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Research Article: Reduced consumption of protein-rich foods follows immune challenge in a polyphagous caterpillar

Short title: Immune challenged caterpillars avoid protein

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Summary

Advances in ecological immunity have illustrated that, like vertebrates, insects exhibit adaptive immunity, including induced changes in feeding behavior that aid the immune system. In particular, recent studies have pointed to the importance of protein intake in mounting an immune response. In this study, we tested the hypothesis that the polyphagous caterpillar, *Grammia incorrupta* (Hy. Edwards, Erebidae), would adaptively change its feeding behavior in response to immune challenge, predicting that caterpillars would increase their intake of dietary protein. We further predicted that this response would enhance the melanization response, a component of the immune system that acts against parasitoids. We challenged the immune system using either tachinid fly parasitoids or a bead injection technique that has been used in studies to simulate parasitism, and measured feeding before and after immune challenge on diets varying in their macronutrient content. To evaluate the effects of diet on melanization, we quantified melanization of beads following feeding assays. Contrary to our prediction, we found that parasitized or injected caterpillars given a choice between high and low protein foods reduced their intake of the high protein food. Furthermore, in a no-choice experiment, caterpillars offered food with a protein concentration that is optimal for growth reduced feeding following immune challenge, whereas those offered a low protein food did not. Although variation in protein intake did not change caterpillars' melanization response, increased

carbohydrate intake did increase melanization, suggesting a prophylactic role for carbohydrates. We discuss alternative mechanisms by which variation in protein intake could negatively or positively affect parasitized caterpillars, including nutritional interactions with the caterpillar's self-medication response.

Key words: ecological immunity, macronutrient, parasitoid, bead injection, illness-induced anorexia

Introduction

Immunity in insects has traditionally been characterized as innate, in contrast to immunity in vertebrates, which has been recognized as having both innate and acquired components (Medzhitov and Janeway, 1998). However, a growing body of empirical work in the field of ecological immunology has shown that immune parameters in insects respond to various ecological factors, and may be induced on timescales relevant to the individual's fitness (Best et al., 2013; Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2003; Schmid-Hempel, 2005; Schulenburg et al., 2009).

In insects, the strongest parallel to adaptive immunity in vertebrates (i.e., immunological memory) is immunological priming, whereby exposure to a pathogen acts in an inoculative manner, either with regard to future exposures in the treated individual, or in offspring (Kurtz and Franz, 2003; Moret and Schmid-Hempel, 2001; Moret and Siva-Jothy, 2003; Tidbury et al., 2011). However, induced immunological defense in insects is not limited to inoculation effects, and may act through pathogen-induced changes in behavior (Adamo, 2004). For example, desert locusts (*Schistocerca gregaria*) infected by a fungal pathogen were only able to produce viable offspring when they were permitted to thermoregulate to fever temperatures (Elliot et al., 2002).

In addition to illustrating that behavioral alteration of the thermal context for metabolic processes can affect immunity (Anderson et al., 2013; Elliot et al., 2002; Inglis et al., 1996), ecological immunity research also highlights the importance of chemical and nutritional inputs to the system (Adamo et al., 2010; Ayres and Schneider, 2009; Cotter et al., 2011; Lee et al., 2006b; Lefevre et al., 2010; nutritional aspects reviewed in Ponton et al., 2013; Siva-Jothy and Thompson, 2002). In a transgenerational example, monarch butterflies infected with a protozoan parasite adaptively select host plants that reduce infection in offspring (Lefevre et al., 2010).

Whereas medication studies focus on the therapeutic effects of plant secondary metabolites on individuals infected with parasites or pathogens (Lefevre et al., 2010; Simone-Finstrom and Spivak, 2012; Singer et al., 2009), nutritional studies focus on the role of primary plant metabolites in mediating these interactions (Adamo et al., 2010; Cotter et al., 2011; Lee et al., 2008; Povey et al., 2009; Srygley et al., 2009).

The insect immune response is composed of cellular and humoral responses that work in concert to defend against internal enemies (Beckage, 2008). Specialized immune cells (hemocytes) respond to signaling cascades initiated by the humoral response to isolate invaders and neutralize them via hemocyte asphyxiation and/or melanization cytotoxicity (Kanost and Gorman, 2008; Strand, 2008). These responses require significant amounts of nutrients and energy (Schmid-Hempel, 2003) and diet composition is an important factor contributing to immune efficiency (Lee et al., 2008; Siva-Jothy and Thompson, 2002).

The effects of dietary nutrients on the insect immune system are typically studied in terms of the macronutrients, protein and carbohydrate (Cotter et al., 2011; Lee et al., 2006b; Lee et al., 2008; Povey et al., 2009; Srygley et al., 2009). Macronutrients mediate normal physiological functioning in insects (Scriber and Slansky, 1981) and may be tightly regulated as insects forage (Behmer, 2009; Raubenheimer and Simpson, 2003; Simpson and Raubenheimer, 1993). Although protein and carbohydrate intake targets reflect the overall physiological requirements of a given species, there is also intra-specific variation in these nutritional optima, based on the insect's sex, genetic line, and physiological condition (e.g., Behmer and Joern, 2008; Cotter et al., 2011; Povey et al., 2009; Simpson and Raubenheimer, 1993). Some physiological functions, including processes involved in insect immunity, may be more protein or carbohydrate intensive than others (Cotter et al., 2011; Lee et al., 2006b; Povey et al., 2009; Srygley et al., 2009). When this is the case, plasticity in macronutrient regulation may facilitate enhancement of the immune response (Lee et al., 2006a; Povey et al., 2009). For example, in a study in which the generalist caterpillar *Spodoptera littoralis* was exposed to a nucleopolyhedrovirus, individuals fed diets with high protein to carbohydrate ratios were shown to have both increased resistance to the pathogen, and stronger constitutive immune function compared to individuals fed carbohydrate-biased diets. This led to the conclusion that protein costs of resistance were greater than energy costs (Lee et al., 2006b). Caterpillars that were

allowed to self-regulate their macronutrient intake made the adaptive dietary change, consuming a greater ratio of protein to carbohydrate than controls (Lee et al., 2006b).

Although Lee and colleagues used a generalist insect herbivore in their study, the variation in chemical and nutritional attributes that can exist within plant populations, and even individuals (Karban and Baldwin, 1997; Mattson, 1980), suggests that the adaptive regulation of macronutrients to enhance immunity is available even to monophagous or oligophagous species. However, the plausibility of this adaptive strategy would seem to depend on the variation in food attributes encountered by individuals in their environments. If so, grazing herbivores would be positioned particularly well to capitalize on both intra- and inter-specific plant variation (Lee et al., 2006a; Lee et al., 2003; Raubenheimer and Simpson, 1999; Raubenheimer and Simpson, 2003).

In this study, we tested the hypothesis that herbivores adaptively alter macronutrient intake in response to immune challenge, predicting that the altered diet increases melanization of hemocytes, a component of the insect immune system that acts against parasitoids (Lavine and Strand, 2002; Strand, 2008). We tested this hypothesis in the grazing caterpillar *Grammia incorrupta* (Hy. Edwards [formerly *geneura* (Strecker)]; Erebidae). The species self-medicates using pyrrolizidine alkaloids (PAs) found in some host-plant species when infected with the larvae of tachinid flies (Bernays and Singer, 2005; Singer et al., 2009). However, this defensive strategy is costly: ingesting large quantities of PAs in the absence of parasitism can result in mortality (Singer et al., 2009). The observation that self-medication occurred during the late stage of parasitoid infection led to the hypothesis that during the early stage, caterpillars alter their nutritional intake to bolster the immune system, and that self-medication behavior ensues if this relatively low cost, first line of defense fails (Smilanich et al., 2011a).

The particular questions addressed in this study are a) whether there is a change in relative intake of protein and carbohydrate following immune challenge in *G. incorrupta*, and b) if that change affects caterpillars' melanization response. Based on results showing the importance of dietary protein in mounting an immune response in general (Lee et al., 2006b; Lee et al., 2008; Povey et al., 2009), and the melanization response in particular (Lee et al., 2006b), we designed experiments to address the specific prediction that immune-challenged caterpillars would increase the proportion of protein in the diet. In the first experiment, we compared macronutrient regulation in individuals that were challenged by injection with Sephadex beads

(Lavine and Beckage, 1996) with that of individuals that were parasitized by tachinid flies. This experiment is unique in using both bead-injection and live endoparasites as immune challenges to test behavioral predictions. It thus provided a rare comparative test of effects of parasitism and bead injection, a presumed surrogate of parasitism used in many other studies. In the second experiment, we allowed caterpillars to self-regulate intake of macronutrients before and after bead injection, predicting that bead-injected caterpillars would choose a more protein-biased diet. In the third experiment, we offered caterpillars either a diet with a protein concentration that is optimal for growth or a low protein diet, prior to, and subsequent to bead injection, anticipating that caterpillars fed the low protein diet would ingest a greater amount of food in order to answer the protein demands of the immune response.

