EDUARDO CARLOS COSTANTIN

INFLUENCE OF DIET AND DENSITY ON IMMUNE AND OTHER LIFE HISTORY TRAITS IN THE PHASE POLYPHENIC VELVETBEAN CATERPILLAR (LEPIDOPTERA: NOCTUIDAE)

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Magister Scientiae*.

Orientador: Simon Luke Elliot

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ABSTRACT

COSTANTIN, Eduardo Carlos, M.Sc., Universidade Federal de Viçosa, February, 2020. **Influence of diet and density on immune and other life history traits in the phase polyphenic velvetbean caterpillar (Lepidoptera: Noctuidae).** Advisor: Simon Luke Elliot.

Recently, the immune system has been considered a functional trait in Life-History theory. As well as other traits, the maintenance and up-regulation of the immune system is costly and could trade-off with reproduction and longevity. Trade-offs can be minimized in well-nourished insects, such as those reared in laboratory on artificial diet, which represents an unrealistic situation. The velvetbean caterpillar *Anticarsia gemmatalis* presents phase-polyphenism related to the investment in immunity, in accordance with the Density Dependent Prophylaxis (DDP) hypothesis. This hypothesis predicts that individuals in high density of conspecifics should increase investment in immunity, as the risk of disease transmission is higher. Thus, the present study aimed to investigate the differences in immune parameters and the costs related to the investment in immunity in this species. We used density (1 larva or 4 larvae/per pot) and diet (artificial diet and soybean leaves) as treatments and collected two immune parameters and seven life-history traits. We predicted that caterpillars reared at high density and fed with soybean leaves will face a pronounced resource allocation conflicts, resulting in an immune investment cost in life-history traits. Our main results showed that one immune parameter and three life-history traits were up regulated when larvae were fed with artificial diet. Meanwhile, the high density impacted negatively just the pupal weight when caterpillars were fed on leaves. Contrary to our expectations, neither longevity nor fecundity was affected by the treatments. We conclude that even with the differences founded in life-history, the prophylactic investment in immunity did not result in fitness costs in our system.

Keywords: Immune ecology. Density Dependent Prophylaxis. *Anticarsia gemmatalis*. Costs of immunity.

RESUMO

COSTANTIN, Eduardo Carlos, M.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Influência da dieta e da densidade na imunidade e em outros traços de história de vida na lagarta-da-soja (Lepidoptera: Noctuidae).** Orientador: Simon Luke Elliot.

Na Teoria da história-de-vida, os traços funcionais são aqueles que caracterizam o ciclo de vida de um organismo e influenciam em seu "fitness". Exemplos clássicos são o tamanho dos indivíduos, sua taxa de crescimento, sua idade na primeira reprodução e o número de descendentes gerados. Mais recentemente, o sistema imune vem sendo incluído como um importante traço funcional nessa teoria, assim como sua modulação por fatores ecológicos e ambientais, como a qualidade e a disponibilidade de alimento. A manutenção desse sistema é custosa e compete por recursos com outras funções, como a reprodução e a longevidade, dando origem aos "trade-offs". Os "trade-offs" podem ocorrer de maneira mais evidente a depender do estado nutricional do indivíduo. A lagarta-da-soja (Anticarsia gemmatalis) é um inseto-praga importante para produção de soja no Brasil e apresenta diferentes fenótipos (verde, preto e intermediário), que são dependentes da densidade de coespecíficos. Os fenótipos diferem em coloração e no investimento em parâmetros imunes, em acordância com a hipótese da Profilaxia Densidade-Dependente (DDP hipótese). A DDP hipótese prediz que em alta densidade há maior risco da transmissão de patógenos, havendo então maior necessidade de se investir em imunidade. Sendo assim, o presente estudo almeja investigar i) as diferenças nos parâmetros imunes; e ii) os custos relacionados ao investimento na imunidade nessa espécie. Nós utilizamos como tratamento a densidade (1 lagarta ou 4 lagartas/por pote) e a dieta (artificial ou folhas de soja). Coletamos, então, dois parâmetros imunes: número de hemócitos e atividade de lisozima na hemolinfa; e sete traços de história de vida: desenvolvimento da lagarta e da pupa, fenótipo, peso da pupa, longevidade, fecundidade e conteúdo gorduroso. Nossa predição é que lagartas criadas em alta densidade e se alimentando de folhas de soja irão apresentar um conflito de alocação de recursos mais pronunciado, resultando em um custo de investimento relacionado à imunidade nos traços de história de vida. Os principais resultados mostraram que o número de hemócitos foi mantido em maiores níveis quando a lagarta foi alimentada com dieta artificial, e três traços de história de vida aumentaram: peso da pupa, tempo de pupa e o conteúdo gorduroso. Enquanto isso, a alta densidade impactou positivamente a atividade de lisozima e negativamente o peso da pupa quando as lagartas foram alimentadas em plantas.

Tanto a fecundidade, quanto a longevidade dos adultos não foi afetada por nenhum dos tratamentos. Dessa forma, concluímos que não há indicação de custos no “fitness” dos indivíduos relacionados a DDP hipótese nesse sistema, mas há diferenças substanciais na história de vida e no número de hemócitos em lagartas criadas em dieta artificial comparadas com aquelas alimentadas com a planta hospedeira.

Palavras-chave: Ecologia imune. Profilaxia densidade dependente. *Anticarsia gemmatalis.* Custos da imunidade.

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INTRODUCTION

Entomopathogens negatively impact the fitness of their hosts, representing a biotic threat. In response to this threat, hosts could react with behavioral (Lee et al., 2006; Povey et al., 2009), physiological (Dubovskiy et al., 2016) and immune (Schmid-Hempel, 2003) defensive strategies. Insects, for example, rely on three lines of defense to face pathogenic challenges: behavioral avoidance, boundary defense and immunity (Siva-Jothy, 2005).

Once pathogens break through the first two lines of defense and enter the insect hemocele, the immune system will respond with cellular and humoral components. Cellular defenses consist of hemocyte-mediated immune responses such as phagocytosis, nodulation and encapsulation (Ratner and Vinson, 1984; Lavine and Strand, 2012), while the humoral defense involves, among others, lysozyme bactericidal activity against gram-positive bacteria (Lockey and Ourth, 1996; Barabas et al., 2012) and the phenoloxidase (PO) cascade, that initiate the melanogenesis and promotes cuticular sclerotization (Sugumaram, 1998). The PO also interacts with the cellular defenses, as it participates in parasite melanisation (Kan et al., 2008).

The immune system is a powerful tool to fight infections, but its maintenance and upregulation can incur fitness costs associated with increased metabolic rates (Freitak et al., 2003), reduced reproduction (Schwenke et al., 2016) or reduced longevity (Armitage et al., 2003). The magnitude of these costs is associated with genetic variation among different populations (Lazzaro et al., 2008) and with a range of ecological factors, such as the availability of nutrient resources (Zuk and Stoehr, 2002) and population density.

Since immunity comes at a cost, insects have developed phenotypically plastic strategies to adjust and regulate their immune systems in contexts where the likelihood of pathogen transmission varies, as when conspecific population densities fluctuate. Insect crowding can occur in natural environments, agroecosystems and mass rearing. Under crowding, it is expected that the risk of pathogen infection among insects increases (Anderson and May, 1981). Given this, the hypothesis known as Density-Dependent Prophylaxis (DDP) predicts that organisms can use the perception of increasing population density as a cue to the risk of becoming infected and should invest more resources in immune defenses (Wilson & Reeson, 1998).

This phenomenon has been observed in different groups of insects, such as Lepidoptera (Wilson and Reeson, 1998; Wilson et al., 2001; Hagen et al., 2006; Kong et al., 2013), Coleoptera (Barnes and Siva-Jothy, 2000) and Orthoptera (Wilson et al., 2002). In most of

these studies, crowded individuals form different phenotypes that include variations in morphology, life history, coloration and behaviors, representing a density-dependent phase polyphenism (Simpson et al., 2011). These changes in the individual phenotype come with differences in immune parameters such as phenoloxidase (PO) enzyme activity, melanotic encapsulation response, lysozyme-like activity (Cotter et al., 2004) and hemocyte number (Wilson et al., 2002).

