross 1993, Barbosa and Caldas 2007, Veldtman et al. 2007). These defenses against natural enemies occur at three levels that are both spatially and temporally separated. The primary level consists of morphological and behavioral defenses (coloration, shelter building, group feeding), which defend against direct predator–prey contact and can deter initial attack from enemies. Once the caterpillar has been detected or attacked, the secondary level provides protection, via hairs, spines, regurgitating, thrashing, or dropping. Tertiary defenses act after enemies have overcome the first two lines of defense and include cellular and humoral mechanisms to resist parasitoids, parasites, and pathogens.

Although a large variety of traits are posited to be defensive, the efficacy of putative defenses varies depending on the enemy (Dyer 1997, Ratcliffe and Nydam 2008). Recent studies suggest that defenses that are effective against generalized predators are not as effective against parasitoids (Gentry and Dyer 2002, Barbosa and Caldas 2007). This difference in defensive efficacies may lead to differential herbivore mortality depending upon the dominant natural enemy in a given community. Parasitoids and nonparasitic predators have different ecological and evolutionary responses to the same prey partly because of variation in how they interact with their prey. Endoparasitoids have a unique life cycle compared to most predators because they spend their entire larval stage developing within their prey (Godfray 1994). Consequently, defenses that are effective against more generalized predators, such as spiders and birds, may be less effective against parasitoid wasps and flies.

While many ecological studies have focused on the efficacy of primary and secondary defenses, there has been less research investigating the effectiveness of tertiary defenses. The encapsulation response is an immediate tertiary defense that is carried out by several groups of specialized cells (e.g., hemocytes) in the hemolymph. Encapsulation is generally composed of both a humoral and a cellular response, although they do not always occur together (reviewed by Strand 2008); the latter response consists of layers of cells adhering to a foreign object, dying, and hardening onto the surface. When the cells die, they undergo melanization, which includes the production of the cytotoxic molecule phenoloxidase, and other free radicals such as quinones.

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The invader is killed through the asphyxiating effects of the encapsulation process and through the cytotoxic effects of the phenoloxidase cascade (Nappi and Christensen 2005).

Parasitoids are one of the highest biotic sources of mortality for insect herbivores, particularly lepidopteran larvae (Hawkins et al. 1997). The encapsulation response, which is ubiquitous, presumably should be effective against parasitoids as well as pathogens and other parasites (Godfray 1994). However, there are many variables that negatively affect the encapsulation response. Some are specific immunosuppressive agents such as polydnaviruses and additional parasitoid-derived immune suppressive factors (ISF) that are associated with certain species or families of parasitoid. These parasitoid-derived substances are injected into the host along with the parasitoid's egg or larva (Vass and Nappi 2000, Lovallo et al. 2002, Kohler et al. 2007). Other factors act in a more general fashion to affect the host immune response. These include the nutritional quality of the herbivore's diet (Ojala et al. 2005, Lee et al. 2006, Klemola et al. 2007), and ingestion or sequestration of plant secondary metabolites (Haviola et al. 2007, Smilanich 2008). Any of these factors can render a caterpillar immunocompromised and less likely to successfully fend off a parasitic egg, larva, or pathogen.

In this paper, we evaluate the effectiveness of the lepidopteran immune response by testing for a correlation between the strength of the encapsulation response and the frequency of parasitism for individuals in nine different families of lepidopteran larvae. We predict that species that are proficient at encapsulation (as measured by the melanization of silica beads) will have lower frequency of parasitism. In addition, melanization ability can be compared to other correlates of parasitism to rank its efficacy. Previous studies have utilized this approach of quantifying associations between caterpillar defenses and successful parasitism to compare defensive efficacies (Gross 1993, Gentry and Dyer 2002, Veldtman et al. 2007). Here we focus on physiological defenses rather than the morphological and behavioral defenses examined in previous comparative studies. Consequently, a second goal of this paper is to compare the effectiveness of the immune response to that of behavioral and morphological defenses. Because the immune response is specifically aimed at internal enemies, we predict that it will provide a better defense against parasitoids than behavioral or morphological defenses.

Methods

Study organisms and field site

Caterpillar species used for this study comprise nine families, 11 subfamilies, and 16 species in the order Lepidoptera (Table 1; see also Plate 1). The work was conducted at La Selva Biological Station (10°25′ N, 84°05′ W) from December 2006 to January 2008. La Selva is a lowland tropical wet forest located on the Caribbean Slope of Costa Rica at the confluence of the Sarapiqui and the Puerto Viejo rivers. All caterpillars were field-collected throughout the reserve and brought back to the laboratory for the immune assay. Species were chosen based upon their relative abundance, the availability of parasitism data, and to represent a wide breadth of lepidopteran families.