Results

Parasitism/Injection Experiment. The feeding behavior of immune-challenged caterpillars differed significantly from that of controls for the two days following immune challenge. There was a significant treatment effect on the total amount of food eaten on each day following parasitism or injection (ANCOVAs Day 1: $F_{2,79} = 9.45$, $P = 0.0002$; Day 2: $F_{2,74} = 9.94$, $P = 0.0001$). The Tukey test shows that feeding was reduced in both injected and parasitized individuals compared to controls (Fig. 1). Contrary to our prediction, reductions in food consumption were principally due to reductions in intake of the high protein food. In particular, parasitized individuals ate significantly less of the high protein diet than controls, a difference that was highly significant on the second day following infection (ANCOVAs Day 1: $F_{2,76} = 1.36$, $P = 0.35$; Day 2: $F_{2,76} = 10.84$, $P < 0.0001$)(Table S1)(Fig. 1). This result is also reflected in the diminished preference for high protein food in parasitized individuals in the second day following infection. Controls ate more high protein food than low protein food on both days (t-tests Day 1: $t = 2.55$, $df = 28$, $P = 0.016$; Day 2: $t = 3.39$, $df = 57$, $P = 0.0021$; Total: $t = 3.07$, $df = 27$, $P = 0.0049$). Injected caterpillars showed the same trend, but it was only significant the second day following infection (t-tests Day 1: $t = 1.81$, $df = 26$, $P = 0.083$; Day 2: $t = 2.55$, $df = 23$, $P = 0.018$; Total: $t = 2.23$, $df = 21$, $P = 0.037$), whereas parasitized caterpillars ate more high protein food the first day following immune challenge, but ate similar amounts of food on the

second day (t-tests Day 1: $t = 3.036$, $df = 28$, $P = 0.0051$; Day 2: $t = 0.62$, $df = 26$, $P = 0.54$; Total: $t = 2.24$, $df = 26$, $P = 0.034$).

Although the consumption data show that reduced feeding is driven by reduced intake of the high-protein food following immune challenge, we did not find a significant difference in the ratio of protein to carbohydrate consumed by different treatment groups (ANCOVAs Day 1: $F_{2,75} = 1.01$, $P = 0.44$; Day 2: $F_{2,75} = 0.31$, $P = 0.75$)(Table S2). However, there was a significant effect of the interaction between caterpillar family and treatment on the ratio of protein to carbohydrate chosen by caterpillars (ANCOVAs Day 1: $F_{4,75} = 6.05$, $P = 0.0003$; Day 2: $F_{4,75} = 3.27$, $P = 0.016$). Although the ANCOVAs did not detect differences in ratios of protein to carbohydrate consumed, the raw amounts of protein and carbohydrate consumed by caterpillars following immune challenge did differ (MANCOVAs Day 1: $F_{4,162} = 3.96$, $P = 0.0043$; Day 2: $F_{4,146} = 4.04$, $P = 0.0039$)(Table S3)(Fig. 2). In particular, planned comparisons showed that the macronutrient intake of parasitized individuals differed significantly from controls on both days following infection (MANCOVAs Day 1: $F_{2,54} = 10.70$, $P = 0.0001$; Day 2: $F_{2,52} = 10.80$, $P = 0.0001$), whereas injected individuals and controls did not differ significantly (MANCOVAs Day 1: $F_{2,52} = 2.39$, $P = 0.10$; Day 2: $F_{2,49} = 1.96$, $P = 0.15$). Differences in the bivariate response between parasitized and control individuals were associated with a reduction in both protein and carbohydrate intake during the first day (ANCOVAs Protein: $F_{1,55} = 10.43$, $P = 0.0021$; Carbohydrate: $F_{1,55} = 19.43$, $P < 0.0001$), and second day following infection (ANCOVAs Protein: $F_{1,53} = 21.57$, $P < 0.0001$; Carbohydrate: $F_{1,53} = 8.43$, $P = 0.0054$)..

Feeding Behavior Before and After Immune Challenge. Consistent with the Parasitism/Injection experiment, there was a treatment effect on the total amount of food consumed by caterpillars in this experiment, which we will refer to as the Choice Experiment (ANCOVA $F_{2,42} = 3.88$, $P = 0.028$)(Table S4). Immune-challenged individuals ate less food than controls, and reduced feeding was underlain by reductions in consumption of high protein foods following immune challenge (Fig. 3, "After immune challenge"). Also in keeping with the Parasitism/Injection Experiment, analyzing data in terms of ratio of protein to carbohydrate in self-chosen diets obscured these differences (Table S5). Treatment itself was not a significant determinant of the macronutrient ratio consumed, nor did we detect an effect of treatment on the change in protein to carbohydrate ratio before and after the time of injection (reflected in the lack of a significant

treatment * time interaction, Table S5). The bivariate analysis revealed a marginally significant change in nutrient regulation following immune challenge (MANCOVAs Before: $F_{4,132} = 1.08$, $P = 0.37$; After: $F_{4,90} = 2.29$, $P = 0.066$). Planned comparisons showed caterpillars in the injected treatment to differ significantly from controls (MANCOVAs Before: $F_{2,44} = 0.82$, $P = 0.45$; After: $F_{2,44} = 6.39$, $P = 0.0037$), whereas differences between sham-injected individuals and controls were marginally significant (MANCOVAs Before: $F_{2,42} = 2.05$, $P = 0.14$; After: $F_{2,42} = 3.02$, $P = 0.059$) following infection. Differences between injected and control individuals were due to reduced intake of both protein and carbohydrate following injection (ANCOVAs Protein: $F_{2,45} = 12.03$, $P = 0.0012$; Carbohydrate: $F_{1,45} = 5.76$, $P = 0.021$), whereas only protein intake was significantly reduced in sham-injected individuals (ANCOVA $F_{1,43} = 5.92$, $P = 0.019$) (Fig. 4).

Caterpillars in this experiment ate significantly more than those in the Parasitism/Injection experiment, which can be explained by their greater size: caterpillars used in the Choice Experiments were 30% larger than those in the Parasitism/Injection Experiment (t-test (caterpillar mass): $t = -11.03$, $df = 168$, $P < 0.0001$). Caterpillars in the Choice Experiment also showed a marked preference for the low protein food in contrast to those in the Parasitism/Injection Experiment, which preferred the high protein food in the absence of immune challenge (Fig. 1).

When caterpillars were allowed to self-regulate their intake of macronutrients, the tendency to eat less food following injection did not improve melanization capability (Fig. 5). The amount of food consumed by caterpillars following injection was positively (though weakly) associated with bead melanization (Fig. 5). Because the amounts of protein and carbohydrate consumed were highly correlated ($r = 0.95$, $P < 0.0001$), it is difficult to discern if one of these macronutrients or the other is responsible for this relationship. However, when protein was correlated with melanization, it yielded a marginally significant result (Spearman's $\rho = 0.45$, $P = 0.074$), whereas when carbohydrate and melanization were correlated a significant result was obtained (Spearman's $\rho = 0.50$, $P = 0.047$).

No-Choice Experiment. The amount of food that caterpillars consumed depended on the immune-challenge treatment (ANCOVA: $F_{2,105} = 5.98$, $P = 0.0045$), the macronutrient content of the diet ($F_{1,105} = 13.03$, $P = 0.0007$), the interaction between time and treatment ($F_{2,105} = 5.69$, P

= 0.0045), and the three way interaction between time point, level of immune challenge and diet ($F_{2,105}$ = 4.16, P = 0.018)(Table S6). Contrary to the prediction that caterpillars would increase intake of low protein food in response to an immune challenge, caterpillars in all three treatment groups consumed the same amount of low protein food after the time of injection (Fig. 6). However, both bead-injected and sham-injected caterpillars consumed significantly less optimal protein food than controls, with the size of the reduction tracking the severity of immune challenge; sham-injected caterpillars ingested 26.9% less optimal protein food, and bead-injected individuals ingested 58.3% less optimal protein food than controls (Fig. 6). In addition, caterpillar families varied significantly in how much food they consumed ($F_{3,105}$ = 6.57, P = 0.0006).

The observed reduction in optimal protein food intake among immune-challenged individuals did not adaptively affect melanization. We did see a negative correlation between amount of food eaten and melanization but it was non-significant (Fig. 5). Instead, the amount of low protein diet consumed prior to injection was significantly and positively associated with the degree to which beads were melanized (Fig. 5).

