FARLEY WILLIAM SOUZA SILVA

PLASTICIDADE FENOTÍPICA E SUSCEPTIBILIDADE A PATÓGENOS EM *Anticarsia gemmatalis* (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*.

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"A ciência não pode prever o que vai acontecer. Só pode prever a probabilidade de algo acontecer." (César Lattes)

"Um pouco de ciência nos afasta de Deus. Muito, nos aproxima."
(Louis Pasteur)

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RESUMO

SILVA, Farley William Souza. M.Sc., Universidade Federal de Viçosa, julho de 2010. **Plasticidade fenotípica e susceptibilidade a patógenos em *Anticarsia gemmatalis* (Lepidoptera: Noctuidae)**. Orientador: Simon Luke Elliot. Co-orientadores: Eraldo Rodrigues de Lima e Fernando Hercos Valicente.

Investimentos profiláticos em defesa a parasitas e patógenos envolvem um custo. Assim, é esperado que organismos os usem somente quando requeridos, por exemplo quando em altas densidades populacionais, onde o risco de transmissão de parasitas e patógenos pode ser aumentado. Se o risco para o individuo aumenta com a densidade populacional, é esperado que hospedeiros evoluam mecanismos de resistência, como proposto pela hipótese da "profilaxia densidade-dependente" (PDD). Testes dessas predições têm sido conduzidos em uma gama de insetos, seja em estudos comparativos onde espécies vivem em diferentes densidades ou em estudos experimentais onde a densidade do hospedeiro é manipulada. Nesse caso, no entanto, testes têm sido restritos a insetos gregários que apresentam o polifenismo de fase densidade-dependente. Por isso, nós escolhemos testar essas predições em uma espécie solitária que apresenta características de insetos gregários, *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). Neste estudo, nós mostramos as repostas profiláticas de larvas de *A. gemmatalis* ao aumento da densidade de co-específicos. Quando em maiores densidades larvais, acompanhada de mudança fenotípica, há um maior grau de encapsulação do filamento de nilon, aumento no número de hemócitos, mas menor melanização. Nós mostramos que essas respostas foram função não só densidade *per se*, mas também do contato entre co-específicos. Dessa forma, nós mostramos que a PDD é um fenômeno mais comum que se pensava, ocorrendo mesmo em espécies conhecidas como sendo solitárias. Além disso, as respostas profiláticas nessa espécie não são só densidade-dependente, mas mais especificamente, contato-dependente. Isto fornece um novo contexto a essa hipótese, já que mesmo espécies solitárias podem apresentar respostas profiláticas ao aumento da densidade populacional.

ABSTRACT

SILVA, Farley William Souza. M.Sc., Universidade Federal de Viçosa, July, 2010. **Phenotypic plasticity and susceptibility to pathogens in *Anticarsia gemmatalis* (Lepidoptera: Noctuidae)**. Advisor: Simon Luke Elliot. Co-Advisors: Eraldo Rodrigues de Lima and Fernando Hercos Valicente.

Prophylactic investment in defense from parasites and pathogens involves a high cost. Thus, it is expected that organisms use this only when required, for example, when at high population densities, where the risk of parasite or pathogen transmission may be increased. If the infection risk for individuals increases with host density, it is expected that hosts subject to high densities evolve mechanisms of resistance, as proposed by the "density-dependent prophylaxis" (DDP) hypothesis. Tests of these predictions have been conducted in a wide range of insects, both in comparative studies, where species live at different densities and in experimental studies, where the host density is manipulated. In the latter case, however, tests have mostly been restricted to gregarious insects that present density-dependent phase polyphenism. For this reason, we chose to test these predictions in a solitary species, *Anticarsia gemmatalis* (Lepidoptera: Noctuidae), that presents features of gregarious insects. We showed a prophylactic response to increased conspecific densities in *A. gemmatalis* larvae. When at increased larval density, with the accompanying phenotypic change, there is a greater degree of encapsulation of the nylon filament, higher hemocyte numbers, but lower melanization. We showed that this response was a function, not only the density *per se*, but also of the contact among conspecifics. Thus, we showed that DDP is a more common phenomenon that previously thought, occurring even in a species known as being solitary. Furthermore, the prophylactic responses in this species are not only density-dependent, but are more specifically contact-dependent. This may provide a new context to the DDP hypothesis; in which even solitary species may present plastic prophylactic responses at crowded moments.

1. LITERATURE REVIEW

1.1. Pathogen transmission

All animals and plants interact with parasites (including pathogens). As a consequence, microrganisms represent an important selective force to their hosts (Wilson & Cotter 2009), with the ecology of these interactions being very diverse (Bonsall 2004), affecting direct and indirectly not only the individual host but also the host population. In nature, disease seems to be a density-dependent factor, with epizootics (i.e., generalized infection within a population) occuring with greater intensity in moments of high host densities (Steinhaus 1958).

The assumption in most parasite-host models is that transmission risk is density-dependent, or increases linearly with the population density (Anderson & May 1981; Wilson & Reeson 1998). Ryder *et al.* (2007) argue that if this transmission is direct, the model will be valid if contact among the hosts increases according with the population density. Thus, the infection rate is given as:

$$vmNS(I/N)$$

where v is probability of transmission by contact, m is a constant of the total population density (N) for a encounter rate density-dependent (mN), and S and I are the densities of susceptible and infected individuals, respectively.

This can be simplified to βSI, where β is the transmission coefficient (efficiency of transmission between "S" susceptible and "I" infective hosts) (Hochberg 1991; Wilson & Cotter 2009). In an analogy between the densities of susceptible and infective animals, and the concentration of two chemical reagents, the model is also called "mass action" (Knell *et al.* 1996; McCallum *et al.* 2001).

On the other hand, alternative models have shown that pathogen transmission in insects sometimes does not correlate linearly with host density (White & Wilson 1999; D'Amico *et al.* 2005; Boots & Mealor 2007). This may be observed in some lepidopteran species such as *Orgyia pseudotsugata* and *Lymantria dispar* (Lepidoptera: Lymantriidae), in which the increase of larval density resulted in a decrease in pathogen transmission, indicating that the transmission rate is not directly proportional to the host density (Dwyer 1991; D'Amico *et al.* 1996). This happens because if the infection risk for individuals increases with the host density, they are expected to evolve mechanisms of resistance through natural selection, following the "density-dependent prophylaxis" (DDP) (Wilson & Reeson 1998).

1.2. Hypothesis of density-dependent prophylaxis

The DDP hypothesis predicts that, (i) the mortality rate of individuals will decrease with prior experience of increased populations; (ii) the investment of each individual in resistance mechanisms is proportional to the increase in population; and (iii) under field conditions, the host mortality induced by parasites will saturate as a function of host density (Wilson & Cotter 2009). For this reason, insects that live at high population densities should be selected to invest more in resistance than those that live at low densities (Reeson *et al.* 1998). However, these conditions of high population density may be stressful to an insect, compromising its immune system and rendering it more vulnerable to pathogens (Steinhaus 1958). Faced with these two hypothesis, Reilly & Hajek (2008) tested the relationship between larval density of *L. dispar* and resistance to nucleopolyhedrovirus [one the most used system to test this hypothesis involve baculoviruses and caterpillars (White & Wilson 1999)], and found that

resistance to the virus decreased with an increase of density, and similarly, the time to death was faster in high densities than in low densities.