The parasitism data set used for this study includes >15 years of data from the La Selva forest (Gentry and Dyer 2002, Dyer et al. 2007). This data set includes host plant and caterpillar associations and identifications as well as frequencies of parasitism for all collected caterpillars. As of January 2008, this data set has generated >100 000 rearings of individual caterpillars. Caterpillars in the La Selva parasitism data set were collected at third and later instars in order to allow increased exposure to larval and pupal parasitoids. Collected caterpillars were then reared on their host plants until they eclosed as adults or until a parasitoid emerged. Caterpillars were collected year-round and in all forest types. We used these data to obtain frequencies of parasitism for the species used in our study. The parasitism variable measured in this manner does not distinguish between parasitoids that avoid species with a given defense or that are killed once they attack. Caterpillars that were used in our experiment were collected in instars 1–3 to decrease the chance of parasitism because we were interested in challenging the immune system in a laboratory setting. The immune response was assessed at instar 5, which is the final instar of all species in this study. For both the parasitism data and the encapsulation experiments, all caterpillars were housed at ambient conditions (mean monthly temperatures at La Selva are 24.7–27.1°C) in plastic bags containing fresh leaves from their host plant.

Injection technique

To quantify the immune response, caterpillars were injected with a foreign object as a proxy for oviposition by a parasitoid (Lavine and Beckage 1996, Lovallo et al. 2002, Rantala and Roff 2007). This technique has been used widely in the literature and can accurately quantify immune function in insects (Rantala and Roff 2007). Since we were interested in testing the strength of the encapsulation response, this method of simulated parasitism is desirable because female parasitoid wasps will often inject calyx fluid or polydnaviruses that suppress encapsulation when ovipositing (Lovallo et al. 2002). By injecting egg-sized foreign bodies into the caterpillars, we control for unwanted variables that would be present if female parasitoids were used. Moreover, this technique enables a controlled comparison of the immune response across taxa. The injection method described here is the method used for all caterpillars. Injections were performed using a glass Pasteur pipette fashioned into a microinjection needle using a Bunsen burner flame. The pipette was heated until the silica became flexible, and was then pulled

TABLE 1. Mean melanization and parasitism for each caterpillar species from La Selva Biological Station, Costa Rica, used for the immune assay.

Caterpillar species (*N*)	Family	Melanization (%)	Parasitism (%) (*N*)	Parasitoid taxa
Antichloris viridis (2)	Arctiidae	73.29	31.25 (28)	Braconidae
Desmia sp. (5)	Crambidae	57.16	50.86 (99)	Tachinidae, Braconidae, Ichneumonidae
Xylophanes pluto (1)	Sphingidae	80.28	31.70 (60)	Tachinidae, Phoridae
Tarchon felderi (2)	Bombycidae	64.90	33.33 (167)	Tachinidae, Braconidae
Achylodes busirus (3)	Hesperiidae	79.32	6.65 (145)	Braconidae, Nematoda
Emesis lucinda (6)	Riodinidae	74.17	12.53 (1461)	Tachinidae, Braconidae, Chalcididae, Nematoda
Papilio thoas (3)	Papilionidae	74.74	9.75 (80)	Tachinidae
Castilia ofella (10)	Nymphalidae	81.20	0 (47)	none
Chlosyne janais (33)		80.14	10.09 (473)	Tachinidae, Nematoda
Chlosyne gaudealis (4)		85.55	12.39 (213)	Tachinidae, Nematoda
Euptychia jesia (13)		79.02	6.87 (510)	Tachinidae, Nematoda
Caligo memnon (15)		74.04	13.53 (327)	Tachinidae, Braconidae
Opsiphanes tamarindi (23)		71.76	35.29 (98)	Tachinidae, Chalcididae, Braconidae
Cyclomia disparilis (6)	Geometridae	64.05	3.06 (356)	Tachinidae, Braconidae
Eois apyraria (10)		90.09	5.12 (650)	Tachinidae, Braconidae, Ichneumonidae, Chalcididae
Eois nympha (19)		91.65	9.43 (241)	Braconidae

Note: Sample size (*N*) indicates the number of individuals injected for immune challenge, and the parasitism sample size (*N*) indicates the total number of caterpillar individuals reared over a 15-yr period.