Discussion

Our findings support the hypothesis that the dietary generalist herbivore, *G. incorrupta*, modifies its macronutrient intake in response to immune challenge. However, contrary to our prediction, immune-challenged caterpillars did not increase their intake of dietary protein. In fact, caterpillars reduced feeding in response to immune challenge, and this reduction was stronger with regard to the high protein food, than the low protein food. Interestingly, control caterpillars in the Parasitism/Injection Experiment preferred high protein food, whereas those in the Choice Experiment preferred low protein food (Figs. 1, 3). We are uncertain as to what underlies this difference in preference but speculate that it may be the result of differences in caterpillar stock used. These differences could be genetic, or could stem from transgenerational environmental effects, since the parents of caterpillars in the Parasitism/Injection experiment were collected from the wild, whereas those used in the Choice and No-choice experiments had been bred for several generations under laboratory conditions. Alternatively, some caterpillars used in the Parasitism/Injection experiment could have been in their penultimate, rather than final larval stadium. *Grammia incorrupta* exhibits life-history plasticity in the number of stadia it

undergoes, and there is a high degree of body size variation within each stadium. Since caterpillars were freeze-killed following feeding assays, we cannot be sure whether they would have undergone an additional stadium. If so, it would not change the relevance of this study, given that tachinid flies readily attack, and are successful on *G. incorrupta* individuals during both stages of their life history (personal observation). The observation that caterpillars in the two experiments converged on the tendency to eat less protein-rich food following immune challenge suggests that this response may be adaptive when circumstances (e.g., genetic background, hormonal milieu) vary.

It may seem counterintuitive that caterpillars could specifically reduce their intake of high protein food following parasitism without significantly changing the ratio of macronutrients in the diet. However, this is a possibility associated with the experimental diets used here. Each time caterpillars ingested some protein, they would necessarily ingest some carbohydrate and vise versa, perhaps swamping out variation in proportional consumption. The relative aversion to high protein foods seen here suggests that, rather than bolstering the immune response as shown in *Spodoptera* species (Lee et al., 2006b; Povey et al., 2009), excess dietary protein may be detrimental to immunity in *G. incorrupta*. However, if the cost of consuming a high protein diet stemmed from a negative effect of protein on the melanization response, we would have expected protein consumption after injection to be negatively correlated with bead melanization, an expectation that was not met by results of the Choice Experiment (Fig. 5). A stringent test of a costly-protein hypothesis would require measuring immune attributes in response to a high protein diet, rather than an optimal-protein or self-regulated diet.

Although protein content of the diet was not positively correlated with melanization, as anticipated, our results do suggest that dietary nutrients interact with the melanization response in *G. incorrupta*. When caterpillars were fed a low protein, carbohydrate-rich diet, the amount of food consumed before injection was positively correlated with bead melanization. However, the same was not true of caterpillars fed the optimal protein diet (Fig. 5). Because protein and carbohydrate were inversely correlated in experimental diets, this could mean either that caterpillars benefitted from increased carbohydrate, or from reduced protein in foods, prior to immune challenge. If protein were detrimental to the melanization response, we would have expected the amount consumed of the optimal protein diet prior to injection to be negatively

correlated with melanization, which was not the case (Fig. 5). This suggests that carbohydrate, rather than protein, may limit the prophylactic action of nutrients toward immunity. A similar result was found in the mosquito, *Anopheles gambiae*, which melanized beads to a greater degree when reared on diets rich in glucose (Schwartz and Koella, 2002). Increased feeding on carbohydrate-rich foods may lead to greater mass of the fat body, the site of production for many immune precursors (Beckage, 2008). If carbohydrate consumption increases the mass of the fat body, this is one mechanism by which melanization capability may have been enhanced.

Interestingly, there was no correlation between the amount of food eaten and the degree of bead melanization when caterpillars were allowed to self-regulate their macronutrient intake prior to injection (Choice Experiment, Fig. 5). This suggests that, in the absence of immune challenge, caterpillars self-regulate to a lower carbohydrate intake target than would provide a prophylactic benefit to the immune system (21P:19C when self-regulated, compared to 15P:25C)(Fig. 5). This could result, for example, if the carbohydrate requirement of the melanization response conflicted with the protein requirement of growth and reproduction. Tradeoffs between the immune system and life-history traits (Adamo et al., 2010; Cotter et al., 2008; Fedorka et al., 2004; Ponton et al., 2011; Zuk and Stoehr, 2002), as well as those between different parameters within the immune system are well documented (Cotter et al., 2004; Cotter et al., 2011; Povey et al., 2009). A potential tradeoff with particular relevance to this system might be that between the balance of nutrients and the balance of beneficial plant secondary metabolites in the insect's diet. Eating a mixture of plants containing different defensive chemicals acts to defend *G. incorrupta* against at least one generalist predator (P. A. Mason et al., unpublished). If the defensive benefit of mixing host plants (in the absence of parasitism) is stronger than the benefit of prophylactic enhancements to the immune system, caterpillars would be expected to mix foods on short timescales, even if doing so would lead to a sub-optimal melanization response, as seen here. The effects of nutrients in conjunction with secondary metabolites can indeed affect dietary preference and the performance consequences thereof (Behmer et al., 2002; Slansky and Wheeler, 1992).

The observation that the amount of food eaten and melanization were only positively correlated when caterpillars were allowed to self-regulate dietary macronutrients (Fig. 5) contrasts with the finding by Cotter and colleagues (2011) that the macronutrient ratio in the diet

affects immune attributes more strongly than the caloric density of food. Instead, it suggests that the quality and quantity of foods may interact to affect immune parameters in *G. incorrupta*. A similar effect was seen in an investigation of how illness-induced anorexia might reduce competing demands of immunity and digestion in the cricket, *Gryllus texensis* (Adamo et al., 2010). Resistance to bacterial infection was reduced when crickets were fed lipid-rich foods, and although crickets reduced feeding following immune challenge, they exhibited the adaptive preference for foods with low lipid content at that time (Adamo et al., 2010).

A number of hypotheses have been put forth to explain anorexic behavior in response to disease (Adamo, 2006; Kyriazakis et al., 1998), and of these, four can be addressed to some extent by this work. One is that parasitoids induce reductions to feeding for their own benefit. Although adaptive parasite manipulation of host feeding behavior has been shown in some systems (Hughes et al., 2012; Moore, 2002), this explanation is unlikely given that bead-injected individuals also exhibited an anorexic response (though one that was less pronounced than that of parasitized individuals). Another hypothesis, that anorexia enhances the immune response, is not supported by our melanization results, however, there are many immune parameters that we did not measure here.

Two related hypotheses regarding disease induced anorexia do seem to be supported by this study, 1) that anorexia allows individuals to be more selective in the foods that they eat, and 2) that anorexia serves to starve parasites. In this study, caterpillars exhibited an anorexic response that differed with respect to different types of foods, supporting the former hypothesis. The latter has been discounted to some extent on the basis that the main prediction of the hypothesis is not generally met in mammals (Kyriazakis et al., 1996; Kyriazakis et al., 1994), namely that the anorexic response should be more pronounced with regard to high quality foods than low quality foods (Kyriazakis 1998). However, our results do meet this expectation; caterpillars exhibited anorexia particularly with regard to protein-rich foods (see also Adamo et al., 2010).

Perhaps reduced ingestion of high protein foods acts to retard the development of parasitoids. Protein levels in the hemolymph respond to dietary protein (Lee et al., 2008; Povey et al., 2009; Thompson et al., 2005), and can affect parasitoid development (Thompson et al.,

2005). If this is the case here, lower protein titers in the hemolymph could translate to slower growth of parasitoids during the early stage of infection, when parasitoid larvae are likely to be most vulnerable to the host's melanization response. An immunological strategy that combines slowing growth of parasitoids by nutritional means with the melanization response could be particularly effective in *G. incorrupta* because a) their grazing feeding strategy allows them to access the necessary nutritional variation, and b) they possess a particularly strong melanization response relative to other caterpillar species (A. M. Smilanich, personal observation). Moreover, such a strategy may incur little cost, given that a lower protein diet can afford *G. incorrupta* a comparable growth benefit to the optimal protein food used here (see Fig. A1, Appendix II).