DDP seems to occur in a wide range of animals, mainly in those that presents variable densities through generations, such as insects (Wilson & Reeson 1998; Wilson *et al.* 2002). When the phenotype of an individual is changed in response to perceived variation in the local density of conspecifics, is known as "density-dependent phase polyphenism" (Whitman & Agrawal 2009; Wilson & Cotter 2009).

1.3. Density-dependent phase polyphenism

For Reeson *et al.* (1998)**,** the observation that in some species, the population in high densities triggers the cuticular melanization, suggests that a higher phenoloxidase (PO) level may increase the resistance, and the cuticular melanization will relate directly with the activity of phenoloxidase in hemolymph. As this prophylactic investment in resistance mechanisms to parasites (immunological, behavioral, chemical or physical) may involves a cost to be maintained, it is expected that organisms under stronger selective pressure will use them only when required (Reeson *et al.* 1998; White & Wilson 1999).

Phase polyphenism is a widespread phenomenon in insects, having been recorded in Lepidoptera, Orthoptera, Coleoptera and Hemiptera (Reeson *et al.* 1998; Wilson *et al.* 2002; Wilson & Cotter 2009), with the "phase" which individuals live in crowding, known as "gregaria", being characterized by individuals with darker or melanized cuticula than individuals of "solitary" phase (Cotter *et al.* 2004a). Examples of insects which have different phases are *Spodoptera littoralis* and *S. exempta* (Lepidoptera: Noctuidae), *Porthetria dispar* (Lepidoptera: Lymantriidae) and *Schistocerca gregaria* (Orthoptera: Acrididae); they differ not only in color, but also in

morphology, physiology and behavior (Leonard 1968; Wilson *et al.* 2001; Elliot *et al.* 2003; Lee *et al.* 2004). When they involve process of sclerotization and pigmentation of insect tegument, the morphological and chromatic transitions respond more slowly to changes in population density than behavioral transitions (Applebaum & Heifetz 1999).

1.4. Mechanisms of insect defense against parasites

Prophylactic investment in immune defense can vary with the population density experienced by the previous generations, with the prior experience of individuals to infection and/or during the early development of insect (Moret & Siva-Jothy 2003; Wilson & Cotter 2009; Elliot & Hart 2010). In lepidopterans, the amount of tactile stimuli received during the larval period define what kind of phenotype the individual will adopt (Reeson *et al.* 1998), although the phenotypic changes are not always visible (Wilson & Cotter 2009).

Regarding acquired immunity in invertebrates, there discussions about it, but both vertebrates and invertebrates have innate immunity (Moret & Siva-Jothy 2003; Kurtz & Armitage 2006). To test the possibility of an insect having adaptive innate immunity, Moret & Siva-Jothy (2003) exposed *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae to an immunological insult, and found a greater survival in later infections. The immune system of invertebrates comprises cellular responses (phagocytosis and encapsulation response by hemocytes), induction of antibacterial peptides and the pro-phenoloxidase (proPO) cascade (Little *et al.* 2005). However, there are high energy and nutrients costs associated with the maintenance of this system. Povey *et al.* (2009) showed that the survival of virus-infected *S. exempta* larvae increases with a high-protein diet, suggesting a high proteic cost associated with its resistance.

The system of the PO enzyme plays a important role in insects, especially in the encapsulation response (Robb *et al.* 2003). This occurs in the hemolymph in response to invasion for a wide range of microrganisms (Wilson *et al.* 2002; Moret & Siva-Jothy 2003). The melanization process involves the enzyme phenoloxidase that oxidizes tyrosine derivatives which subsequently produce melanin (Wilson *et al.* 2001; Cotter & Wilson 2002). PO has been isolated from the cuticule and hemolymph of some insects (Cotter & Wilson 2002; Moret & Schmid-Hempel 2009), and has also been linked to melanization in the midgut, in response to parasites that enter the host orally (Wilson *et al.* 2001).

1.5. *Anticarsia gemmatalis*: biological aspects, importance and control methods

Anticarsia gemmatalis Hübner (Lepidoptera: Noctuidae), known as the velvetbean caterpillar, is the key pest of soybean (*Glycine max*) in Brazil. As a holometabolous species, it goes through four development stages. The larval period may last around 26 days, with up to six instars (Andrade *et al.* 2004). Larvae present variable colors, depending on rearing density, varying from green to black and reaching up to 48 mm in length (Anazonwu & Johnson 1986; Fescemyer & Hammond 1986). Pupae coloration varies from green to brown and they measure about 18 mm in length and four mm in width. In this stage, the insects are found underground for a seven day period, until they emerge as adult moths. Adults live about 15 days and present, on average, a 35 mm wingspan, with the color varying from gray to brown, with no apparent sexual dimorphism (Andrade *et al.* 2004). Their reproductive process, including mating and oviposition, occurs at night (Hoffmann-Campo *et al.* 2000).

Each female is capable of laying 1000 eggs during its lifespan, and after eclosion, the larvae may cause significant losses to soybean fields, ranging from

defoliation to the complete destruction of the plant (Hoffmann-Campo *et al.* 2000; Praça *et al.* 2006). Much effort is invested in controlling this pest. Soybean is economically the most important crop in Brazil, responsible for significant expansion to new areas in the central and northern regions of the country (Piubelli *et al.* 2006). Some countries have invested in technologies and strategies that improve soybean productivity, such as the utilization of insect-resistant cultivars. These cultivars produce chemicals (secondary metabolites) which can have behavioral (antixenosis, i.e. occurring before feeding, with adverse plant effects on insect behavior) and/or physiological (antibiosis, i.e. occurring after feeding, with negative plant effects on insect growth and development) effects on insects (Piubelli *et al.* 2005).

Some of these chemical compounds act directly on the digestive system of the velvetbean caterpillar, inhibiting proteases (the main enzyme classes involved in the insect's digestion). However, the velvetbean caterpillar is able to bypass the effects caused not only by natural inhibitors found in soybean plants, but also by synthetic inhibitors (Visotto *et al.* 2009). For this reason, the choice of soybean cultivars resistant to this pest is so important.

Insecticide applications are another strategy commonly used to control this pest throughout soybean crops. However, this strategy has several negative points, such as residues in the crop, high cost, negative environmental impacts (Piubelli *et al.* 2006), and perhaps most importantly, selection for insecticide resistance within the insect population. Thus, biological control arises as an important component of soybean integrated pest management (IPM). In this stratategy of IPM, several kinds of biological control agents are used, such as parasitoids, predators and entomopathogens (e.g. fungi, bacteria and viruses). Currently, entomopathogens have an important role in the main control programs, either by direct use (as bioinsecticides) or indirectly (through genetic

engineering, e.g. transgenic soybean expressing genes that produce toxins against *A. gemmatalis* larvae).