apart, so that a small diameter tip could be made by cutting the pulled pipette at the desired tip size. Silica beads (DEAE Sephadex A25, 40–120 μm in size; Sigma-Aldrich, St. Louis, Missouri, USA) were dyed using a 0.1% mixture of Congo red dye in order to facilitate bead retrieval (Lavine and Beckage 1996) and allowed to dry overnight in the hood. Once dry, the beads were suspended in Ringer's solution and stored in the refrigerator until injections were performed. Before caterpillars were injected, they were cooled in the freezer (−17°C) for 3 min to slow their metabolism and prevent thrashing movements. Approximately 5–15 beads suspended in Ringer's solution were injected into the caterpillar at the base of the third proleg. The injection site was covered using New-Skin Liquid Bandage (Medtech Products, Jackson, Wyoming, USA) to prevent desiccation and infection. Caterpillars were then returned to their food and given 24 h to encapsulate, then freeze-killed.

To retrieve the beads, caterpillars were dissected in Ringer's solution and melanization was compared between treatments by photographing beads using a camera mounted on a dissection microscope (Discovery v.8, AxioCam software; Carl Zeiss, Oberkochen, Baden-Wurttemburg, Germany). All photographs were taken at 80× magnification. Because the beads were dyed red before injecting them into the caterpillars, we were able to obtain an approximate 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 value of an image on a scale ranging from 0 to 255, where 0 is pure gray, and 255 is pure red. The lower the *r* value, the greater the degree of melanization will be. Using Adobe Photoshop (version 6.0), the *r* value was measured for all beads retrieved from a caterpillar and a mean *r* value was calculated for each individual caterpillar. The *r* value score was transformed into a percentage of melanization (1 − (*r* value/maximum *r* value)) for ease of interpretation.

Statistical analyses

All analyses were performed using SAS statistical software (SAS Institute 2008). We tested for a correlation between the mean percentage parasitism (number of individuals parasitized for each species collected over 15 years) and the mean percentage melanization of each species (average transformed *r* value for each species). We also performed separate correlation analyses to examine the relationship between melanization and the percentage parasitism from each parasitoid taxon (Hymenoptera, Diptera, Nematoda). Although the time scales for measuring parasitism and melanization were dramatically different, parasitism for host species in the La Selva database does not vary significantly among seasons or years and is a good predictor of other variables measured on different time scales (Gentry and Dyer 2002, Stireman et al. 2005).

We used a logistic regression (PROC LOGISTIC; SAS Institute 2008) to test if melanization was a good predictor of the likelihood of parasitism. In addition, because shelter building, group feeding, and caterpillar chemistry are the best predictors of parasitism frequency of caterpillars at La Selva (Gentry and Dyer 2002), we included these defenses in two additional logistic regression models (the values per species for these additional variables were determined in Gentry and Dyer 2002); one model included melanization, group feeding, and shelter building while the other model included melanization, group feeding, and chemistry.