Because parasitized caterpillars in this study succumbed to parasitoid infection (data not shown), we conclude that anorexia alone is insufficient to overcome parasitoids. However, it is possible that anorexia acts in conjunction with melanization and/or self-medication to defend caterpillars against parasitoid infection. *Grammia incorrupta* caterpillars self-medicate using pyrrolizidine alkaloids during the late stage of parasitoid infection (approximately 96 hours after oviposition), enhancing their survival (Singer et al., 2009; Smilanich et al., 2011a). If the efficacy of self-medication is contingent on the condition (e.g., size) of parasitoids at that time-point, the effects of caterpillar diets on parasitoid development could have major fitness consequences under natural circumstances, when caterpillars can harness both macronutrient and chemical variation in plants. This hypothesis is consistent with the expectation that generalist herbivores should be positioned particularly well to employ complex, immunity-enhancing behavioral strategies (Lee et al., 2006a; Lee et al., 2003; Raubenheimer and Simpson, 1999; Raubenheimer and Simpson, 2003).

Although both parasitized and unparasitized, immune-challenged caterpillars exhibited anorexia, we also observed a difference in nutrient intake between parasitized and injected caterpillars in the Parasitism/Injection Experiment (Fig. 1). One possible explanation is that parasitoids had taken control of host nutrient intake for their own benefit (Hughes et al., 2012; Moore, 2002). As we did not measure the effects of diet on parasitoid fitness here, this hypothesis is difficult to evaluate. Another possibility is that parasitism disrupted the caterpillar's regulation of nutrient intake. Thompson and Redak (2005) showed such a breakdown in *Manduca sexta* caterpillars in response to wasp parasitism by using choice experiments

364 employing multiple pairs of foods that differed in their macronutrient content. Using this design
365 they were able to conclude that parasitized individuals fed indiscriminately, whereas controls
366 maintained a macronutrient intake target regardless of the macronutrient ratios in the pairs of
367 foods offered (Thompson and Redak, 2005). Our experimental design precludes using this
368 method to draw such a conclusion; however, if nutrient regulation had broken down in response
369 to parasitism, we would expect greater variance in the amounts of each food eaten by parasitized
370 and control individuals. To test this, we applied Brown-Forsythe tests for unequal variances to
371 the proportion of high protein food eaten each day following parasitism, and found that variances
372 did not differ among treatments (Day 1: $F_{2,82} = 1.166$, $P = 0.20$; Day 2: $F_{2,76} = 2.21$, $P = 0.12$).
373 Nonetheless, differences in the extent to which feeding was affected in parasitized and injected
374 individuals illustrates that at least some part of the cue inducing this change is biotic.

375 **Conclusions**

376 Contrary to findings from similar studies, immune-challenged caterpillars reduced their
377 intake of high protein food. Prior to immune challenge, greater intake of carbohydrate-biased
378 diets improved the melanization response. After immune challenge, increased feeding on diets
379 with self-selected macronutrient ratios improved melanization, whereas eating more of diets with
380 fixed macronutrient ratios did not. This suggests that immune function is affected by the
381 interaction between food quality (macronutrient ratio) and quantity in *G. incorrupta*. We
382 hypothesize that these dietary attributes may also interact with developing parasitoids, and their
383 susceptibility to anti-parasitoid resistance from both the melanization response, and self-
384 medication by their hosts. These findings reinforce the notion that the immune response,
385 including its behavioral components, can be expected to differ depending on the host, the
386 pathogen or parasite, and numerous other ecological considerations.

387

388 **Materials and Methods**

389 *Study System*. Caterpillars of *Grammia incorrupta* (Erebidae) are grazing generalist herbivores,
390 feeding on over 80 species of plants in 50 different plant families (Singer and Stireman, 2001).
391 This species inhabits arid grasslands and woodlands of the Southwestern US and Northwestern
392 Mexico (Schmidt and Sperling, 2008). Host-plant switching is a common behavior and moving

between individual host plants over the course of a day is a regular occurrence (Singer et al., 2002). This grazing dietary strategy benefits the species by improving its physiological efficiency (P. A. Mason et al., unpublished), as well as providing defense against natural enemies (Singer et al., 2004; Singer and Stireman, 2003). On average, 15% of *G. incorrupta* caterpillars in natural populations experience mortality from parasitoids, with the majority of parasitism coming from tachinid fly species, including *Exorista mella* and *Chetogena* species, and to a lesser extent from hymenopteran parasitoids (Stireman and Singer, 2002). Given the nutritional variation that individuals are likely to encounter by using such a broad range of host plants, it seems likely that grazing individuals could also adaptively alter their diet to support the immune system.

These experiments took place in the Singer lab at Wesleyan University. The Choice and No-Choice experiments were performed in the fall of 2008, and the Parasitism/Injection Experiment was performed during the summer of 2009. Caterpillars used for the experiments were taken from a laboratory breeding colony, initiated from caterpillars originally collected in southeastern Arizona, USA. Colony individuals were reared on a nutritious, wheat-germ based rearing diet (Yamamoto, 1969), as were individuals used in experiments prior to feeding on experimental diets. All caterpillars used in experiments were housed in 167.2 ml clear plastic cups (Russell Hall Co., Meriden, CT, USA).

Parasitism/Injection Experiment. The purpose of this experiment was to test a) for changes in feeding behavior in response to immune challenge, and b) whether the Sephadex bead injection technique (described below), which has been used in prior studies to mimic parasitoid infection in *G. incorrupta* and other species (Lavine and Beckage, 1996; Smilanich et al., 2011a; Smilanich et al., 2011b), elicits the same feeding behavior in *G. incorrupta* as parasitism by a tachinid fly. We predicted that, when allowed to self-regulate, both parasitized and injected caterpillars would consume more of the high protein food than controls. We are confident that the fly species used here attacks *G. incorrupta* during the final larval stadium in the wild because we obtained flies for the laboratory colony by collecting *G. incorrupta* caterpillars in their final stadium upon which fly eggs were visible.

After the final larval molt, we weighed caterpillars and distributed them among three treatments: those that would act as controls, those that would be injected with beads, and those

that would receive parasitoid eggs. The low protein food contained 15% protein and 25% carbohydrate, by dry weight, and the high protein food contained 35% protein and 5% carbohydrate, by dry weight (see Appendix I for complete list of ingredients). We varied macronutrient ratios, rather than raw amounts, because protein and carbohydrate concentrations in plants are often inversely correlated (Bernays and Chapman, 1994) and their consumption by insect herbivores non-independent (Raubenheimer and Simpson, 1999; Raubenheimer and Simpson, 2004; Simpson and Raubenheimer, 1993; Simpson et al., 2004). Presenting food to caterpillars in this manner allowed caterpillars to self-regulate to a target ratio.

Caterpillars in the parasitism treatment were exposed to tachinid flies, either *Chetogena edwardsi* or *C. tachinomoides*, on the day of their final larval molt. We used two closely related fly species in these experiments because both were present in our tachinid colony at the time and we could not reliably distinguish the two species during experiments. After the experiment, we received confirmation from a taxonomic expert (J.O. Stireman) on the identities of tachinid specimens saved from the experiment. Although it is possible that these congeners elicit different feeding responses in *G. incorrupta*, we did not test that experimentally. Caterpillars were exposed to flies for several minutes until they had received 1-3 eggs. Three attempts were permitted, and then caterpillars were inspected more closely in a clear plastic vial to ensure that at least one egg was present. Since it takes 48 to 60 hours for *Chetogena* larvae to hatch from eggs and burrow through the cuticle (Smilanich et al., 2011a), caterpillars in the injection treatment were injected two days after the final larval molt, so that the moment of injection would approximate the moment that parasitoids entered caterpillars (injection technique described under *Immune Assay* below). We measured the amounts of each food block eaten by caterpillars in all three treatments for two days following the time of immune challenge in order to assess whether injected caterpillars grouped with controls or parasitized caterpillars in how much food, and the ratio of protein to carbohydrate that they consumed. To do this, we weighed initial amounts of food provided to caterpillars on both feeding days and converted these to dry weights using a wet-dry conversion curve. Dry weights of food that remained after 24 hours (food was removed after each of the two feeding days) were then subtracted from initial dry weights to determine the dry mass of food eaten each day.

Feeding Behavior Before and After Immune Challenge. In this experiment we tested whether caterpillars regulate macronutrient intake differently before and after immune challenge. We predicted that caterpillars would bias macronutrient intake towards protein following injection by ingesting a greater amount of the high protein food than controls. As in the previous experiment, injection with Sephadex beads represented the challenge to the immune system (Lavine and Beckage, 1996). Unlike in the previous experiment, we included a sham injection group in which individuals were injected with only isotonic Ringer's solution and no beads to control for the wound response to injection (Smilanich et al., 2011a). We predicted that immune-challenged individuals would regulate their macronutrient ratio toward a higher protein intake in response to the immune challenge. On the third day of the final larval stadium, caterpillars were offered blocks of both low protein and high protein foods (15P:25C and 35P:5C dry weight respectively), and allowed to self-regulate their macronutrient intake for 24 hours prior to bead-injection, sham, and control treatments. After the time of immune challenge, caterpillars were given fresh food blocks and allowed to feed for an additional 24 hours. The third day and fourth day of the stadium were chosen for feeding assays because they represent the middle of the final larval stadium, when caterpillars feed most (pers. obs.). For comparison, the timing of immune challenge was one day later in this experiment than in the Parasitism/Injection experiment. Because some caterpillars did not eat for several days after molting, we allowed the day number to vary to ensure that caterpillars had initiated feeding before receiving the immune challenge. Amounts of food eaten were determined as described above, and injected individuals were freeze-killed at the end of the feeding trial and dissected later to determine bead melanization.