In the presence of pathogens, DDP is adaptative as the investment in immune parameters predicted by this hypothesis enhances the resistance to entomopathogenic microorganisms (Wilson at al., 2002; Barnes and Siva-Jothy, 2000; Silva et al., 2013). However, once DDP is adopted, it is maintained even in the absence of parasites, so the risk of error associated with the perception of population density needs to be low (Wilson and Cotter, 2008). This is especially true as assuming the metabolic costs of mounting an immune response can negatively impact life history traits independently of the damage caused by the pathogen (Ahmed et al., 2002; Ardia et al., 2012;).

Another crucial variable that influences immune responses and their implications for the organism's life history is the availability and quality of food resources that the individual experiences. Differences in the host plant (Vogelweith et al., 2011), amount of protein consumption (Wilson et al., 2018) and quality of the protein (Lee et al., 2008; Alaux et al., 2010) modulate an individual's immune status. This modulation occurs because humoral (lysozyme activity; PO activity) and cellular (hemocyte density) components of the insect immune system can be expressed differently in relation to the ratio and amount of protein and carbohydrate ingested (Cotter at al., 2010). In addition to the effect on components of the immune system, the nutritional status of an individual could also impact the presence or absence of costs in the immune system activation. For example, in workers of the bumblebee *Bombus terrestris*, when they were immunologically challenged and starved, their lifespan was reduced compared to those that had access to food (Moret and Schmid-Hempel, 2000).

Herbivorous insects like lepidopteran larvae rely mostly on plants as food to complete their life cycle and reproduce. However, plants may impose nutrient limitations that reduce herbivore performance (Wetzel et al., 2016) and challenge them physiologically with secondary metabolites (Schoonhoven et al. 2005; Forbey et al. 2009). For example, concentrations of the flavonoids rutin and gestinin in soybean *Glycine max* (L.) Merr. can confer some degree of resistance to herbivory, and consequently impact life history traits of lepidopteran larvae (Piubelli et al., 2005).

In contrast to what occurs in nature, for laboratory studies on immune ecology and the DDP hypothesis using lepidopteran larvae, the standard rearing procedure is to use artificial diet (e.g. Cotter et al., 2004; Silva et al., 2013). The prevalent use of artificial diet is justified for the standardization of the individual's nutritional status, and there are significant savings in labor, cost, time and space to maintain the colonies and perform experiments. This diet does not contain secondary metabolites and is produced with high-quality protein (casein) and carbohydrate, plus a vitamin solution that contains several essentials amino acids.

Taking into consideration the nutritional aspects of the artificial diet and plant-based diets, when these two have been compared: larval development, pupal weight and female fecundity were enhanced in lepidopteran species on artificial diet (McMorran, 1965; Abdullah et al., 2000, Gupta et al., 2004) representing an impact on life history traits and, possibly, fitness. With respect to immune function, assuming that this trait also demands energy and protein from food, the nutritional differences of artificial diet and plants will probably impact their expression in these larvae.

If the different biological systems of an organism, say the immune system and the reproductive system, compete internally for the same resources, the allocation of the resource for one of these could constrain the other. In terms of prophylactic defense against parasites, as the different quality of food ingested can circumvent allocation trade-offs between immunity and other life-history traits (Singer et al., 2014), the use of artificial diet to investigate the impact of immune investment can represent an unrealistic situation that masks resource allocation conflicts.

The velvetbean caterpillar, *Anticarsia gemmatalis* Hübener. 1818 (Lepidoptera: Noctuidae), is an important pest of soybean production in Brazil. Larvae of these species cause damage to the plant by consuming the leaves and, consequently, reducing photosynthesis and productivity (Moscardi et al., 2012). Caterpillars present a color polyphenism related to the presence of conspecifics, ranging from green to black, the green phenotype being more frequent in solitary caterpillars while the black phenotype is often manifested in caterpillars reared with conspecifics. The change in color is associated with upregulation in immune defenses and resistance to pathogens (Silva et al., 2013), as has been seen in other insects, notably other noctuid caterpillars (Wilson & Reeson., 1998) and locusts (Wilson et al., 2002)

In the context of the DDP hypothesis and resource allocation conflicts, our aim is to investigate: (i) the differences in immune parameters; and (ii) the costs related to the

investment in immunity in *A. gemmatalis*. We used density (1 or 4 larvae per pot) and diet (artificial diet or soybean leaves) as treatments to measure two immune parameters and seven life history traits. We predict that caterpillars raised at high density and fed with soybean leaves will face a pronounced resource allocation conflicts, presenting a physiological cost related to immune investment.

MATERIAL AND METHODS

1. Insect colony

The *Anticarsia gemmatalis* colony was established in the Laboratory of Insect-Microbe Interactions at the Universidade Federal de Viçosa in 2013 with insects from the Laboratory of Biological Control at EMBRAPA/CNPMS. These insects are kept in the laboratory under controlled conditions of 25±2 °C temperature, 65±10% relative humidity and 12:12h light cycle. The moths were housed in groups of ca. 80 pairs in wooden cages (measuring 30 × 30 × 30 cm) lined with paper sheets (where oviposition occurs) and fed *ad libitum* with a liquid diet consisting of honey, beer, saccharose, ascorbic acid and nipagin – methodology adapted from Hoffmann-Campo et al. (1985). The eggs were collected every 72h and kept in plastic pots (1 liter) containing plugs of artificial diet until they hatched. Upon hatching, larvae were transferred to plastic pots (100 ml) at densities of 1 or 4 individuals per pot. These densities are known to trigger color phenotypic changes (Silva et al., 2013), then they were chosen to maintain the expression of the solitary (1 individual) and the high-density phenotype (4 individuals).

2. Artificial diet and soybean leaves

The artificial diet used to feed the larvae to maintain the colony was the same used to perform the experiments. It is composed of textured soy protein, bean, wheat germ, beer yeast, nipagin, ascorbic acid, sorbic acid, formaldehyde, agar-agar, casein and vitamin solution (adapted from Hoffman-Campo et al., 1985). Casein is a high-quality protein, thus we consider the artificial diet as a high-quality diet.

Soybean (*Glycine max* (L.) Merr.) plants used in this study were of the variety “Williams 82”. The seeds were obtained from the Laboratory of Genetic and Genomic Plant-Pathogen Interactions, Universidade Federal de Viçosa (LGGIP-UFV). Sowing occurred weekly in 500 ml plastic cups containing a mixture of substrate for plants, sand and soil. The cups were placed in cages covered with gauze to avoid prior contact with other herbivores. Plants were fertilized one week after seed germination with macronutrients following recommendations from EMBRAPA (2008). The cages were placed outside and plants were watered once a day. The leaves used in the experiment had no symptoms of disease and were obtained from the plants before they reached the R4 stage (full pod).

3. *Experimental design, color phenotypes and pupal weights*

We conducted two experiments with the same experimental design, in which the independent variables were larval density and diet, as described below. They were conducted one after the other, for logistical reasons. In both experiments, data on larval color phenotypes were collected in addition to pupal weight. These variables, besides being of interest themselves, allowed us to check the two experiments for consistency and also to determine (in the case of color polyphenism) that the density treatment was having its expected effect. The first experiment was conducted to perform immune assays and the second to collect data on life-history traits.

Newly emerged larvae were placed in 100 ml pots and assigned to a 2×2 factorial design, in a completely randomized design, as follows: density (1 or 4 larvae per pot) and diet (artificial or soybean leaves). While the artificial diet was added only at the beginning of the experiment, the leaflets had their petioles wrapped in a damp cotton and were replaced as necessary – pots were checked twice a day and, approximately one leaflet was replaced for solitary larvae and two leaflets for high density larvae a day. To simulate similar manipulation conditions, pots containing artificial diet were also manipulated at the same frequency as the pots containing leaves.