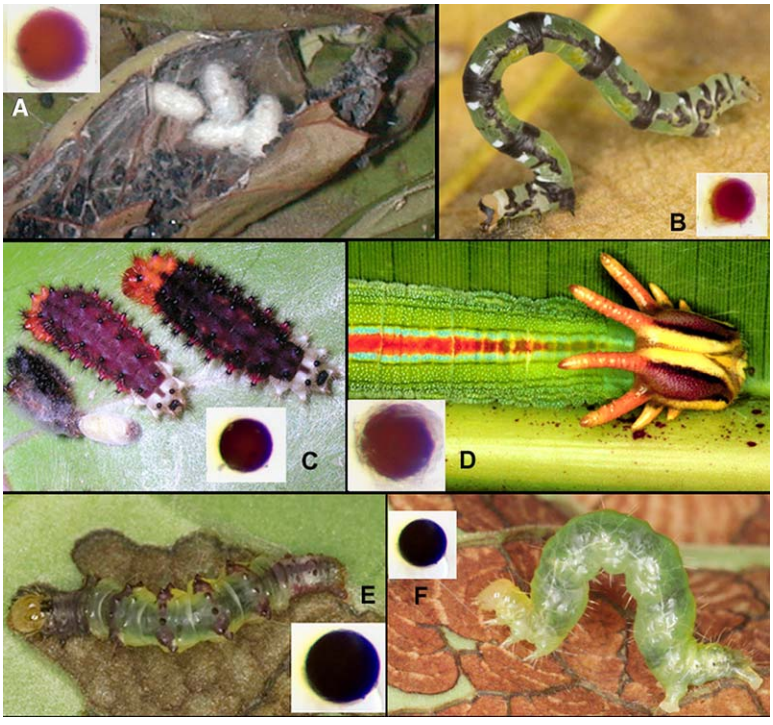

PLATE 1. A sample of species that have the lowest, intermediate, and highest levels of melanization. Inset pictures are representative beads from each species. (A) *Desmia* sp. (braconid cocoons), (B) *Cyclomia disparilis*, (C) *Emesis lucinda* (and one braconid cocoon), (D) *Opsiphanes tamarindi*, (E) *Eois apyraria*, and (F) *Eois nympha*. Photo credits: (A) and (D), G. L. Gentry; (B), Eduardo del Solar; (C), Humberto Garcia; (E) and (F), L. A. Dyer.

We utilized the same approach as Gentry and Dyer (2002): (1) the dependent variable used in the models was a dichotomous variable (parasitized vs. not parasitized); (2) each individual caterpillar was counted as a random sample from a group of individuals, each with specific characteristics; and (3) because all variables could not be included in one model, several models were utilized to rank predictor variables with standardized parameter estimates (SPE). For each logistic regression model, we utilized a subset of the parasitism data including 2559 results (i.e., adult or parasitoid) representing balanced data across all of the species used for encapsulation data (sample sizes per species varied from 43 to 253 individuals). The maximum likelihood method was used to fit the linear logistic regression models, likelihood ratios were used to assess the overall fit of the model, and the Wald chi square was used to assess individual parameter estimates.

RESULTS

The melanization response was variable between individuals ($s^2 = 79.5$), between species ($s^2 = 84.7$), and between families ($s^2 = 89.7$) of Lepidoptera. The families Arctiidae and Geometridae had the highest percentage melanization, and the Crambidae and Bombycidae had the lowest percentage melanization. Table 1 summarizes the percentage melanization and the frequency of parasitism for each species.

We found a significant negative correlation between percentage melanization and percentage parasitism for the tropical species of Lepidoptera (Fig. 1a, Pearson correlation coefficient, $r = -0.59$, $N = 16$, $P = 0.02$). When family was used as the unit of replication the correlation was stronger (Pearson correlation coefficient, $r = -0.79$, $N = 9$, $P = 0.01$). This negative correlation indicates that species or families with a weak immune response are more likely to be successfully parasitized. The mechanism by which this occurs is unknown. One possibility is that caterpillars with weak immune responses are unable to successfully encapsulate parasitoid eggs. An alternative explanation is that individuals with a strong immune response are avoided by parasitoids. Still another explanation is that individuals in poor physiological condition have a compromised immune response, and also have weaker behavioral defenses against parasitoids. Nevertheless, there is a strong positive correlation between immune response and frequency of parasitism.

When percentage parasitism was examined for different parasitoid taxa (Diptera, Hymenoptera, and Nematoda), we found that the relationship between parasitism and melanization differed significantly among parasitoids. There was a negative correlation