No-Choice Experiment. In this experiment, we tested whether there would be differences in caterpillars' consumption of two diets that differed in their macronutrient ratio after immune challenge. We predicted that immune-challenged caterpillars would increase protein consumption through compensatory feeding on the low protein diet (Raubenheimer and Simpson, 1993; Slansky and Wheeler, 1992). Therefore, we expected greater consumption of the low protein diet than the high protein diet among immune-challenged individuals. As in the Choice Experiment described above, we challenged the immune system using bead injection during the fourth day of the final larval stadium, and compared feeding responses between injected individuals, sham-injected and control groups. Conducting a no-choice test in

conjunction with the choice test described above would also permit us to differentiate between preference for a given food type, and aversion to the alternative.

All individuals were subjected to a no-choice feeding assay for 24 hours prior to, and 24 hours subsequent to, the time of injection. Caterpillars were offered either a low protein, or an optimal protein food (15P:25C and 25P:15C dry weight respectively), so that mixing foods was not a possibility. We consider 25P:15C an optimal ratio because preliminary experiments showed that a) it afforded *G. incorrupta* the greatest growth on average among five experimental diets that varied in their macronutrient ratios (Fig. S1, Appendix II), and b) caterpillars chose a similar ratio when allowed to self-select a macronutrient intake target (Fig. S2, Appendix II).

On the third day of the seventh larval stadium, individuals were randomly assigned to injection, sham, or control groups as well as optimal protein diets or low protein diets. Each treatment level received 20 individuals. After 24 hours of feeding, individuals were injected with Sephadex beads or sham injected, then returned to their respective diets to continue feeding for another 24 hours. We measured amounts of food eaten on each day using the method described above. Injected individuals were freeze-killed at the end of the feeding trial and dissected later for retrieval of beads (see Immune Assay below).

Immune Assay. To measure the melanization response to dietary nutrition, *G. incorrupta* caterpillars were injected with Sephadex beads (Sephadex A25, 40-120 μm; Sigma-Aldrich, St. Louis, MO, USA) as a proxy for parasitism (Lavine and Beckage, 1996; Smilanich et al., 2009a; Smilanich et al., 2009b). We predicted an increase in the melanization response in individuals with an optimal ratio of dietary protein to carbohydrate. Sephadex beads were dyed red using 0.1% Congo red (dye content 35%; Sigma-Aldrich, St. Louis, MO, USA) and were suspended in Ringer's solution so that 5-10 beads could be injected into the base of the third proleg. Injections were done using Pasteur pipettes (Sigma-Aldrich, St. Louis, MO, USA) that we had stretched under heat in order to create tiny glass needles (Lavine and Beckage, 1996). Caterpillars were then returned to their test diets and freeze-killed at the end of the feeding trial (after an additional 24 hours). To retrieve beads, caterpillars were dissected in 95% methanol and beads were photographed using a camera mounted on a dissection microscope focused at 80x magnification (Carl Zeiss Discovery V.8, AxioVision software; Carl Zeiss Microscopy, LLC, Thornton, NY, USA). Since the beads were dyed red before injecting them into the caterpillars, we quantified

melanization by measuring the red value, a scale ranging from 0-255, where 0 = pure gray, and 255 = pure red, for each bead. The lower the r-value, the blacker the bead, indicating increasing levels of melanization. Using Adobe Photoshop (version 6.0), 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, so that high values indicate a greater degree of melanization and vice versa (Smilanich et al., 2009a; Smilanich et al., 2009b).

Statistical Analysis

Parasitism/Injection Experiment: We used ANCOVA to assess differences in amounts of food eaten following immune challenge. This was done for total food eaten, and for high protein and low protein foods separately. Models included treatment, family (treated as a random effect), initial mass, and significant two-way interactions. We used Tukey tests to identify differences in amounts of foods eaten by caterpillars in different treatments. To assess changes in preference associated with immune challenge, we used paired t-tests.

To identify differences in nutrient regulation in the two days following immune challenge, we analyzed the ratio of protein to carbohydrate consumed, and the bivariate response, amounts of protein and carbohydrate consumed. The ratios of protein to carbohydrate consumed were log transformed and analyzed using ANCOVA with the same factors in the models as we used for the consumption data. We analyzed amounts of protein and carbohydrate consumed using MANCOVAs (main effect: treatment; covariate: initial mass) to identify treatment differences in self-regulated macronutrient intake, and performed planned comparisons to discern which treatment(s) differed from the control. We also performed univariate planned comparisons to identify whether intake of protein, carbohydrate, or both were responsible for significant differences between treatments.

Choice Experiment: We used the same analytical procedures in the Choice Experiment as in the Parasitism/Injection experiment, with a few modifications. Because, in this case, we measured consumption before and after immune challenge, we used repeated measures ANCOVAs to analyze both consumption data, and protein to carbohydrate ratios. Repeated measures ANCOVA models included the independent variables immune challenge (bead-injected, sham-

injected, control), time (before injection, after injection), and the time*treatment interaction. We did not include family as a covariate in these models, or in those for the No-Choice experiment because there were too few individuals of the same family used in the experiments for meaningful interpretation of family effects. We did not use t-tests to evaluate changes in food preference associated with immune challenge because the set of caterpillars used in this experiment exhibited clear preferences for low protein foods regardless of dietary treatment. Differences in the strength of this preference are reflected in results of Tukey tests applied to consumption data.

No-Choice Experiment: We analyzed the amounts of food ingested by caterpillars before and after injection using repeated measures ANCOVA, with the same variables indicated for the Choice Experiment, with the addition of the variable diet (low protein, optimal protein). Tukey tests were used to identify treatment differences in amounts of foods eaten. Because caterpillars ate only one diet in this experiment, protein and carbohydrate intake were perfectly correlated with the amount of food consumed, precluding separate analyses of how each macronutrient affected melanization.

Injection Assay: To assess the effect of the amounts of foods eaten on melanization when caterpillar body size was accounted for, we regressed amount of food eaten over caterpillar mass, and used residuals in Spearman's rank correlations with melanization data.

All statistics were calculated using JMP statistical software (JMP, 2007). Full models and their results can be found in Tables S1-S6 of the supplementary materials.

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Author Contributions

M.S.S. is responsible for conception of the project. All authors were involved in experimental design, P.A.M. and A.M.S executed the experiments, and all authors were involved in the interpretation of results. P.A.M. wrote the manuscript, and M.S.S. and A.M.S. edited it.

Competing Interests

No competing interests declared.

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Figure Legend

Figure 1. Amount of high protein and low protein foods consumed by caterpillars for the first (above) and the second (below) 24-hour period following the time of immune challenge in the Parasitsm/Injection Experiment. Least square means were derived from the repeated measures ANCOVA, detailed in Table S1. Letters above bars correspond to Tukey tests performed on total amounts of foods (italics), amounts of high protein foods (capital) and amounts of low protein foods (lower case) eaten. Letters are absent above individual bars in the top panel because amounts of individual foods did not differ significantly across treatments. Columns or pairs of columns not sharing a letter of the same case or style are statistically distinct. Error bars show standard errors and numbers at the base of columns indicate sample sizes.

Figure 2. Bivariate least square means (+/- 1 SE) of protein and carbohydrate intake for caterpillars in the Parasitism/Injection Experiment in the first day (above) and the second day (below) following immune challenge. Least square means account for variation in family, its interaction with treatment, and the initial masses of caterpillars. Symbols where trajectories terminate represent the intake points (non-cumulative) reached each day following immune challenge. The broken line indicates the trajectory if caterpillars had eaten equal amounts of protein and carbohydrate. For statistical comparison of intake points, see Table S3.

Figure 3. Amount of high protein and low protein food consumed by caterpillars in control, sham-injected, and injected treatments in the 24 hours before (above) and after (below) the time

of immune challenge in the Choice Experiment. Least square means were derived from the repeated measures ANCOVA, detailed in Table S4. Letters above bars correspond to Tukey tests performed on total amounts of foods (italics), amounts of high protein foods (capital) and amounts of low protein foods (lower case) eaten. Letters are absent above individual bars in the top panel because amounts of individual foods did not differ significantly across treatments. Columns or pairs of columns not sharing a letter of the same case or style are statistically distinct. Error bars show standard errors and numbers at the base of columns indicate sample sizes.