The phenotypic classification was done in both experiments on the tenth day after larval eclosion. As described by Fescemyer & Hammond (1986) and Silva et al. (2013) there are visual differences between the phenotypes, especially the body color and the head capsule. The green phenotype has an olive-green body color and head capsule colors ranging from green to yellow; the intermediate phenotype has patches of black coloration arranged apparently randomly on the dorsum and subdorsum and head capsule color ranged from yellow to orange; the black phenotype has a velvety dark body and a yellow-orange head capsule. To classify the larvae, we visually observed the olive-green and the black discrepant coloration first, then the individuals that did not fit in one of these categories were classified as intermediate. This visual classification was used (rather than using digitalized images) as we wished to avoid overmanipulating the larvae. This manipulation could affect the development and trigger the change in the phenotype (D.L. Viol, *personal communication*).

The pupal weight was also obtained for both experiments, from each individual on the second day after they had pupated, using an analytical balance (Bel Engineering).

4. *Experiment I - Immune assays*

On the tenth day after larval eclosion, caterpillars of the immunoassay test group were weighed and two immune parameters were assessed using collected hemolymph: hemocyte number and lytic activity. In respect to the high density treatment, we randomly selected the individual to perform the immune experiment.

4.1 Hemocyte numbers

A sample of 2·5µl of hemolymph was collected from each individual by puncturing a small hole beside the first prolegs using a thin needle and added to an Eppendorf tube with 20 µl of anticoagulant buffer (98mM NaOH, 186mM NaCl, 17mM NaEDTA and 41mM citric acid, pH 4.5). Two aliquots of 10 µl of the suspension were added to each side of a Neubauer improved chamber and total hemocytes were counted under a microscope. The final value was the mean of the two aliquots, providing the numbers of hemocytes per microliter (adapted from Ibrahim & Kim, 2006).

4.2 Lytic activity

Lysozyme antibacterial activity was measured through the inhibition zones formed in agar plates containing the bacterium *Micrococcus lysodeikticus* ATCC 4698 (Cotter & Kilner, 2010). Plates were prepared with 1.5% agar, 0.75g of *M. lysodeikticus* in lyophilized form, 100 ml distilled water and 50 ml of 2M potassium phosphate buffer 2M (pH 6.4). Holes were punched in the agar after it had hardened with the aid of glass capillary tubes (1.5 mm diameter). Two aliquots of 1 µl of hemolymph per sample were pipetted into the holes of agar plates. Plates were incubated at 33°C for 24 hours and then photographed. The areas of clear zones formed in the vicinity of the holes were measured with the aid of ImageJ software and were transformed to diameters (mm).

5. *Experiment II – Life-history traits*

To assess the costs of immune defenses and the influence of diet in *A. gemmatalis*, we assessed the following life history parameters:

5.1 Larval and pupal development

To characterize larval development, we observed: (i) caterpillar development time (egg to pupa) and (ii) pupal time. We checked the pots every day and defined that the larvae had pupated when they changed color to brown and adopted a barrel shape. The individuals were also sexed using a stereomicroscope (Olympus SZ61) based on the sexual dimorphism presented in the pupal phase, according to Hoffman-Campo et al. (1985).

5.2 Longevity and potential fecundity

Newly emerged adults from life-history experiment were placed in 300ml pots with a falcon tube cap containing a piece of cotton moistened with líquid diet. The diet was replaced every two days and the moths could feed freely. A piece of gauze was used to cover the pot, thus avoiding escape and excessive humidity. To determine adult longevity, the number of dead adults in each of the treatment groups was counted and recorded daily until all adults died. The moths were considered dead when they seemed to be immobile in the pot and did not move even after being touched with tweezers. Potential fecundity was determined by recording the number of eggs laid in the pot plus the number of eggs remaining in the female oviducts. Since mating in this species seems to occur only under grouping conditions (E.C.C., *personal observation*) fecundity was estimated from virgin females. Fecundity estimation from virgin females has already been done by Kemp & Rutowski (2004) and Calvo & Molina (2005) in different species of Lepidoptera.

5.3*Fat content*

Fat content was estimated from the technique described by Plaistow and Siva-Jothy (1996). We dried males and females in a drying oven, at 60 °C, for 48 h. After this drying period, we weighed the thorax and abdomen using a Shimadzu ATX 224 microbalance (precision 0.1 mg) and placed the body parts in a microtube containing 3 ml chloroform for 48 h. Then we returned the fatless body parts to a drying oven for an additional 24 h and reweighed them. Drying oven and chloroform time periods were adjusted based on previous tests. We calculated fat content as the percentage of fat extracted in chloroform in relation to the moth thorax and abdomen initial weight.

6. *Statistical analysis*

The effects of density and diet on immunity parameters and life-history traits of *A. gemmatalis* were verified using Generalized Linear Models (GLM) followed by an analysis of variance (ANOVA) and survival analysis. The analyses were carried out by fitting a full model and then simplifying it by excluding the non-significant interaction and terms. The final model was accepted as the simplest model that was not significantly different from the full model. For data that did not fit in the normal distribution and were overdispersed, the negative binomial distribution with log-link function was used (hemocyte numbers). When data fit the normal distribution, the Gaussian family was used (lytic activity, number of eggs, fat content and pupal weight). The models were compared using an F-test ($p>0.05$) A survival analysis with a Weibull distribution was conducted for parameters such as development time of larvae, pupal period and longevity of the moth. For model comparison in the survival analysis we used the $\chi 2$ test ($p>0.05$). All analysis was done using the Software R (version 3.4.1).

RESULTS

1. *Phenotype frequency and pupal weight in Experiments I and II*

To check the similarities between the two experiments, we assessed phenotype frequencies and pupal weights. *Anticarsia gemmatalis* expressed different color phenotypes – black, intermediate or green, according to the rearing density: 1 or 4 larvae per pot (Figure 1). In both experiments, when reared alone (D1) the green phenotype was prevalent – over 45%, while the black phenotype was the least expressed. Meanwhile, at high densities (D4) the black phenotype was predominant – over 57%, and the green phenotype the least expressed.

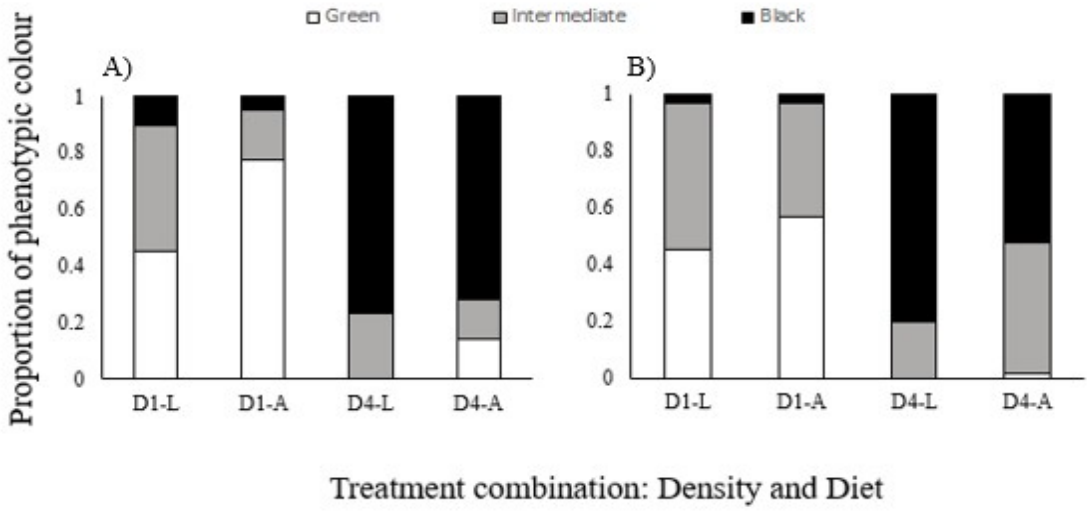

Figure 1. Proportions of green, intermediate and black phenotypes in caterpillars of the noctuid moth *Anticarsia gemmatalis* as a function of density (1 or 4 larvae per pot, "D1" or "D4" respectively) and diet (artificial diet "A" or soybean leaves "L") used in two experiments: (A) Experiment I and (B) Experiment II (see text for details). The color of each caterpillar was defined visually on the tenth day after egg eclosion.