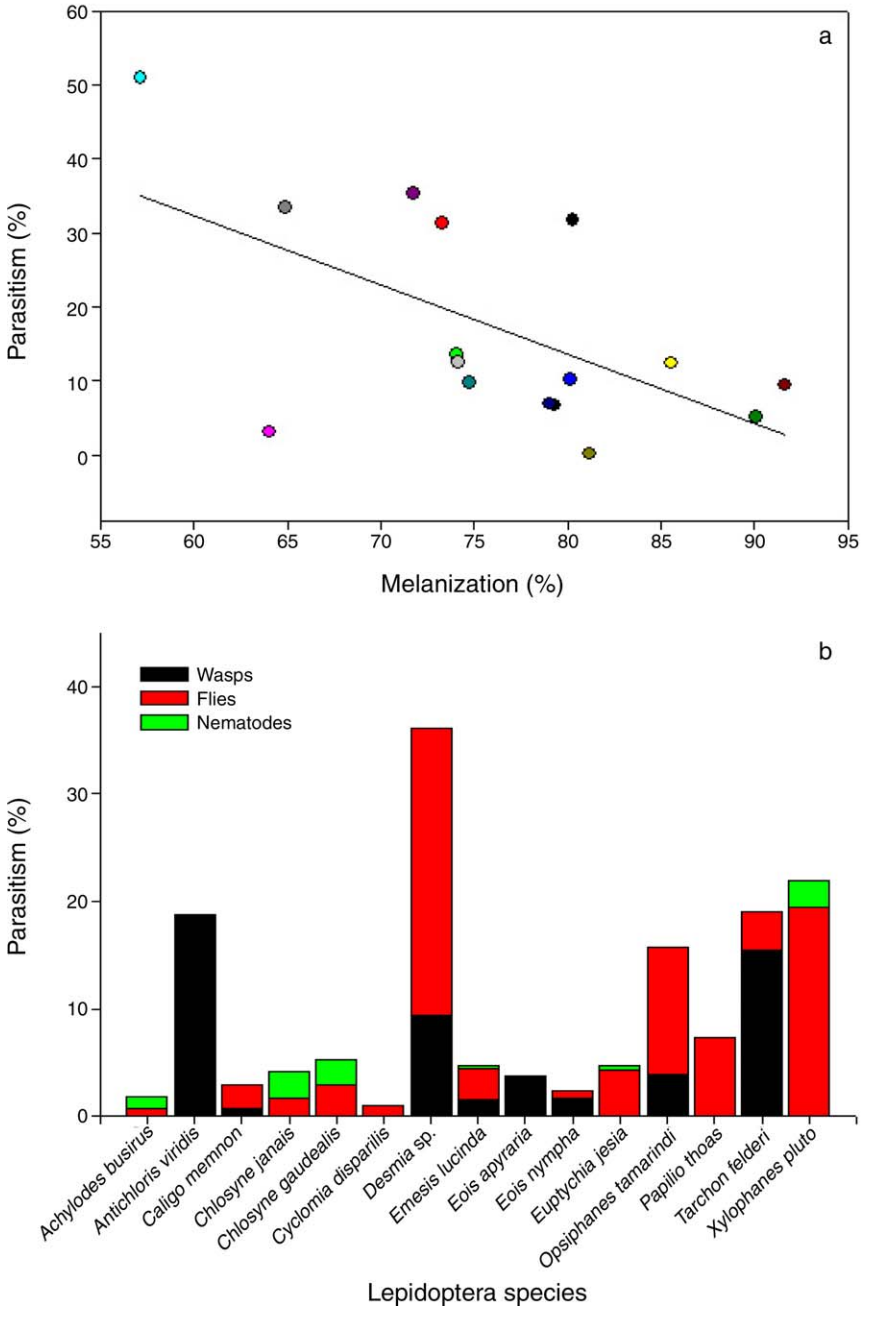

FIG. 1. (a) Negative correlation between the percentage of parasitism and the melanization response of 16 species of tropical Lepidoptera. Each circle represents the mean percentage parasitized and mean melanization response for each species. (b) Frequency of parasitism, by parasitoid taxa (wasps, flies, and nematodes), for each caterpillar species used in our study (the same species as in panel a). Frequencies were calculated as the number of parasitism events from a specific parasitoid taxon divided by the total number of parasitism events for that caterpillar species.

between melanization and parasitism for wasps (Pearson correlation coefficient, $r = -0.634$, $N = 10$, $P = 0.048$) and flies ($r = -0.581$, $N = 13$, $P = 0.037$) as we expected, but no correlation for the phylum Nematoda ($r = 0.089$, $N = 6$, $P = 0.866$). When the frequency of parasitism from a specific parasitoid taxon for each lepidopteran species was calculated, we found that the majority of parasitism was from flies (48%) followed by wasps (37%), and then nematodes (22%) (Fig. 1b).

The logistic regression revealed that the melanization response is a significant predictor of parasitism ($\chi^2 = 56.0$, df = 1, $P < 0.0001$; SPE = 7.4). When feeding modes were included in the logistic regression ($\chi^2 = 59.7$, df = 3, $P < 0.0001$), melanization (SPE = 6.3) was a

better predictor of parasitism than group feeding (SPE = 1.3) and shelter building (SPE = 0.01). When chemical defense was included ($\chi^2 = 14.0$, df = 3, $P < 0.003$), melanization (SPE = 2.1) was a better predictor than group feeding (SPE = 1.4) and chemistry (SPE = 0.9).