The first example of an entomopathogen is *Bacillus thuringiensis* (Bt) which is a common soil bacterium that produces crystalline proteins (i.e. endotoxins encoded by *cry* genes) which are used to control certain insect pests, mainly Lepidoptera (Beron & Salerno 2006; Valicente *et al.* 2010). Control of the velvetbean caterpillar with Bt products may be a viable alternative (Bobrowski *et al.* 2002), but the main interest in this bacteria is because of its endotoxin production. Bt delta-endotoxins are specifically toxic to insect larvae (Saadaoui *et al.* 2009) and are activated under alkaline conditions of the lepidopteran midgut, resulting in the formation of cation channels that lead to osmotic imbalance, cell lysis and death of the insect (Hernandez-Martinez *et al.* 2009; McPherson & MacRae 2009). Thus, soybean encoding delta-endotoxins may be very useful to control *A. gemmatalis* (Homrich *et al.* 2008ab) with little or no negative effect on predatory arthropods (McPherson & MacRae 2009). Soybean cultivars expressing Bt toxins have not yet been commercialized, although experimental lines have been developed (Homrich *et al.* 2008a).

Nomuraea rileyi is an entomopathogen fungus that occurs frequently and offers high levels of natural control. It is an important candidate for velvetbean caterpillar IPM (Sujii *et al.* 2002). This entomopathogen has a wide host range, having been recognized as the main mortality factor in several lepidopteran populations, at 90% larval mortality (Boucias *et al.* 2000). When it attacks the velvetbean caterpillars, *N. rileyi* presents a white color due to its vegetative growth. As soon as appropriate humidity conditions occur, the fungus sporulates and turns from white to green. The spores produced on the dead caterpillars are spread by wind, infecting other individuals present in the crop and multiplying the pathogen. To avoid disrupting this natural control, it has been

recommended that monitoring be undertaken of the progress of the disease in velvetbean caterpillar populations, to avoid unnecessary applications of chemical products (Hoffmann-Campo *et al.* 2000).

Within the natural control of velvetbean caterpillar with entomopathogens, special attention has to be given to the *A. gemmatalis* multicapsid nucleopolyhedrovirus (AgMNPV), also known as *Baculovirus anticarsia*.This virus occurs naturally in velvetbean caterpillar populations in Brazil (Moscardi 1999) and from 1977, it was investigated in tests, indicating it as a potential control agent in soybean integrated pest management programs (Moscardi 1989). Currently, velvetbean control by AgMNPV is the largest program in the world using a virus against pest insects, being employed in an area of approximately 1,750,000 ha/year in Brazil (1999 value) (Moscardi 1999).

This virus belongs to the family Baculoviridae, which is subdivided in two subfamilies (Eubaculovirinae, including both occluded nuclear polyhedrosis viruses, NPV, and granulosis viruse, GV, and Nudibaculovirinae, comprised of nonoccluded baculoviruses). Eubaculovirinae is divided into two genera (*Nucleopolyhedrovirus* and *Granulovirus*). Virions of NPV and GV are occluded in polyhedral or capsular proteinaceous occlusion bodies (OB), respectively (Moscardi 1999).

In general, larval infection occurs orally by the ingestion of polyedra that are released into the insect midgut, cross the peritrophic membrane and fuse with the membrane of the midgut epithelial cells, initiating the replication cycle (Levy *et al.* 2007). After that, the virus reachs the hemolymph, and later, other organs and tissues, such as hemocytes, fat body and muscles (Savio & Pinotti 2008). According to Savio & Pinotti (2008), in this process, the larvae lose their ability to feed (on the fourth day after infection) and mobility, and die around the seventh day after infection. Infection in the field generally occurs by horizontal transmission, with the healthy larvae feeding on

leaves contaminated by frass or cadavers of other larvae. Following applications, such infected larvae may serve as inoculum for secondary virus transmissions, which may be affected by abiotic factors (e.g. rain and wind) or the movement of natural enemies (e.g. predators and parasitoids) (Moscardi 1999).

Although AgMNPV is an important component of the soybean integrated pest management, the use of this virus in laboratory in selection experiments has led to the emergence of resistent larvae to AgMNPV (Moscardi 1999; Piubelli *et al.* 2005). This is concerning, since this resistance is similar to that to chemical insecticides (Negreiro *et al.* 2009).

2. INTRODUCTION

Parasites represent an important selective force to their hosts (Wilson & Cotter 2009). The costs of this interaction may be expressed in different ways, such as a reduction in reproductive fitness, reduced survival or inhibition of metamorphosis (see Hu *et al.* 2008; Dawes *et al.* 2009; Pruijssers *et al.* 2009). Both empirical and theoretical studies have discussed the higher risk of pathogen transmission for insects experiencing high conspecific densities (Steinhaus 1958; Anderson & May 1981; Reeson *et al.* 1998; Wilson *et al.* 2002; Ruiz-Gonzalez *et al.* 2009). If the infection risk for individuals increases with host density, it is expected that host subject to high densities evolve mechanisms of resistance, as proposed by "density-dependent prophylaxis" hypothesis (Wilson & Reeson 1998). In the case of insects that undergo plastic phenotypic changes when at high densities (i.e., density-dependent phase polyphenism), they may invest more in prophylactic mechanisms according to a predictable infection threat at crowded moments (Wilson & Cotter 2009). As an example, Barnes & Siva-Jothy (2000) found that *Tenebrio molitor* beetles reared at high larval densities had higher degrees of cuticular melanization and lower mortality when exposed to an entomopathogenic fungus.

One aspect of these phenotypic adjustments is changes in immune parameters of insects, such as an increase in phenoloxidase (PO) activity and encapsulation response (Reeson *et al.* 1998; Wilson *et al.* 2001; Cotter *et al.* 2004a; Cotter *et al.* 2004b). The immune system of insects comprises cellular defenses (phagocytosis, encapsulation, and melanization; all mediated by hemocytes) and humoral defenses (antimicrobial peptides and products generated by enzymatic cascades, such as PO) (Strand 2008), although these two responses do not always occur separately. The melanization process involves the PO enzyme which oxidizes tyrosine derivatives producing melanin (Wilson *et al.*

2001; Cotter & Wilson 2002). PO has been isolated from cuticule and hemolymph of some insects (Cotter & Wilson 2002; Moret & Schmid-Hempel 2009), being also linked to melanization in the midgut of polyphenic insects, in response to parasites that enter the host orally (Wilson *et al.* 2001).

Phase polyphenism is a widespread phenomenon in insects, have been recorded in Lepidoptera, Orthoptera, Coleoptera and Hemiptera (Reeson *et al.* 1998; Barnes & Siva-Jothy 2000; Wilson *et al.* 2002; Wilson & Cotter 2009), with the "phase" which individuals live in crowding, known as "gregaria", being caracterized by individuals with darker or melanized cuticula than individuals of "solitary" phase (Cotter *et al.* 2004a). The polyphenism may result in changes not only in color, but also in morphology, physiology and behavior of insects (e.g. Leonard 1968; Wilson *et al.* 2001; Elliot *et al.* 2003; Lee *et al.* 2004). In the desert locust *Schistocerca gregaria*, the amount of tactile stimuli received during the nymphal period will define what phenotype the individual will adopt (Simpson *et al.* 2001). These authors identified by repeatedly touching with aid of paintbrush on outer face of nymphs' hind femur, where the mechanical stimulation elicited of gregarious phase behavior in solitarious phase. Phase polyphenism is a phenomenon commonly found in species that live gregariously, although it can also occur in those that live in isolation.