Pupal weight was affected by density and diet in both experiments in a similar way - experiment I (diet: $F_{[1,159]}$= 154.44, p<0.0001; density: $F_{[1,160]}$= 7.65, p=0.0064) and experiment II (diet: $F_{[1,264]}$= 259.87 , p<0.0001; density: $F_{[1,265]}$= 22.83, p < 0.0001) (Figure 2). In experiments I and II, pupae fed with artificial diet as larvae were 22% and 20% heavier (respectively) than those fed with soybean leaves. With respect to density, pupae reared alone (D1) were approximately 6% heavier than those one reared at high densities (D4) in both experiments.

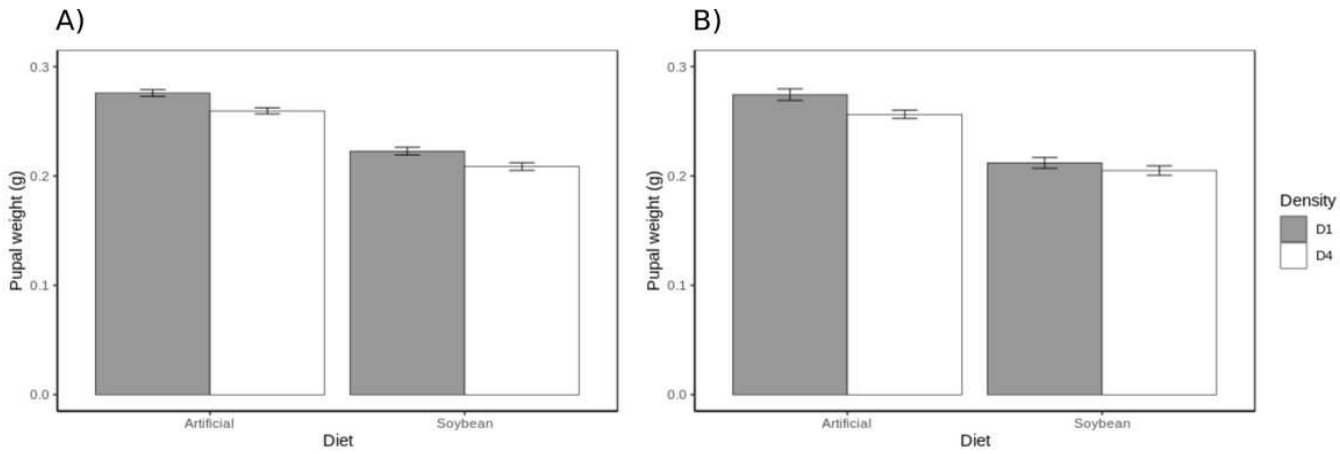

Figure 2. Pupal weight of the noctuid moth *Anticarsia gemmatalis* as a function of density (1 or 4 larvae/per pot: "D1" or "D4" respectively) and diet (artificial diet or soybean leaves) in both experiments: (A) Experiment I and (B) Experiment II. The pupal weight from each individual was obtained on the second day after they had pupated. The bars represents the 25th and 75th percentiles, the central line represents the median and upper and lower lines denote the maximum and minimum values for each group. Dots show outliers.

2. *Experiment I – Influence of density and diet on immune parameters*

2.1 Hemocyte number

Only diet had an effect on the number of hemocytes ($Z_{[1,150]}$= -3.526, p= 0.0004) (Figure 3). The mean value of circulating hemocytes in hemolymph was 20% lower in larvae fed with soybean leaves (2.232 ± 139.1 cells µl^{-1}) (we give means ± SE throughout) in comparison to artificial diet (2.808 ± 200.7 cells µl^{-1}), independent of density.

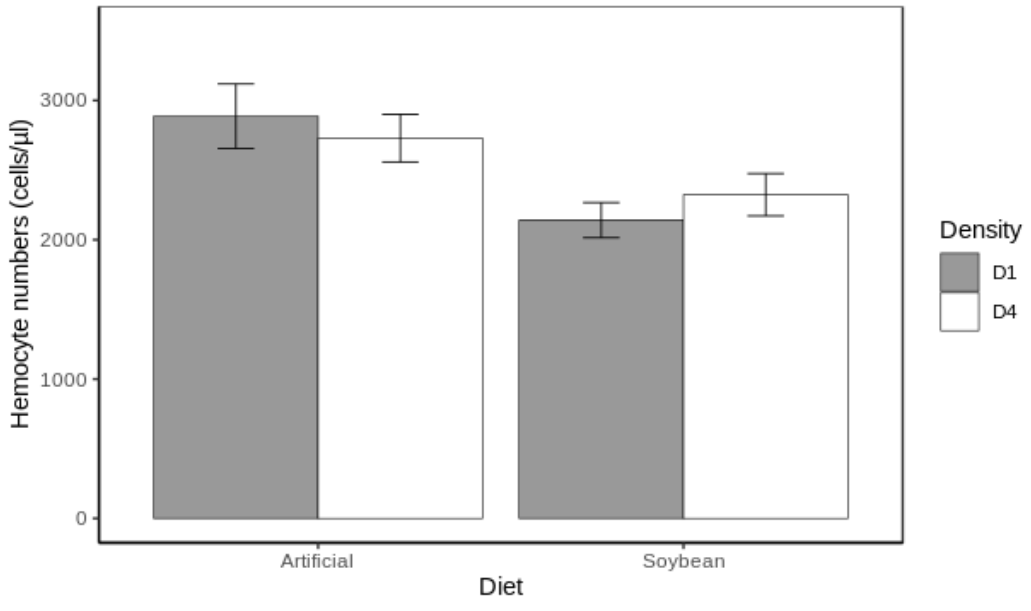

Figure 3. Hemocyte number (cells μl^{-1}) in *Anticarsia gemmatalis* as a function of density (1 or 4 larvae/per pot: “D1” or “D4” respectively) and diet (artificial or soybean leaves).We used caterpillars on the 10th day after the beginning of the experiment. A sample of 2·5µl of hemolymph was collected from each caterpillar and added to 20 µl of anticoagulant buffer. Two aliquots of 10 µl of the suspension were added to each side of a Neubauer improved chamber and total hemocytes were counted under a microscope. The error bars represent the standard errors.

2.2 Lytic activity

The lytic activity of hemolymph showed no significant differences in relation to larval diet ($F_{[1,151]}$= 2.36, p= 0.1217), but did differ with density ($F_{[1,150]}$= 7.54, p= 0.0057) (Figure 4). The mean diameter of inhibition zones for solitary larvae (D1) was 7.265 (± 0.22 mm), while for high density (D4) individuals, this was 7.88 (± 0.20 mm).