Figure 4. Bivariate least square means (+/- 1 SE) of protein and carbohydrate intake for caterpillars in the Choice Experiment for the day before (above) and the day following (below) immune challenge. Least square means account for variation in family, its interaction with treatment, and the initial masses of caterpillars. Symbols where trajectories terminate represent the intake points (non-cumulative) reached for the 24-hour period before and after immune challenge. The broken line indicates the trajectory if caterpillars had eaten equal amounts of protein and carbohydrate. For statistical comparison of intake points, see Table S3.

Figure 5. Correlations between food consumption (corrected for caterpillar size) and melanization of beads in *G. incorrupta* before and after immune challenge when fed three experimental diets (LP = low protein, OP = optimal protein, and Choice = self regulated between high and low protein foods). Trendlines are only drawn, and statistics provided when the Spearman's rank correlation was significant. Scale was omitted from some panels for clarity, but in each case, the x-axis ranges from -200 to 200, and the y-axis ranges from 0-80%.

Figure 6. Amount of food consumed by caterpillars in control, sham-injected, and injected treatments in the 24 hours before (above) and following (below) immune challenge in the No-Choice Experiment. Least square means were derived from the repeated measures ANCOVA, detailed in Table S6. Capital and lower-case letters correspond to Tukey tests performed on data from high protein and low protein fed groups separately. Columns not sharing a letter of the same case are statistically distinct. Error bars show standard errors and sample sizes appear at the base of each column.