As an example, *Anticarsia gemmatalis* (Lepidoptera: Noctuidae), despite being a solitary species (i.e., adults lay their eggs singly and larvae live solitarily), presents density-dependent phase polyphenism (Fescemyer & Hammond 1986). Thus, as in other insect species, their body color varies from green, when larvae are reared in isolation (solitary), to black when they are reared in high densities (crowded) (Anazonwu & Johnson 1986; Fescemyer & Erlandson 1993). Besides polyphenism, the density may directly affect biological parameters in *A. gemmatalis*, such as larval

development and wet and dry weights and lipid and protein contents in adults (Fescemyer & Hammond 1986, 1988). This species, popularly known as the velvetbean caterpillar, is one the most important pests in soybean agrosystems (Zavala *et al.* 2001; Bobrowski *et al.* 2002; Piubelli *et al.* 2006), being widely distributed and causing yield losses in all areas where soybean is cultivated (Sosa-Gomez 2004). *A. gemmatalis* is mainly controlled by chemical insecticides, although it is also widely controlled by two entomopathogens: multicapsid nucleopolyhedrovirus (AgMNPV) (a species-specific pathogen) and *Bacillus thuringiensis* (specific to Lepidoptera) (Moscardi 1999; Bobrowski *et al.* 2002; Beron & Salerno 2006).

The aim of this study was to test the "density-dependent prophylaxis" hypothesis in a solitary species, *A. gemmatalis*. The reason is that *A. gemmatalis* lives solitarily, but presents features of gregarious insects that exhibit density-dependent phase polyphenism. To date, no studies have considered such intermediate systems. We first showed prophylactic responses to increased conspecific densities in *A. gemmatalis* larvae. We then measured some immune parameters as factors mediating the defense of larvae reared at different densities, concomitantly with larval phenotype, and directly tested susceptibility to the two pathogens mentioned above. Our results suggest that the prophylactic responses observed in this species are not only density-dependent, but are more specifically "contact-dependent".

3. MATERIALS AND METHODS

3.1. Anticarsia gemmatalis *density treatments*

A stock rearing of *Anticarsia gemmatalis* was maintained on artificial diet according to Hoffman-Campo *et al.* (1985), at 25±5 °C, 70±5 % relative humidity and 12h photophase. For the experiments, adults were allowed to oviposit on sheets of sulphite paper and eggs were separated. Hatching occurred within 24h of oviposition, and larvae were held in 100ml opaque plastic pots, lidded and with airholes, at four densities: 1, 2, 4 or 8 larvae/pot. They were kept in a climate-controlled chamber (25±1 °C, 60±3 % relative humidity and 12h photophase) until further use. This procedure was adopted in four separate assays (below), and insects were used at 10 days post-eclosion, approximately 4th instar. Throughout, only one insect, randomly chosen, was ever used from a given pot, to reduce pseudoreplication.

3.2. Frequency distribution of phenotypes

Anticarsia gemmatalis larvae expressed different color phenotypes - black, intermediate or green, according to rearing density. The phenotypes were determined according to coloration of larvae head capsules and body. The black phenotype has a dark body with dark spots of body color on dorsum, and a yellow-orange head capsule; the intermediate has black spots arranged on the dorsum and subdorsum, with head capsule color ranging from yellow to orange; the green phenotype has an olive-green body color with prominent black spots on it, and head capsules color ranging from green to yellow (see details in Fig. 1, and Fescemyer & Hammond 1986). We performed tests showing the frequency of larvae expressing these phenotypes in the four separate experiments below.

3.3. Encapsulation responses

When subjected to invasion by a parasite, one of the immune defenses of insects is a triggering of the encapsulation response (Cerenius *et al.* 2008). To determine if the density of conspecifics affects this, larvae were submitted to a challenge that simulates the presence of a parasite. Before the experiment, larvae were weighted to ensure that the corporal conditions were homogeny and the immune parameter were just a function of treatments. Thus, a piece of sterile nylon filament (2 mm length and 0,12mm Ø) was inserted through the first thoracic segment (dorsal region) of 30 larvae/density (two larvae died before the experiment, so n=118). After 24h, the larvae were dissected and the nylon filaments were mounted on slides. The slides were photographed (camera Power Shot A640 coupled to light microscope Zeiss Axioskop 40) and two variables were analyzed: capsule area formed around the nylon and capsule melanization, with the aid of IMAGEJ 1.42q software. Capsule area was assessed by selecting (polygon command) in pictures of the cells layer formed. To melanization measure, the pictures are converted to grayscale ranging from 0 to 255, allowing quantification as the mean value of this grayscale for each image (methodology adapted from Dubovskii *et al.* 2010).

3.4. Hemocyte counts

Another measure of an insect's immunity is hemocyte counts. As in the experiment above, larvae were weighted before to be used. Hemolymph extraction was conducted with 30 partially surface-sterilized (70% ethanol) larvae per density, by puncturing a small hole beside the first prolegs. The exuded hemolymph was collected with the aid of a pipette and was held in an Eppendorf tube with 20 µl anticoagulant buffer (98mM NaOH, 186mM NaCl, 17mM Na_2 EDTA and 41mM Citric acid, pH 4.5) plus 12 µl of

Giemsa stain. Two aliquots of 8 µl of hemolymph suspension were added in each side of a Neubauer improved chamber (Bright-Line, Precicolor (HBG), Germany) and hemocytes were counted under a microscope. The final value was the mean of the two aliquots, providing the cell numbers per microliter (adapted from Ibrahim & Kim 2006).

3.5. Susceptibility to Baculovirus anticarsia *(AgMNPV)*

The virus used in this assay, *Baculovirus anticarsia* or AgMNPV (kindly provided by CNPSo-EMBRAPA), is the principal ingredient of the BaculovirusAAE® bioinsecticide. AgMNPV was chosen as a model in this study because it occurs naturally in populations of *A. gemmatalis*, besides being used in one of the world's most successful programs of biological control (Moscardi 1999). Twenty-four hours before inoculation with AgMNPV, 30 larvae from each density treatment were individualized and starved. Thereafter, square soybean leaf pieces (15 x 15 mm) were inoculated with 20 µl of virus suspension (6 x 10^6 polyhedra/larvae) obtained from the formulated product plus Tween 80 (surfactant diluted to 0,01%) and offered to the larvae to feed for a 24h period. This viral concentration was chosen as a discriminatory dose in preliminary tests, as it started to kill larvae around the fifth day after inoculation. In the control group, 30 larvae/density were fed with soybean leaf pieces inoculated with 20 µl of distilled water plus Tween 80. The leaf piece is easily consumed by one larva in a single day (ensuring ingestion of a uniform number of viral particles and therefore infection); larvae that did not consume the entire leaf piece in this period were excluded from the experiment. Virus-inoculated larvae were kept as above and mortality was assessed daily until death or pupation.

3.6. Susceptibility to Bacillus thuringiensis *subsp.* kurstaki *(Bt)*

The bacterium used in this assay, *Bacillus thuringiensis* subsp. *kurstaki*, is widely used as the principal ingredient of a lepidopteran bioinsecticide (Dipel® - registred product to *A. gemmatalis*). This experiment was conducted according to the same methodology as that used for AgMNPV (above). Small soybean leaf pieces were inoculated with 20 µl of Bt suspension (3 x 10^4 spores/larvae) obtained from the formulated product, and offered to the larvae to feed for a 24h period. This bacterial concentration was chosen after preliminary tests.