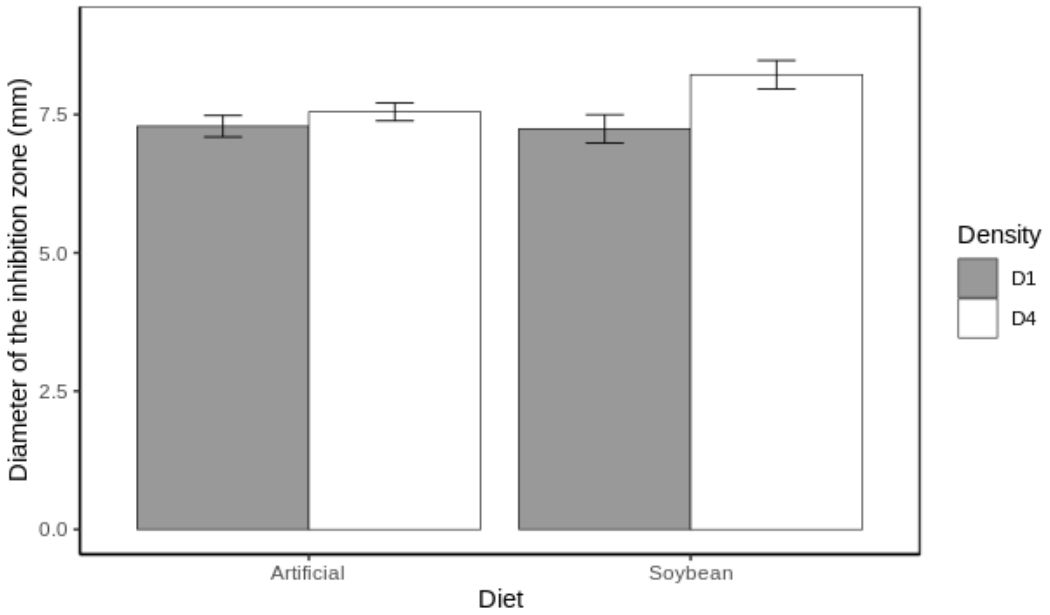

Figure 4. Diameter of inhibition zone area (mm) formed by the lysozyme activity from hemolymph in *Anticarsia gemmatalis* as a function of density (1 or 4 larvae/per pot: “D’=1” or “D4” respectively) and diet (artificial or soybean leaves). Two aliquots of 1 µl of hemolymph per sample were pipetted into the holes of agar plates containing the bacterium *Micrococcus lysodeikticus.* The areas of clear zones formed in the vicinity of the holes were measured with the aid of ImageJ software and transformed to diameters. The error bars represent the standard errors.

3. *Experiment II - Influence of density and diet in Life history-traits*

3.1 Development times - larvae and pupae

Larval development times did not vary with diet ($\chi^2_{[1,255]}$= 4.00, p= 0.0504) or density ($\chi^2_{[1,254]}$= 1.31, p = 0.2921). Pupal times, though, did vary with diet ($\chi^2_{[1,250]}$= 69.93, p<0.0001), with larvae reared on artificial diet remaining more time as pupae (10.4 ± 0.584 days) than those reared on soybean leaves (9.72 ± 0.605 days). Means are shown in Table 1.

3.2 Longevity of adults

The longevities of adult moths were not affected by none of our treatments, diet ($\chi^2_{[1,128]}$=1.31, p= 0.252) and density ($\chi^2_{[1,127]}$=0.02, p= 0.0941) (Table 1).

Table 1. Duration (days ± standard error; SE) of *Anticarsia gemmatalis* development stages (larval period and pupal period) and longevity of the moth as a function of density (1 or 4 larvae/per pot: “D1” or “D4” respectively) and diet (artificial diet “A” or soybean leaves “L”)

Treatment combination	Duration (days) ± SE					
	Larval period	n	Pupal period	n	Longevity of the moth	n
D1 – L	13.19 ± 0.094	66	9.71 ± 0.074	66	18.44 ± 0.964	32
D1 – A	13.40 ± 0.092	65	10.49 ± 0.079	65	18.81 ± 0.979	31
D4 – L	13.19 ± 0.107	67	9.73 ± 0.073	67	18.00 ± 0.934	33
D4 – A	13.35 ± 0.090	69	10.31 ± 0.064	69	19.81 ± 0.988	32

3.3 Potential fecundity

The potential fecundity of the females did not differ in relation to diet ($F_{[1,51]}$= 0.005, p= 0.943) or density ($F_{[1,52]}$= 0.288, p = 0.590).

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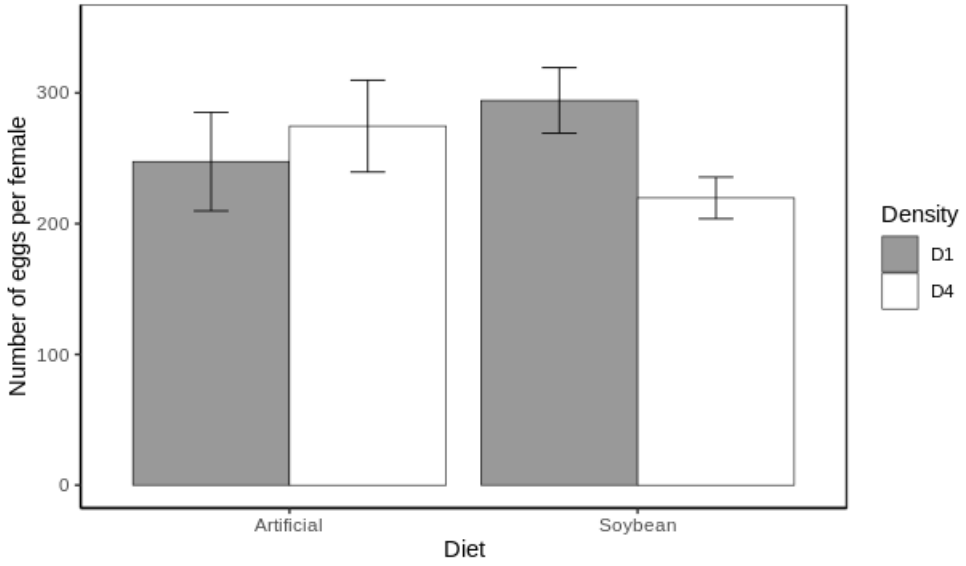

Figure 5. Potential fecundity of *Anticarsia gemmatalis* represented by the number of eggs per female as function of density (1 or 4 larvae/per pot: "D'=1" or "D4" respectively) and diet (artificial diet or soybean leaves). Fecundity was determined by recording the number of eggs laid in the pot plus the number of eggs remaining in the female oviducts on the day of death. The error bars represent the standard errors.

3.4 Fat content

The fat content in the moths was affected only by diet ($F_{[1,125]}$= 144.50, p< 0.0001). Moths that in their larval period were fed with artificial diet had greater percentages of fat in their bodies (24.8%) than those reared with soybean leaves (14.6%).

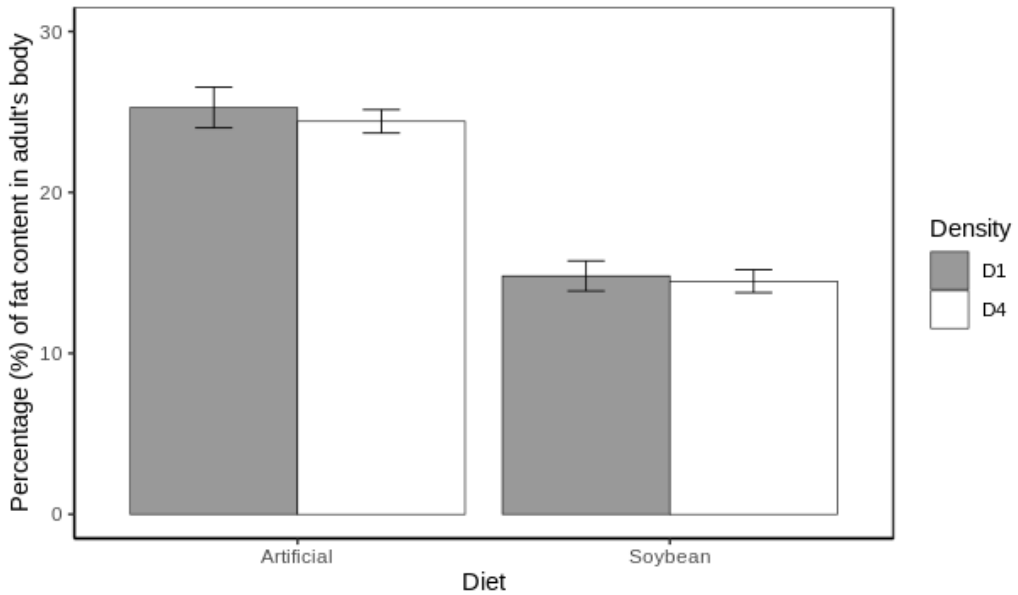

Figure 6. Percentage of fat content in moths of *Anticarsia gemmatalis* as a function of density (1 or 4 larvae/per pot: “D1” or “D4” respectively) and diet. The individuals were dried, the thorax and the abdomen were weighed and placed in 3 ml of chloroform for 48 h. The fatless body parts returned for drying and were reweighed. The fat content was calculated as the percentage of fat extracted in chloroform in relation to the moth thorax and abdomen initial weight. The error bars represent the standard errors.