Discussion

The significant correlation between strength of melanization and frequency of parasitism supports the idea that the tertiary level of defense, which includes the melanization response, is an effective defense for caterpillars against immature parasitoids. Previous work has shown that parasitoids are not deterred by the same combination of defenses that work against more generalized predators like birds, spiders, and social wasps (Dyer and Gentry 1999). Gentry and Dyer (2002) found that caterpillars that are shelter builders, group feeders, and chemically defended had the highest likelihood of being parasitized, while caterpillars that regurgitated were best defended against wasp parasitoids. Barbosa and Caldas (2007) found that green coloration in caterpillars was associated with higher frequency of parasitism, unlike the other defensive traits that were measured. These studies mostly focused on traits that do not adequately protect caterpillars, but that enhance their likelihood of parasitism, while our study found a defensive trait that does work against parasitoids. In fact, the presence of an efficient immune response may be the most effective protection that caterpillars have against parasitoids, especially because not all caterpillars have effective suites of primary and secondary defenses but do have the ability to encapsulate. This is true whether parasitoids are avoiding good encapsulators or are killed via melanization once they have attacked. An interesting parallel is reported by Barbosa and Caldas (2007), who showed that brown coloration in caterpillars was associated with reduced parasitism. Darker coloration in caterpillars may be associated with higher levels of melanization because both rely on similar biochemical processes (Wilson et al. 2001, but see Cotter et al. 2008).

There was considerable variation in the melanization response between certain families of caterpillars. Species in the butterfly superfamily (Papilionoidea) all had high melanization responses that were similar to each other (Table 1). Additionally, the sister taxon to the butterflies, Hesperioidea, had similar melanization rates. Variation in the immune response among different orders of insects is unknown. However, the adaptive value of the immune response is likely to be dependent upon the suite of natural enemies that are most abundant or causing the highest mortality for a given herbivore population in a community. In populations suffering from high parasitoid mortality, natural selection should favor strong encapsulators. Several studies have shown that variation in the immune response is mostly due to quantitative genetic effects, with additional variation caused by environmental stress, diet, life history traits, feeding efficiency, and predation pressure (Kraaijeveld and Godfray 1997, Rolff and Siva-Jothy 2003, Schmid-Hempel and Ebert 2003, Rantala and Roff 2007, Smilanich 2008). Thus it is possible that enhanced encapsulation could evolve in taxa under strong selective pressure from parasitoids.

When we tested for a relationship between melanization and parasitism for hymenopteran and dipteran parasitoids, we found that the strength of the melanization response is not correlated with susceptibility to specific taxa of parasitoids. Other studies demonstrated that certain defenses work better against hymenopteran parasitoids than against dipteran parasitoids and vice versa (Mallampalli et al. 1996). For example, Gentry and Dyer (2002) found that avoidance behaviors such as thrashing, dropping, or biting were effective against hymenopteran parasitoids, but not against dipteran parasitoids. The fact that many dipteran parasitoids use indirect methods, such as microtype eggs or planidial larvae to infect their host, buffers the female parasitoid from coming into direct contact with the caterpillar, thus avoiding these defensive behaviors. For melanization, the attack behavior of the parasitoid is irrelevant because once the larvae/eggs are inside the caterpillar's hemocoel, the response is universal. Interestingly, there was no correlation between melanization and nematode attack, indicating that the immune response may not be an effective defense against nematodes.

The immune response can be compromised and does not always provide protection from parasitoids, which have evolved elaborate mechanisms for avoiding the immune response. For example, many female parasitoid wasps inject calyx fluid from the ovaries during oviposition (Godfray 1994). This fluid includes substances such as polydnaviruses that function to diminish the immune response (Lovallo et al. 2002). Some larval tachinids (*Ormia ochracea*) completely bypass the immune response by traveling to the abdominal cavity once they are inside the host, thereby, avoiding immune specific cells (Bailey and Zuk 2008). Factors associated with an herbivore's host plant such as nutritional quality (e.g., Ojala et al. 2005, Klemola et al. 2007, Yang et al. 2008) and secondary chemistry (Haviola et al. 2007, Smilanich 2008) can also affect the strength of the immune response. The effect of these host plant factors on the immune response is complex and is variable between studies. In our investigation, the difference in parasitism rates between caterpillar species may reflect variation in the immune response due to effects of host plants in addition to intrinsic genetic factors.

Based on the predictive power of melanization vs. other defensive traits associated with parasitism, it is clear that the immune response is one of the most important defenses against parasitoid wasps and flies. In addition to the utility of this predictive power for ecology, it may be important for applications such as biological control efforts, which have traditionally been hindered by a lack of herbivore characteristics that reliably predict predation and parasitism (Dyer and

Gentry 1999). Because all insects have the ability to encapsulate or melanize, focusing on variation in this particular defense to enhance ecological prediction or the success of biocontrol is likely to achieve successful results. It is clear that some species of caterpillars are better at melanizing than others. If the causes of this variation can be determined for key herbivores and crop pests, then agriculturalists and ecologists alike can make better predictions of an herbivore's susceptibility to parasitism.

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