3.7. Statistical procedures

Tests of frequency distribution of larval color phenotypes were realized with tests of independence incorporating *G*-tests (Sokal & Rohlf 1995; Elliot *et al.* 2003). The effects of density and phenotype on immunity parameters of *A. gemmatalis* were verified using generalized linear models (GLM with normal data) in R statistical software (R Development Core Team 2008). Models were analyzed to verify the significance, and following the analysis, data overdispersion was checked to determine whether the distribution was the most suitable. Finally, model simplification proceeded with the combination of categorical variables (i.e., phenotypes) found not to be significantly different (Crawley 2007). Data on the survival of *A. gemmatalis* larvae infected with virus or bacteria were analyzed using Kaplan-Meier survival curves in SPSS v.8. Data were censored when larvae pupated before the experiment end. The *logrank* test was used to test the equality of survival distributions and to perform pairwise comparisons among levels in each treatment (i.e. density or larval phenotype).

4. RESULTS

4.1. Frequency distribution of phenotypes

Anticarsia gemmatalis larvae expressed different color phenotypes - black, intermediate or green, according to rearing density – 1, 2, 4 or 8 per pot (see Figure 1). In all experiments, the frequency of the black phenotype increased with rearing density, while the frequency of the green phenotype decreased with rearing density. The frequency of the intermediate phenotype also decreased with density in all experiments, except in the last (i.e., Bt resistance), where it remained almost constant. Tests of independence show the frequency of larval color to be associated with rearing density in all four experiments (Values of G for: encapsulation response, hemocyte numbers, virus resistance and Bt resistance experiments are 77.60, 104.51, 78.66 and 79.77, respectively, all greater than $\chi^2_{0.001[6]}=22.46$ for $p<0.001$). Furthermore, when density 1 is compared with the remainder (densities 2, 4 and 8 pooled), the test of independence shows the larval phenotype to depend, more specifically, upon the presence or absence of conspecifics (Values of G for: encapsulation response, hemocyte numbers, virus resistance and Bt resistance experiments are 69.89, 90.30, 77.28 and 71.65, all greater than $\chi^2_{0.001[2]}=13.82$ for $p<0.001$).

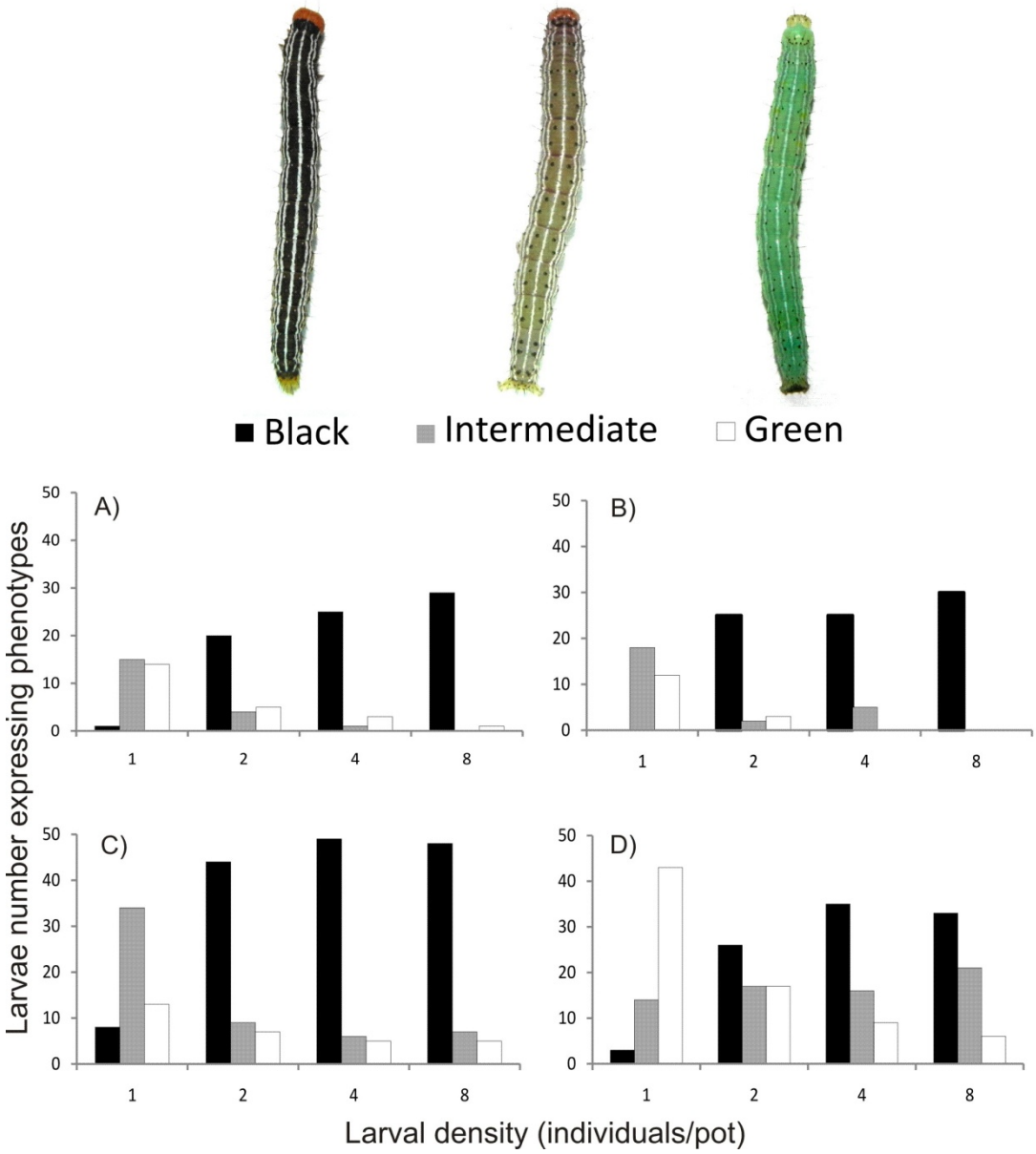

Figure 1. Frequency distribution of *Anticarsia gemmatalis* larvae expressing color phenotypes according to rearing density. Larvae were reared at four densities – 1, 2, 4 or 8 per cup and expressed the phenotypes black, intermediate or green as a result (see statistical tests in text). The black phenotype has a dark body with dark spots of body color on dorsum, and a yellow-orange head capsule; the intermediate has black spots arranged on the dorsum and subdorsum, with head capsule color ranging from yellow to orange; the green phenotype has an olive-green body color with prominent black spots on it, and head capsules color ranging from green to yellow. Frequency distributions are shown for data collected in each experiment: (A) Encapsulation response; (B) Hemocyte numbers; (C) Susceptibility to *Baculovirus anticarsia*; and (D) Susceptibility to *Bacillus thuringiensis*. The frequency of black phenotype increases with rearing density, while the frequency of green decreases. The frequency of intermediate phenotype also decreased with density, except in the last experiment (i.e., susceptibility to Bt), where it remained almost constant.