DISCUSSION

Although previous studies have tested the influence of food quality (Klemola et al., 2007 Lee et al., 2008; Singer et al., 2014; Wilson et al., 2018) and density-dependent prophylaxis (Wilson et al., 2002; Cotter et al., 2004; Ruiz-Gonzáles et al., 2009; Kong et al., 2013) in the context of immune ecology, we could find no studies that investigate the differences between artificial diet and host plant. We showed that for the velvetbean caterpillar *Anticarsia gemmatalis* one immune parameter (hemocyte number) was upregulated when the artificial diet (a more proteinaceous food) was consumed, and three life history traits increased: pupal weight, pupal time and fat content present in the adult, irrespective of rearing density. In concern to the density treatment, the high density impacted positively the lytic activity and negatively the pupal weight in caterpillars fed on plant material.

The higher densities of hemocytes in the hemolymph of larvae fed in artificial diet with high quality protein found in *A. gemmatalis* is consistent with other studies of Lepidoptera (Shikano et al., 2009; Vogelweith et al., 2016), although we believe this to be the first study to compare artificial versus natural diets specifically. The hemocytes are maintained and generated in immature stages of insects by hemopoietic tissue and mitotic division by granular and spherule cells (Ratcliffe et al., 1985). Producing and maintaining higher numbers of hemocytes circulating in the hemolymph is energetically demanding (Lazzaro, 2015) so it is expected that the individuals that have access to more balanced food can sustain this cellular defense at higher levels.

Studies of the activity of the antimicrobial protein lysozyme have given contradictory results in species that present density-dependent prophylaxis. In the caterpillar *Spodoptera littoralis* crowded individuals had less lytic activity in hemolymph (Cotter et al., 2014) than solitary individuals, while in the locust *Schistocerca gregaria,* crowded individuals had more lytic activity in hemolymph (Wilson et al., 2008). In our study, the lysozyme antibacterial activity was slightly higher in larvae raised at high density only when they were fed with the host plant. Plants carry a range of microbes that could be ingested by herbivores, include some originally from the soil and carried endophytically to other plant tissues (Chi et al., 2005). Those microorganisms when in contact with the insect gut may trigger some immune parameter, thus interaction of this mechanism and the presence of the conspecifics may be responsible for the slight increase of lysozyme activity in these group of larvae.

Pupal mass was dependent on both density and diet, our results being similar to those found in the same species using artificial diet and soybean leaves as diet in different densities

(Fescemyer and Erlandson, 1993). Carbohydrates and proteins of a given diet are essential nutrients for a herbivorous insect (Behmer, 2009) and impact life history traits such as pupal mass in a holometabolous insect (Nash & Chapman, 2014). Although we did not quantify the amount of carbohydrates and proteins in the two diets used here, the use of bean, casein and vitamin solution in the artificial diet likely explains the differences in pupal mass. The density treatment also impacted pupal weight, but the effect was more discrete than the diet treatment. Since the immune parameters that we measured were not substantially different between the high and lower densities, it was not clear if these reduced weight in pupae reared in high density was due to the investment in other immune response, such as the phenoloxidase activity (Ruuhola et al., 2010) or larval competition in the earlier instars.

Rearing of larvae on artificial diet or soybean leaves resulted in adults with considerable differences in fat contents; this was expected since larvae reared on artificial diet became heavier pupae. The fat content in insects plays an important role in their life history and could influence adult longevity and female fecundity (reviewed by Arrese & Soulage, 2010). In a pilot experiment with no carbohydrate offered to adults, we observed that the moths from the soybean leaf treatment all died by the third day after emergence from their pupae when, while those from the artificial diet were all alive (E.C.C., *personal observation*). These results and our personal observation indicate to us that the fat content is determinant for adult longevity in *A. gemmatalis.*

Contrary to our expectations, neither longevity nor fecundity of the moths were affected by larval diet and density. The quality of food that holometabolous insects experience in their immature stages impacts the adult longevity (Lang et al., 2017; Araujo et al., 2012) and female fecundity (Awmack & Leather, 2002 for a review; Moreau et al., 2006 for an example). However, this did not translate to *A. gemmatalis* when artificial diet and soybean leaves were compared and adults were offered a source of carbohydrate *ad libitum*. We suspect that those individuals that come from the soybean diet treatment can compensate the reduced energy reserves acquired in the larval phase (fat content) by compensatory feeding in the adult stage as has been described in other holometabolous insects (Mevi-Schutz & Erhardt, 2005; Muller & Muller, 2016). This applies in a laboratory setting with food offered *ad libitum*, apparently not in the same setting when offered no food (see above) and in the field, where the insects must expend energy and run risks of predation to ingest carbohydrate, we may expect some middle ground where the potential for compensatory feeding is limited but not impossible. Regarding density, the lack of differences in fecundity (Mensah &

Gatehouse, 1998) was also found in *Spodoptera exempta* reared at high and low larval densities. Adult compensatory feeding behavior could also have masked the costs of larval immune investment or there were no evident costs of density dependent prophylaxis in our system.

Our results showed that (i) the artificial diet made with high-quality protein affects immune parameters; and (ii) besides the decrease in pupal weight, pupal time and fat content when the individuals were fed in the host plant, there's no indication of fitness costs of the DDP hypothesis in *A. gemmatalis.*

As mentioned by Sørensen, et al., (2012) the artificial diet, among other laboratory conditions, could lead to changes in phenotypic traits in an insect laboratory population. Indeed, we find that not only three life-history traits have significantly changed in individuals that experienced artificial diet in their larval period, but also one component of the immune system. These differences must be taken into account when interpreting results from life-history traits such as pupal weight or fat content in mass rearing conditions. In respect to the insect immune system, its complexity makes difficult to any experiment to covers all variables, so it is possible that other components such as phenoloxidase activity could also be modified by artificial diet.

Similar to what was found for *Spodoptera litorallis* by Cotter et al. (2004), there was no indication of physiological life-history costs related to DDP hypothesis in our system. Host plants is regularly considered suboptimal food for insects, especially due to nitrogen limitation (Mattson 1980; Schoonhoven et al. 2005). If there was any fitness cost related to prophylaxis in this system, it is more likely to appear in those individuals who fed in plant material. In *A. gemmatalis*, larval development, pupal time, longevity of the moth, potential fecundity and fat content were not affected by the density treatment even when the larvae were fed with soybean leaves.

Although our hypothesis that caterpillars reared in high density and fed with soybean leaves presents a more pronounced cost of immune investment was not supported, we propose that a more detailed approach in terms of immune parameters and fitness evaluation is needed. Our study brings a more comprehensive understanding about the immune ecology and the influence of environmental factors as density and food resource. We also highlight the differences of a synthetic and natural food in an experimental context where life-history and immunity parameters were investigated.

REFERENCES

Abdullah, M. D., Sarnthoy, O., Chaeychomsri, S., Sarnthoy, O. (2000) Comparative study of artificial diet and soybean leaves on growth, development and fecundity of beet armyworm, *Spodoptera exígua* (Hubner) (Lepidoptera: Noctuidae). *J. Nat. Sci*. 34: 339-344.

Ahmed, A. M, Baggott S. L., Maingon R., Hurd, H. (2002) The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito Anopheles gambiae. Oikos 97:371–77

Alaux, C., Ducloz, F., Crauser, D. (2010) Diet effects on honeybee immunocompetence. *Biol. lett.* 6: 562-65.

Anderson, R. M. & May, R. M. (1981) The population dynamics of microparasites and their invertebrate hosts. *Philos. T. R. Soc. B.*, 291: 451–524.

Araújo, M., Gil, L. H. S., Silva, A. (2012). Larval food quantity affects development time, survival and adult biological traits that influence the vectorial capacity of *Anopheles darlingi* under laboratory conditions. *Malar. J., 11(1), 261*.