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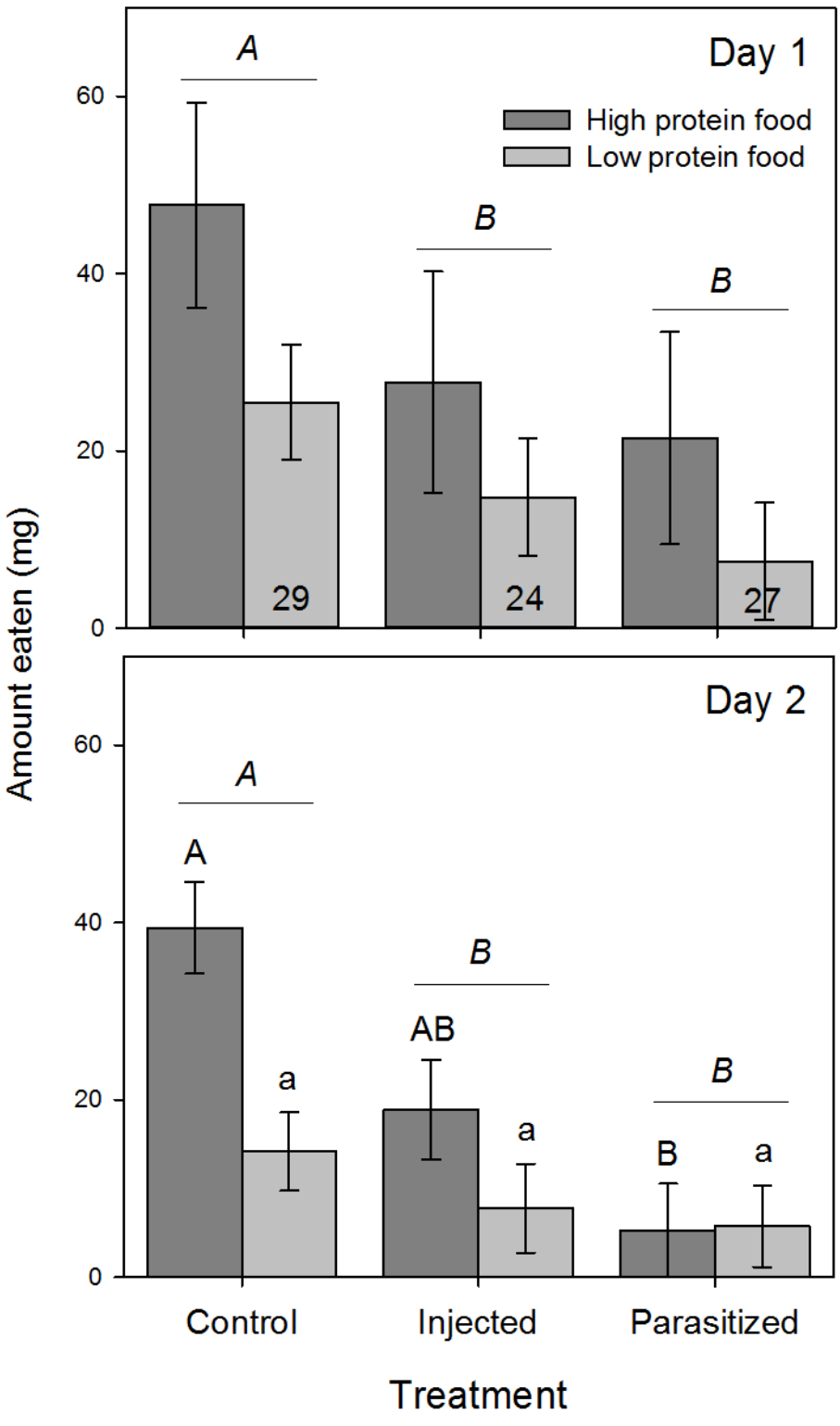
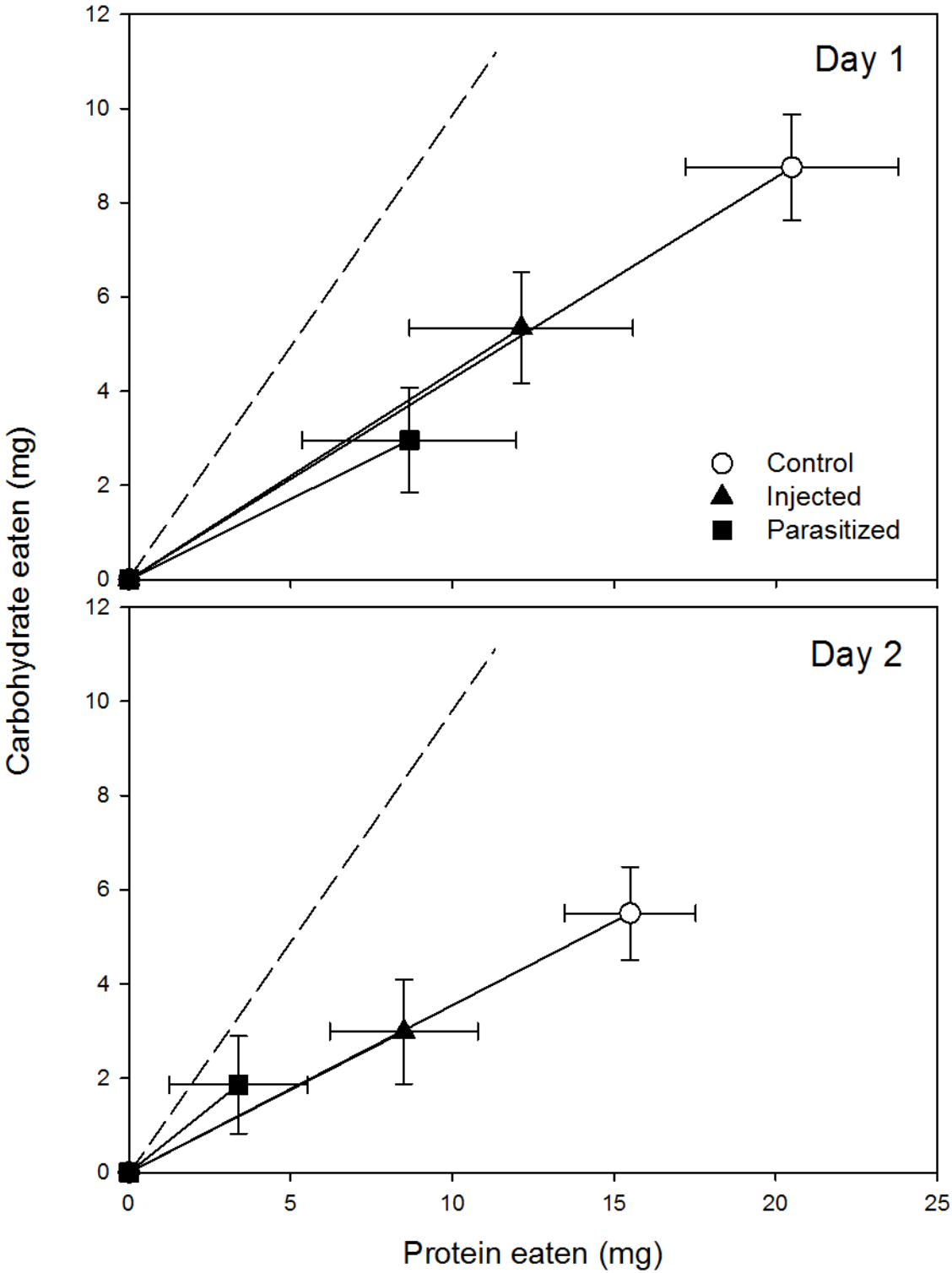
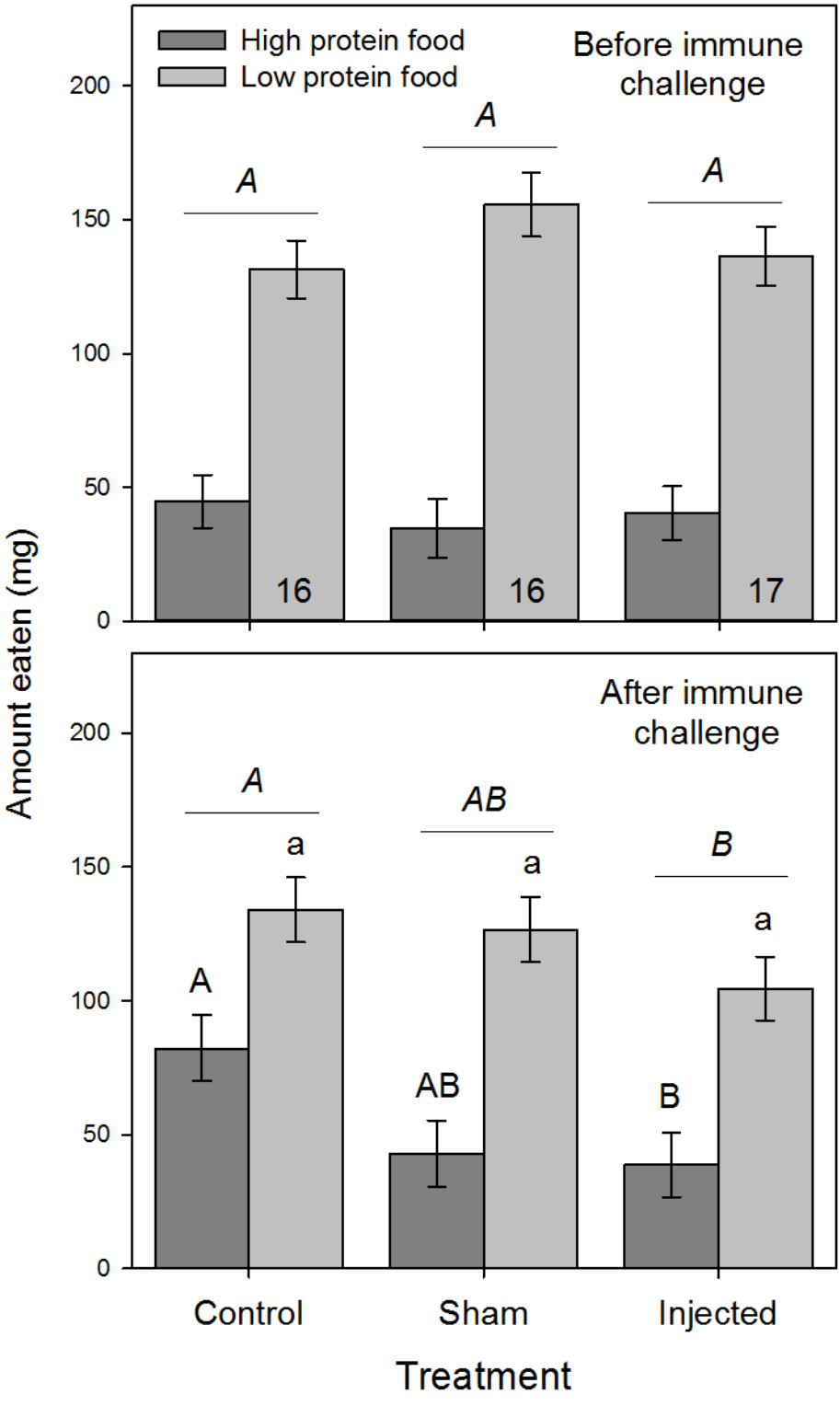
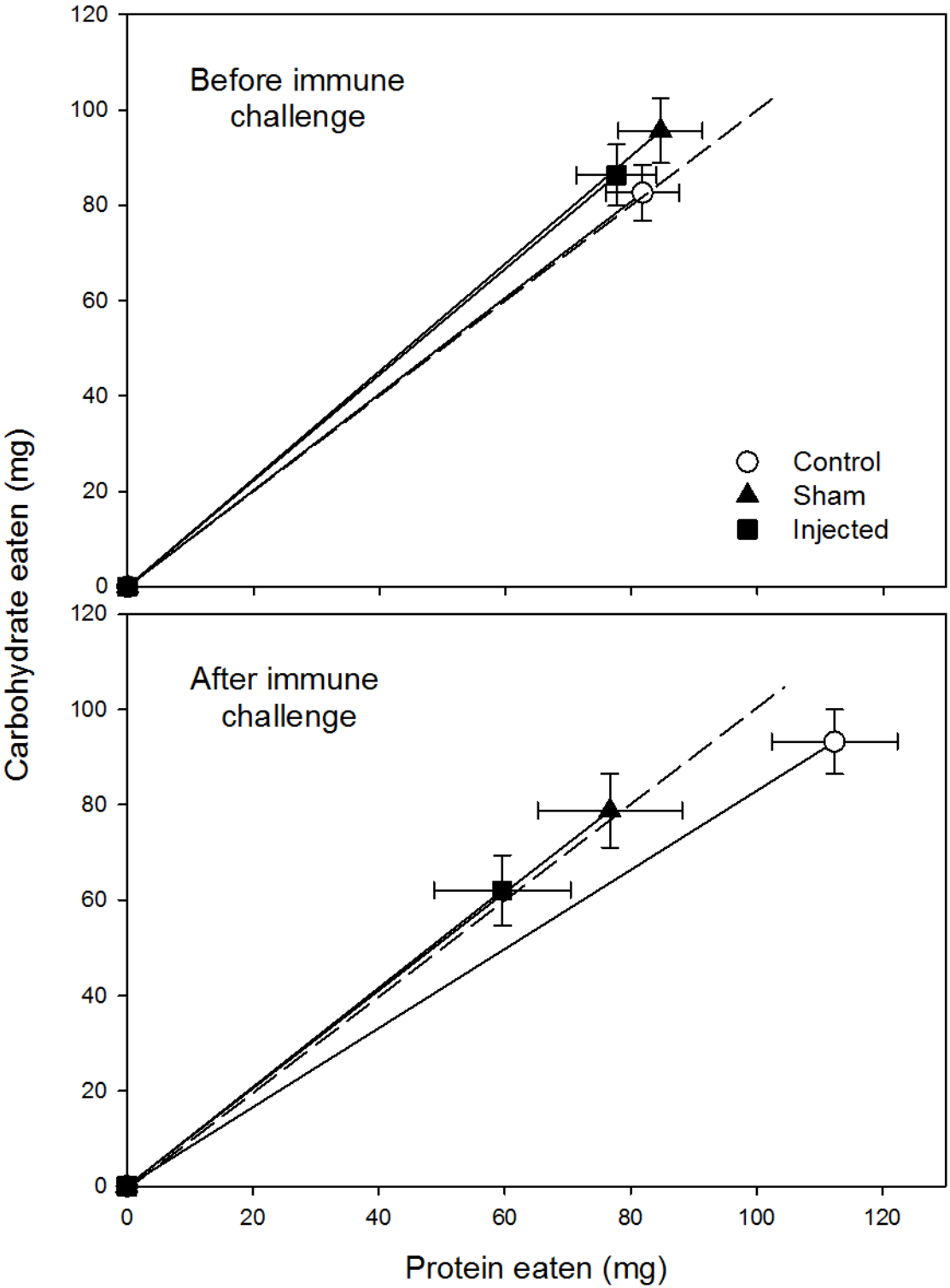
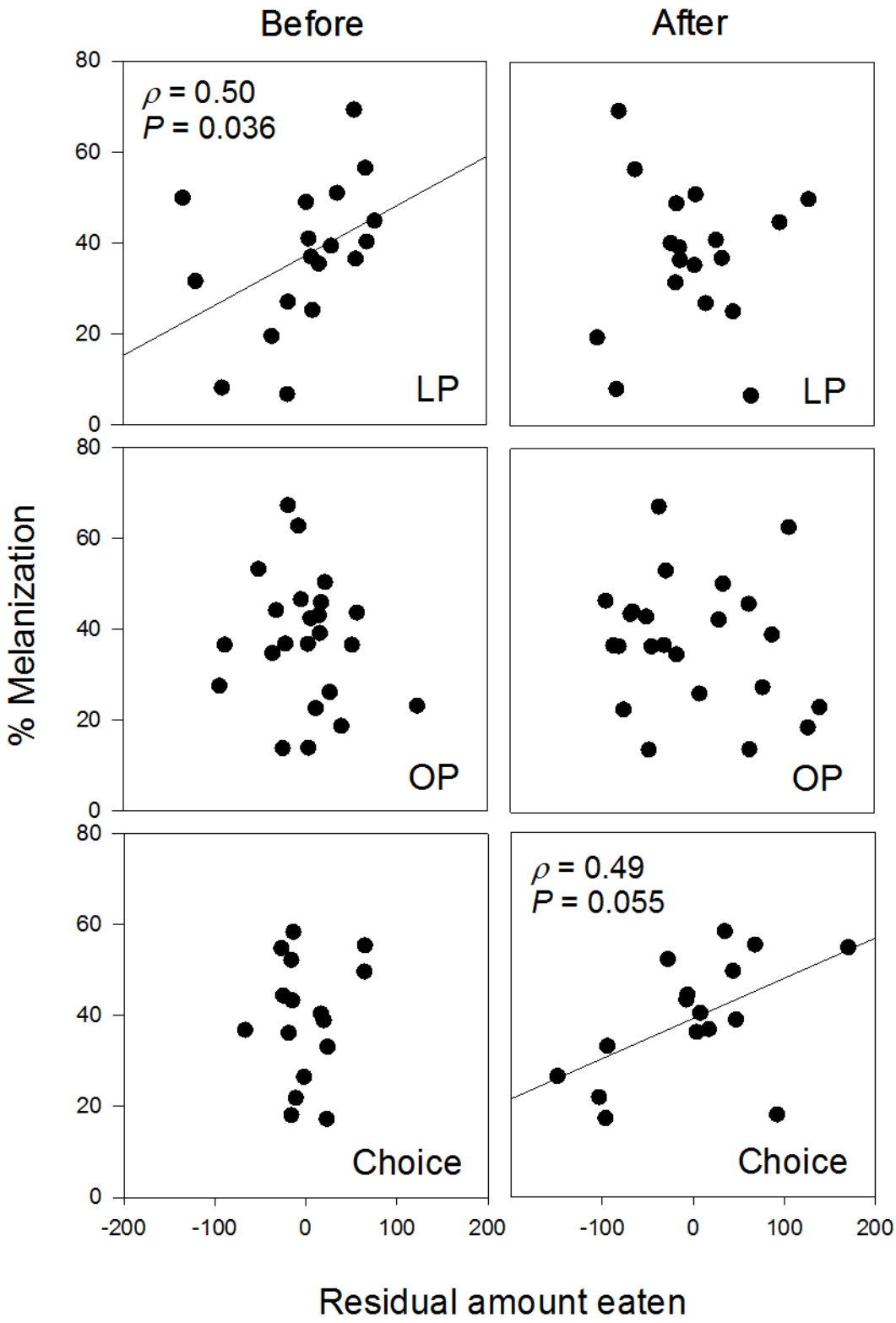
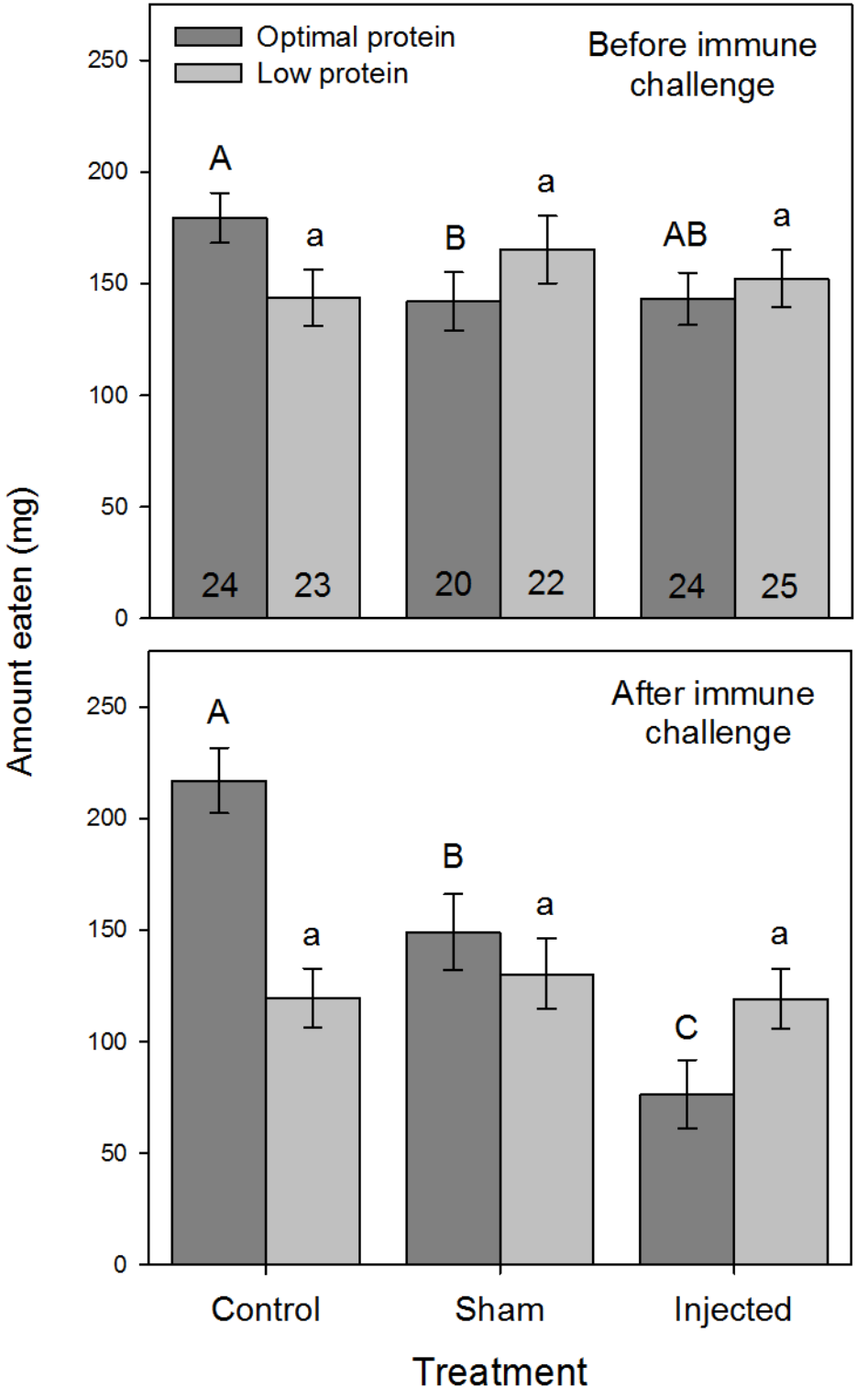