4.2. Encapsulation response

The degree of encapsulation of the nylon filament increased with larval density (Table 1; Figure 2-A). Note that larval weight did not vary with treatment ($F_{[1,116]}$=0.0026, p=0.9595), then, we did not include it in the models. To verify if the overall response was principally a function of density *per se*, or the presence or absence of conspecifics, we analyzed the densities separately. The response was observed when we compared the two lowest densities, 1 *versus* 2 larvae/pot but not when we excluded density 1 from the overall analysis (Table 1; Fig. 2-B). Besides density, we analyzed the encapsulation response associated with larval phenotype (i.e. phenotype as the independent variable). Both the complete model (all phenotypes) and the simplified model (black *versus* intermediate + green phenotype) showed that there is a higher encapsulation response in the black phenotype than the intermediate and green phenotypes, whether we included all densities (Table 1; Fig. 2-C) or densities 1 *versus* 2 larvae/pot in the analysis (Table 2; Fig. 2-D).

Another immune parameter assessed in this experiment was the capsule melanization formed around the nylon filament. In this case, however, melanization decreased with larval crowding (Table 1; Fig. 3-A). Interestingly, the trigger for the change appears to be the same: when we analyzed only density 1 *versus* 2 larvae/pot (Table 1), the increased melanization occured in solitary larvae (Fig. 3-B). When analyzed by larval phenotype, we found that melanization was higher in intermediate and green than in black larvae, both in the complete and the simplified models (Table 1; Fig. 3-C).

Table 1. Effect of rearing density (1, 2, 4 or 8 larvae/pot) and resultant phenotype (black, intermediate or green) on immune parameters in *Anticarsia gemmatalis* larvae. Data were analyzed according to density or phenotype. To verify if the overall immunological response was a function of density *per se*, or contact among larvae, we analyzed the data comparing all densities (1, 2, 4 or 8 larvae/pot), densities with density 1 removed, and density 1 *versus* 2. Variables were amalgamated when the mean values were not significantly different. (*p<0.05, **p<0.01,***p<0.001, *n.s.* = not significant).

Encapsulation	d.f.	residual d.f.	deviance	p	
General model (all treatments)					
By density	1	116	2160	0.01404	*
By phenotype	2	115	5609	0.0002891	***
Black vs. (intermediate + green)	1	116	5593	5.356e-05	***
Density 1 *versus* 2					
By density	1	57	2579.6	0.005537	**
By phenotype	2	56	2823.8	0.01496	*
Black vs. (intermediate + green)	1	57	2770.5	0.003946	**
Densities 2, 4 and 8					
By density	1	86	1	0.9613	*n.s.*
By phenotype	2	85	1053	0.2456	*n.s.*
Melanization					
General model (all treatments)					
By density	1	116	1383.1	0.01656	*
By phenotype	2	115	2855.1	0.002320	**
Black vs. (intermediate + green)	1	116	2611.7	0.0008655	***
Density 1 *versus* 2					
By density	1	57	2088.6	0.01398	*
By phenotype	2	56	1739.9	0.08451	*n.s.*
Densities 2, 4 and 8					
By density	1	86	26.0	0.6332	*n.s.*
By phenotype	2	85	224.7	0.3723	*n.s.*

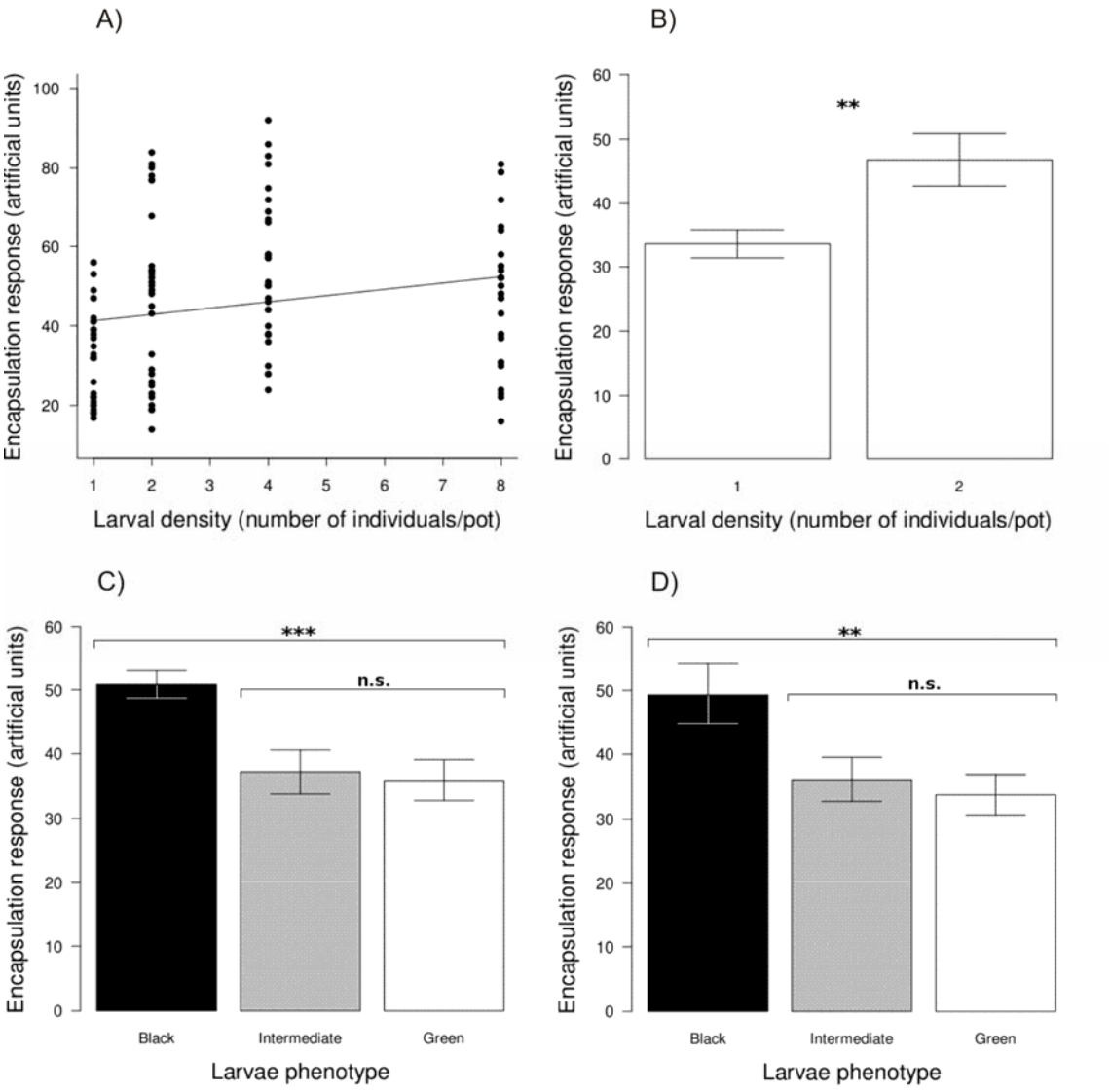

Figure 2. Encapsulation responses of *Anticarsia gemmatalis* larvae according to rearing density and resultant phenotype. Larvae were reared at four densities, 1, 2, 4 or 8 per cup, and expressed the phenotypes black, intermediate or green as a result (see Fig. 1); data are shown as responses to these two variables. To assess the immune defense, a piece of sterile nylon filament was inserted through the first thoracic segment of 30 larvae/density. (A) General model (all treatments) by density: Encapsulation increases with density; shown is curve of best model fit. (B) Density 1 *vs.* 2: Encapsulation (means and standard errors) is lower in solitary larvae than in those in the presence of conspecifics. (C & D) General model (all treatments) or Density 1 *vs.* 2 by phenotype - black *vs.* (intermediate + green): More encapsulation occurred in black than intermediate and green phenotypes, whether all densities are considered (C) or only densities 1 and 2 (D). Variables were amalgamated when the mean values were not significantly different (*F*-tests: **p*<0.05, ***p*<0.01, ****p*<0.001, *n.s.*= not significant; see table 1 for details).