Ardia, D. R., Gantz, J. E., Schneider, B. C., Strebel, S. (2012) Costs of immunity in insects: An induced immune response increases metabolic rate and decreases antimicrobial activity. *Funct. Ecol*. 26: 732–739.

Armitage, S. A. O., Thompson, J. J. W., Rolff, J., Siva-Jothy, M. T. (2003) Examining costs of induced and constitutive immune investment in *Tenebrio molitor*. *J. Evol. Biol*. 16:1038–44.

Arrese, E. L., & Soulages, J. L. (2010) Insect Fat Body: Energy, Metabolism, and Regulation. *Annu. Rev. of Entomol*., 55(1), 207–225.

Barnes, A. I. & Siva-Jothy, M. T. (2000) Density-dependent prophylaxis in the mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. *Proc. R. Soc. Lond. B* 267, 177–182.

Behmer, S.T. (2009) Insect herbivore nutrient regulation. *Annu Rev Entomol* 54:165–87.

Calvo, D. & Molina, J.M. (2005) Fecundity–Body Size Relationship and Other

Reproductive Aspects of *Streblote panda* (Lepidoptera: Lasiocampidae*). Ann. Entomol. Soc. of Am*., 98(2), 191–196.

Chi, F., Shen, S. H., Cheng, H. P., Jing, Y. X., Yanni, Y. G., Dazzo, F. B. (2005) Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. *Appl. Environ. Microbiol.*, 71:7271-7278.

Cotter, S. C., Hails, R. S., Cory, J. S., Wilson, K. (2004) Density-dependent prophylaxis and condition-dependent immune function in lepidopteran larvae: a multivariate approach. *J. Anim. Ecol.* 73: 283–293.

Cotter, S. C., Kruuk, L. E. B., Wilson, K. (2004) Costs of resistance: genetic correlations and potential trade-offs in an insect immune system. *J. Evol. Biol*. 17:421–29

Cotter, S. C., Kilner, R. R (2010) Sexual division of antibacterial resource defence in breeding burying beetles, *Nicrophorus vespilloides*. *Journ. of Anim. Ecol*., v. 79, n. 1, p. 35–43

Cotter, S. C., Simpson, S. J., Raubenheimer, D., Wilson, K. (2010) Macronutrient balance mediates trade-offs between immune function and life history traits. *Funct. Ecol., 25(1), 186–198*

De Witt, T.J., Sih, A., Wilson, D.A. (1998) Costs and limits to benefits as constraints on the evolution of phenotypic plasticity. *Trends Ecol. Evol.*, 13:77-81.

Dubovskiy, I. M., Grizanova, E. V., Whitten, M. M., Mukherjee, K., Greig, C., Alikina, T., Kabilov, M., Vilcinskas, A., Glupov, V. V., Butt, T. M. (2016) Immuno-physiological adaptations confer wax moth *Galleria mellonella* resistance to *Bacillus thuringiensis. Virulence* 7, 860–870.

Fescemyer, H. W. & Erlandson, C.M. (1993) Influence of diet on the density-dependent phase polymorphism of velvetbean caterpillars (Lepidoptera,Noctuidae). *Environmental Entomology*, v.22, n.5, p.933-41.

Fescemyer, H. W. & Hammond, A. M. (1986). Effect of Density and Plant Age on Color Phase Variation and Development of Larval Velvetbean Caterpillar*, Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae). *Environ. Entomol., 15(4), 784–789*

Forbey, J. S., Harvey, A. L., Huffman, M. A., Provenza, F. D., Sullivan R., Tasdemir, D. (2009) Exploitation of secondary metabolites by animals: a response to homeostatic challenges. *Integr. Comp. Biol.,* 49:314–28.

Freitak, D., Ots, I., Vanatoa, A., Hõrak, P. (2003) Immune response is energetically costly in white cabbage butterfly pupae. *Proc. R. Soc. Lond., B* 270:220–222.

González-Santoyo, I., Córdoba-Aguilar, A. (2012) Phenoloxidase: a key component of the insect immune system. *Entomol Exp. Appl.* 142:1-16.

Grupta, G. P., Rani, S., Birah, A., Raghuraman, M. (2005) Improved artificial diet for mass rearing of the tobacco caterpillar, *Spodoptera litura* (Lepidoptera: Noctuidae). *Int. J. Trop. Insect. Sci.*, 25:55-58.

Hagen, S. B., Sørlibråten, O., Ims, R. A., Yoccoz, N. G. (2006) Density dependent melanism in winter moth larvae (Lepidoptera: Geometridae): a countermeasure against parasitoids? *Environ. Entomol.,* 35:1249–1253.

Hillyer, J. F. (2016) Insect immunology and hematopoiesis, *Dev. Comp. Immunol*., 58:102-118.

Hoffman, J. A., Zachary, D., Hoffmann, D., Brehelin, M. (1979) Postembryonic development and differentiation: hemopoietic tissues and their functions in some insects. In: A.P Gupta (Ed.*), Insect Hemocytes*, Cambridge Univ. Press, Cambridge, pp. 29-66

Hoffmann-Campo, C. B., Oliveira, E. B. de., Moscardi, F. (1985) Criação massal da lagarta da soja. Embrapa. In: EMBRAPA/CNPSo, Editor: Londrina, PR. pp. 23.

Hoffmann-Campo, C. B., Moscardi, F., Oliveira, L. J., Sosa-gómez, D. R., Panizzi, A. R., Corso, I. C., Oliveira, E. B. (2000) Pragas da soja no Brasil e seu manejo integrado. In: Embrapa Soja Londrina, p. 70.

Ibrahim, A. M. A., Kim, Y. (2006) Parasitism by *Cotesia plutellae* alters the hemocyte population and immunological function of the diamondback moth, *Plutella xylostella*. *J. Insec. Physiol.,* 52:943–950.

Kan, H., Kim, C. H., Kwon, H. M., Park, J. W., Roh, K. B. (2008) Molecular control of

phenoloxidase-induced melanin synthesis in an insect. *J. Biol. Chem.,* 283:25316–25323.

Kemp, D. J. & Rutowski, R. L. (2004) A survival cost to mating in a polyandrous butterfly, *Colias eurytheme*. *Oikos*, 105, 65–70.

Klemola, N., Klemola, T., Rantala, M.J., Ruuhola, T. (2007) Natural host-plant quality affects immune defence of an insect herbivore. *Entomol. Exp. App*l., 123:167-176.

Kong, H. L. (2013) Density-dependent prophylaxis in crowded Beet Webworm, *Loxostege sticticalis* (Lepidoptera: Pyralidae) larvae to a parasitoid and a fungal pathogen. *Int. J. Pest Manage.,* 59:174–179.

Lang, B., Igdobe, S., McMullen, K., Drury, F., Qureshi, A., Cator, L. (2017) The effect of larval diet on adult survival, swarming activity and copulation success in male *Aedes aegypti* (Diptera: Culicidae). *J. Med. Entomol.,* 55:1-7.

Lavine, M. D., Strand, M. R. (2002) Insect hemocytes and their role in immunity. *Insect Biochem. Mol. Biol*., 32:1295–1309.

Lazzaro, B. P., Flores, H. A., Lorigan, J. G., Yourth, C. P. (2008) Genotype by environment interactions and adaptation to local temperature affect immunity and fecundity in *Drosophila melanogaster*. *PLoS Pathog*., 4:e1000025.

Lazzaro, B. P. (2015) Adenosine signaling and the energetic costs of induced immunity. *PLoS Biol.,* 13:e1002136

Lee, K. P., Cory, J. S., Wilson K., Raubenheimer D., Simpson, S. J. (2006) Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. *Proc. R. Soc. B.,* 273:823–829.

Lee, K.P., Simpson, S.J., Wilson, K. (2008) Dietary protein quality influences melanization and immune function in an insect. *Funct. Ecol*., 22:1052– 1061.

Lockey, T. D. & Ourth, D. D. (1996) Purification and characterization of lysozyme from hemolymph of *Heliothis virescens* larvae. *Biochem. Biophys.* Res. Commun., 220:502-508.