Day 1
High protein food
Low protein food
A
B
B
29
24
27
Day 2
A
B
B
A
AB
B
a
a
a
Amount eaten (mg)
0
20
40
60
Control
Injected
Parasitized
Treatment


Day 1
Day 2
Control
Injected
Parasitized
Carbohydrate eaten (mg)
Protein eaten (mg)
0
2
4
6
8
10
12
0
5
10
15
20
25


High protein food
Low protein food
Before immune challenge
200
150
100
50
0
A
A
A
16
16
17
After immune challenge
A
AB
B
a
a
a
A
AB
B
Amount eaten (mg)
Control
Sham
Injected
Treatment


Before immune challenge
After immune challenge
Control
Sham
Injected
Carbohydrate eaten (mg)
Protein eaten (mg)
0
20
40
60
80
100
120


Before
After
ρ = 0.50
P = 0.036
LP
LP
OP
OP
Choice
ρ = 0.49
P = 0.055
Choice
% Melanization
Residual amount eaten
0
20
40
60
80
-200
-100
0
100
200


Optimal protein
Low protein
Before immune challenge
After immune challenge
Amount eaten (mg)
250
200
150
100
50
0
A
a
B
a
AB
a
24
23
20
22
24
25
A
a
B
a
C
a
Control
Sham
Injected
Treatment