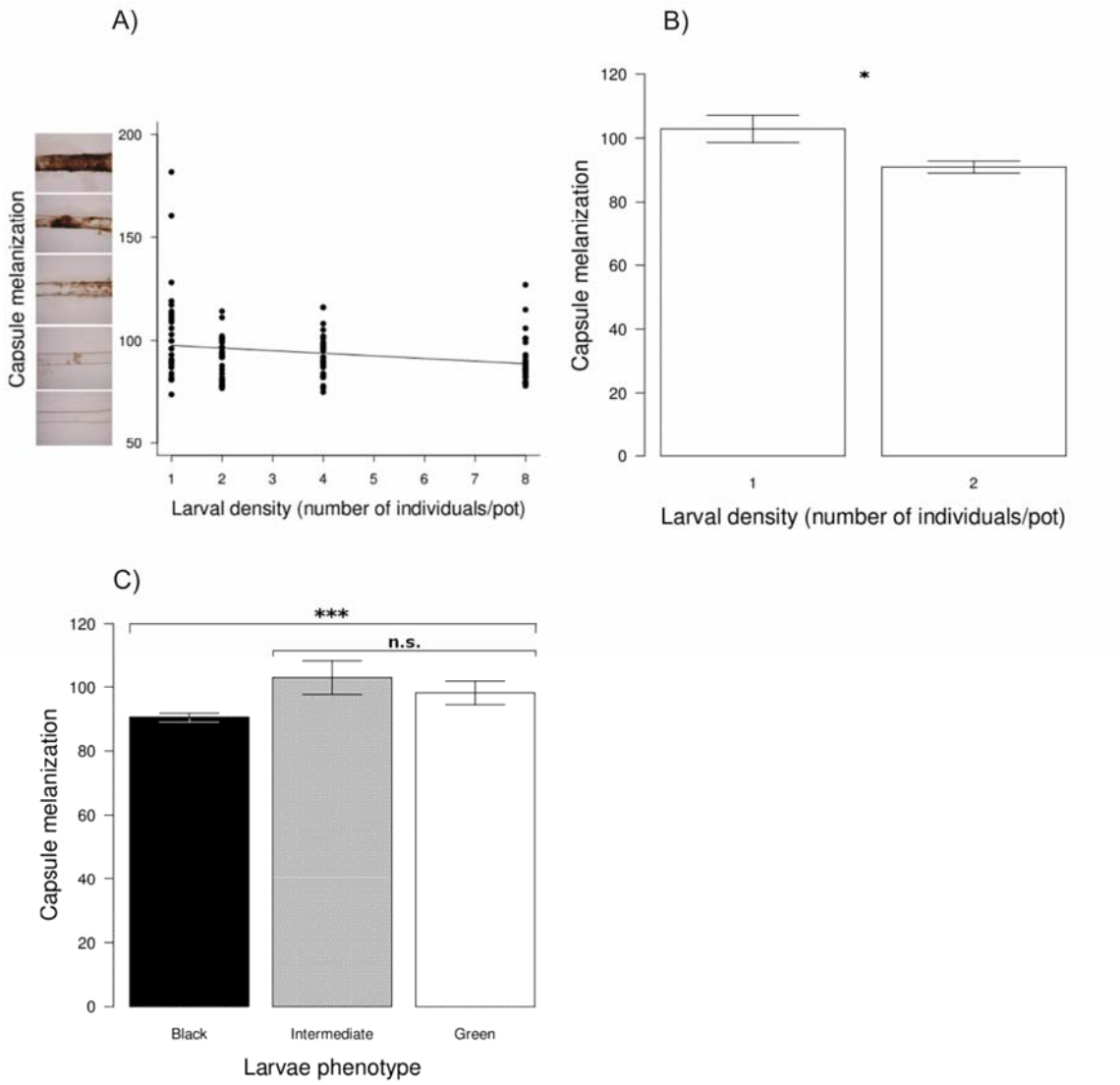

Figure 3. Melanization in *Anticarsia gemmatalis* larvae according to rearing density and phenotype. Larvae were reared at four densities – 1, 2, 4 or 8 per cup and expressed the phenotypes black, intermediate or green as a result (see Fig. 1); data are shown as responses to these two variables. To assess the melanization, a piece of sterile nylon filament was inserted through the first thoracic segment of 30 larvae/density, and after 24h, the nylon filaments were mounted on slides where the melanization was assessed with aid of software. (A) General model (all treatments) by density: Melanization decreases with density; shown is curve of best model fit. (B) Density 1 *vs.* 2: Melanization (means and standard errors) is higher in solitary larvae than in those in the presence of conspecifics (density 1 *versus* 2). (C) General model (all treatments) by phenotype - black *vs.* (intermediate + green): Less encapsulation occured in black than intermediate and green phenotypes. Variables were amalgamated when the mean values were not significantly different (*F*-tests: **p*<0.05, ****p*<0.001, *n.s.*= not significant; see table 1 for details).

4.3. Hemocyte counts

Larvae reared in contact with conspecifics had higher hemocyte numbers than those reared alone (Table 2; Fig. 4-A). In contrast with the previous assays, this was a result of the rearing density: firstly, there was no difference between densities 1 and 2; secondly, when analyzed with no density 1 the increase in hemocyte counts persisted (Table 2; Fig. 4-B). Finally, hemocyte numbers did not vary according to larval phenotype (Table 2). The larval weight did vary with treatments ($F_{[1,118]}$=11.361, p<0.05); however this term was not included in the model, because it did not affect hemocyte numbers ($F_{[1,118]}$ =0.9045, p=0.3435).

Table 2. Effect of rearing density (1, 2, 4 or 8 larvae/pot) and resultant phenotype (black, intermediate or green) on hemocyte numbers in *Anticarsia gemmatalis* larvae. Data were analyzed according to density or phenotype. To verify if the overall immunological response was a function of density *per se*, or contact among larvae, we analyzed the data comparing all densities (1, 2, 4 or 8 larvae/pot), densities with density 1 removed, and density 1 *versus* 2. (*p<0.05, **p<0.01, *n.s.*= not significant).