Mattson, W. J. (1980) Herbivory in relationto plant nitrogen content. *Annu. Rev. Ecol. Syst.*11:119–61

Mensah, B. A. Mattson WJ. 1980. Herbivory in relationto plant nitrogen content.Annu. Rev. Ecol.Syst.11:119–61& Gatehouse, A. G. (1998) Effect of larval phase and adult diet on fecundity and related traits in *Spodoptera exempta*. *Entomol Exp. Appl.,* 86:331–336

Mevi-Schutz, J. & Erhardt, A. (2005) Amino acids in nectar enhance butterfly fecundity: a long awaited link. *Am. Nat.*, 165:411–419.

Moreau, J., B. Benrey., Thiery, D. (2006) Assessing larval food quality for phytophagous insects: are the facts as simple as they appear? *Funct. Ecol*., 20:592-600.

Moret, Y., Schmid Hempel, P. (2000) Survival for immunity: The price of immune system activation for bumblebee workers. *Science,* 290:1166–1168.

Morran, M. C. (1965) A synthetic diet for the spuce budworm, *Choristoneura fumiferana* (Clem.) (Lepidoptera:Tortricidae*). Can. Entomol.*, 97:58–62.

Moscardi, F., Bueno, A. F., Sosa-Gómez, D. R., Roggia, S., Hoffman-Campo, C. B., Pomari, A. F., Corso, I. V., Yano, S. A. C. (2012) Artrópodes que atacam as folhas da soja. In: Hoffman-Campo, C. B., Corrêa-Ferreira, B. S., Moscardi, F. (Ed.). S*oja: manejo integrado de insetos e outros artrópodes-praga.* Brasília: Embrapa. p.213-309

Müller, T., Müller, C. (2016) Adult beetles compensate for poor larval food conditions. *J. Insect. Physiol.,* 88:24–32

Nash, W. J. & Chapman, T. (2014) Effect of dietary components on larval life history characteristics in the medfly (*Ceratitis capitata*): Diptera, Tephritidae). *PLoS One.*, 9:e86029

Piubelli. G. C., Hoffmann-Campo C. B, Moscardi F., Miyakubo, S. H., De Oliveira, M. C. N. (2005) Are chemical compounds important for soybean resistance to *Anticarsia gemmatalis*? *J. Chem. Ecol*. 31:1509–25.

Plaistow, S. J. & Siva-Jothy, M. T. (1999) The ontogenetic switch between odonate life history stages: effects on fitness when time and food are limited. *Anim. Behav.,* 58:659–667.

Povey, S., Cotter, S. C., Simpson, S. J., Lee, K. P., Wilson, K. (2009) Can the protein costs of bacterial resistance be offset by altered feeding behaviour? *J. Anim. Ecol.,* 78:437–446.

Ratcliffe, N. A., Rowley, A. F., Fitzgerald, S. W., Rhodes, C. P. (1985) Invertebrate immunity: basic concepts and recent advances. *Int. Rev. Cytol*., 97:186–350.

Ratner, S., Vinson, S. B. (1983) Phagocytosis and encapsulation: cellular immune responses in Arthropoda. *Am. Zool*., 23:185-94.

Ruiz-Gonzalez, M. X., Moret, Y., Brown, M. J. F. (2009) Rapid induction of immune density-dependent prophylaxis in adult social insects. *Biol. Letters*, 5:781–783.

Ruuhola, T., Yang, S., Rantala, M. J. (2010) Increase in substrate availability down regulates PO activity in *Epirrita autumnata*. *Chemoecology*, 20:11–18.

Schmid-Hempel, P. (2003) Variation in immune defence as a question of evolutionary ecology. *Proc. R. Soc. B*., 270:357–366.

Schoonhoven, L. M, Van Loon, J. J. A., Dicke, M. (2005). Insect-Plant Biology. 2nd ed. New York: Oxford University Press.

Schwenke, R. A., Lazzaro, B. P, Wolfner, M. F. (2016) Reproduction-immunity trade-offs in insects. *Annu. Rev. Entomol.*, 61:239–256.

Shikano, I., Ericsson, J. D., Cory, J. S., Myers, J. H. (2010) Indirect plant-mediated effects on insect immunity and disease resistance in a tritrophic system. *Basic Appl. Ecol*., 11:15-22.

Silva, F.W., Viol, D.L., Faria, S.V., Lima, E., Valicente, F.H., Elliot, S.L. (2013) Two's a crowd: phenotypic adjustments and prophylaxis in *Anticarsia gemmatalis* larvae are triggered by the presence of conspecifics. *PLoS ONE*, 8:e61582.

Simpson, S. J., Sword, G. A., Lo, N. (2011) Polyphenism in insects. *Curr. Biol*., 21:R738–R749.

Singer, M. S., Mason, P. A., & Smilanich, A. M. (2014) Ecological immunology mediated by diet in herbivorous insects. *Integr. Comp. Biol.*, 54(5), 913–921.

Siva-Jothy, M. T., Moret, Y., Rolff, J. (2005) Insect immunity: an evolutionary ecology perspective. *Adv. Insect Physiol.*. 32:1201-1248.

Sørensen, J. G., Addison, M. F., Terblanche, J. S. (2012) Mass-rearing of insects for pest management: Challenges, synergies and advances from evolutionary physiology. *Crop Protect.,* 38, 87–94.

Sugumaran M. (1998) Unified mechanism for sclerotization of insect cuticle. *Adv. Insect Physiol.,* 27: 229–334.

Tisdale, R. A. & Sappington, T. W. (2001) Realized and potential fecundity, egg fertility, and longevity of laboratory-reared female beet armyworm (Lepidoptera: Noctuidae) under different adult diet regimes. *Ann. Entomol. Soc. Am*. 94:415–19

Vogelweith, F., Moret, Y., Monceau, K,. Thiéry, D., Moreau. J. (2016) The relative abundance of hemocyte types in a polyphagous moth larva depends on diet. *J. Insect. Physiol.,* 88:33–39.

Vogelweith, F., Thiéry, D., Quaglietti, B., Moret, Y., Moreau, J. (2011) Host plant variation plastically impacts different traits of the immune system of a phytophagous insect. *Funct. Ecol.,* 25:1241–1247.

Wetzel, W. C., Kharouba, H. M., Robinson, M., Holyoak, M., Karban, R. (2016) Variability in plant nutrients reduces insect herbivore performance. *Nature,* 539:425–427.

Wilson, J. K., Ruiz, L., Davidowitz, G. (2019) Dietary protein and carbohydrates affect immune function and performance in a specialist herbivore insect (*Manduca sexta*). *Physiol. Biochem. Zool.,* 92:58–70.

Wilson, K. & Reeson, A. F. (1998) Density-dependent prophylaxis: Evidence from Lepidoptera-baculovirus interactions? *Ecol. Entomol.,* 23:100–101.

Wilson, K. & Cotter, S.C. (2009) Density-dependent prophylaxis in insects. In: Whitman DW, Ananthakrishnan TN, editors. Phenotypic plasticity of insects: mechanisms and consequences. Science Publishers Inc. pp. 137–176.

Wilson, K., Cotter, S. C., Reeson, A. F., Pell, J. K. (2001) Melanism and disease resistance in insects. *Ecol. Lett.,* 4:637–649.

Wilson, K., Thomas, M. B., Blanford, S., Doggett, M., Simpson, S. J., Moore, S. L.

(2002) Coping with crowds: density dependent disease resistance in desert locusts. *Proc. Nat. Acad. Sci., USA* 99:5471–5475.

Zdybicka-Barabas, A., Mak, P., Klys, A., Skrzypiec, K., Mendyk, E., Fiołka. M. J., Cytryńska. M. (2012) Synergistic action of *Galleria mellonella* anionic peptide 2 and lysozyme against Gram-negative bacteria. *Biochim. Biophys*. *Acta*, 1818:2623–2635.

Zuk, M. & Stoehr, A.M. (2002) Immune defense and host life history. *Am. Nat*. 160:S9–S22.