Hemocyte numbers	d.f.	residual d.f.	deviance	*p*	
General model (all treatments)					
By density	1	118	7355043	0.006951	**
By phenotype	2	117	1804220	0.4193	*n.s.*
Density 1 *versus* 2					
By density	1	58	2667	0.9502	*n.s.*
By phenotype	2	57	642924	0.6254	*n.s.*
Densities 2, 4 and 8					
By density	1	88	5601333	0.02431	*
By phenotype	2	87	1002446	0.6436	*n.s.*

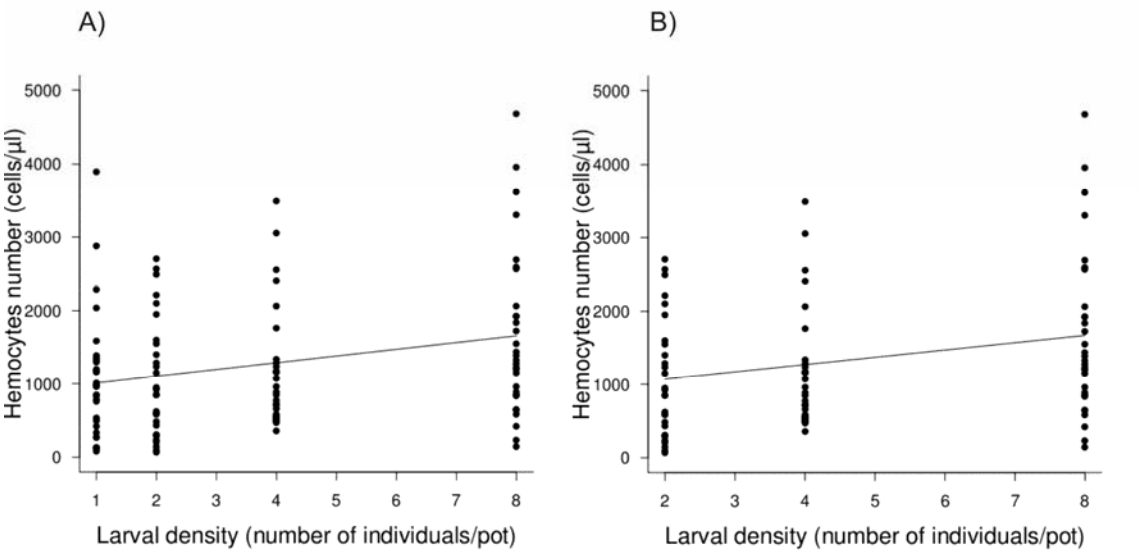

Figure 4. Hemocyte numbers of *Anticarsia gemmatalis* larvae according to rearing density. Larvae were reared at four densities – 1, 2, 4 or 8 per cup. To count the cell numbers, a hemolymph aliquot was extracted from each larva (30 larvae/density), placed in a Neubauer improved chamber, and counted under a microscope. (A & B) General model (all treatments) or Densities 2, 4 and 8: Hemocyte number increase with density; shown is curve of best model fit, whether all densities are considered (A) or only densities 2, 4 and 8 (B). (See table 2 for analyses.)

4.4. Susceptibility to Baculovirus anticarsia *(AgMNPV)*

Throughout, comparisons between control and infection treatments were highly significant ($p<0.0001$). Survival curves of inoculated and uninoculated *A. gematalis* larvae are shown in Fig. 5. Larvae of the uninoculated control did not present signs of infection and did not die from virosis, the majority them pupated before the experiment end. The survival among inoculated larvae in different density treatments was equally and linearly distributed, i.e., larval survival time increased with the rearing density ($Z=16.19$, $p=0.001$). The mean survival time of inoculated larvae reared alone (1 individual/pot) was 198 (± 8, standard error) hours, while those reared in contact with conspecifics (2, 4 or 8 individuals/pot) survived for mean times of 220 (± 7), 220 (± 8) and 234 (± 9) hours, respectively. Hence, we did pairwise comparisons among pathogen treatments, showing that contact with conspecifics increased larval survival (comparing

density 1 with 2, *log-rank* Z=5.40, p=0.0202; 1 with 4, Z=5.29, p=0.0214; and 1 with 8 larvae/pot, Z=12.89, p=0.0003). Meanwhile, there were no statistically significant differences between the remaining density treatments (comparing density 2 with 4, Z=0.01, p=0.9035; 2 with 8, Z=2.75, p=0.0970; and 4 with 8 larvae/pot, Z=3.67, p=0.0553).

When we analyzed survival of inoculated insects by phenotype, larvae that presented the green phenotype (most of the larvae reared alone) survived for less time (a mean of 199 ± 11 hours) than the black phenotype (means of 218 ± 8 and 221 ± 5 hours respectively; *log-rank* Z=3.99, p=0.0458), while neither differed significantly from the intermediate phenotype (comparing intermediate with black phenotype, Z=0.42, p=0.5145; intermediate with green phenotype, Z=1.50, p=0.2206).

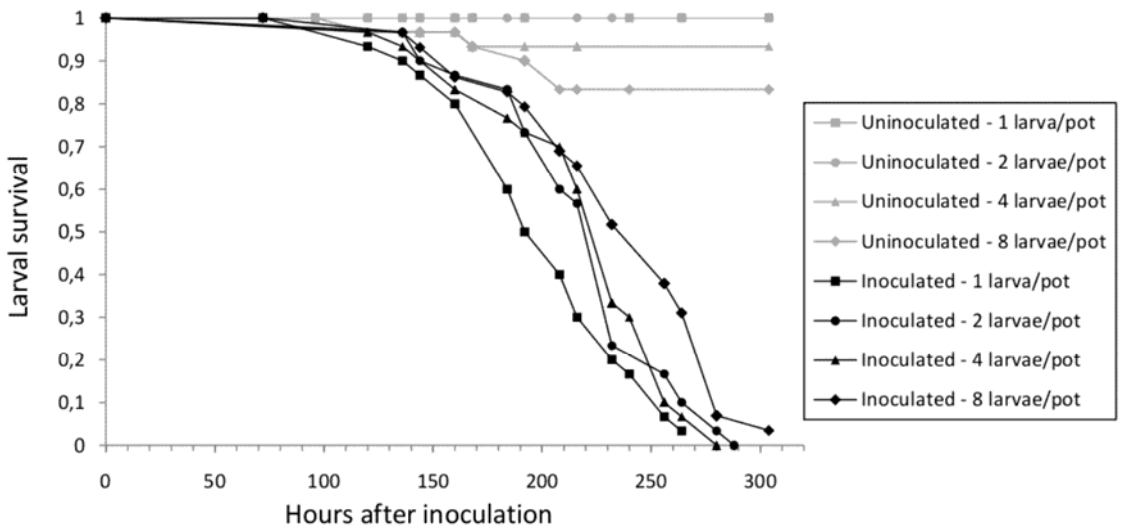

Figure 5. Survival curves of *Anticarsia gemmatalis* larvae uninoculated and inoculated with *Baculovirus anticarsia*. Larvae were reared at four densities – 1, 2, 4 or 8 per cup. The group of pathogen treatment were inoculated with 20 µl of virus suspension (6 x 10^6 polyhedra/larvae), while those of control treatment were inoculated with 20 µl of distilled water. The larval mortality was assessed daily, and the larvae that pupated before the experiment end were included in analysis as censored data. Survival analyses are presented in the text.

4.5. Susceptibility to Bacillus thuringiensis

Throughout, comparisons between control and infection treatments were highly significant ($p<0.0001$). Survival curves of inoculated and uninoculated *A. gematalis* larvae are shown in Fig. 6. The survival among larvae in different treatments was not equally and linearly distributed, i.e., larval survival did not vary with the rearing density ($p>0.05$). Pairwise comparisons among pathogen treatments also revealed no differences ($p>0.05$). Survival was also not different among the larval phenotypes (comparing black with intermediate phenotype, *log-rank* $Z=0.19$, $p=0.6604$; black with green phenotype, $Z=0.13$, $p=0.7149$; intermediate with green phenotype, $Z=0.52$, $p=0.4724$).

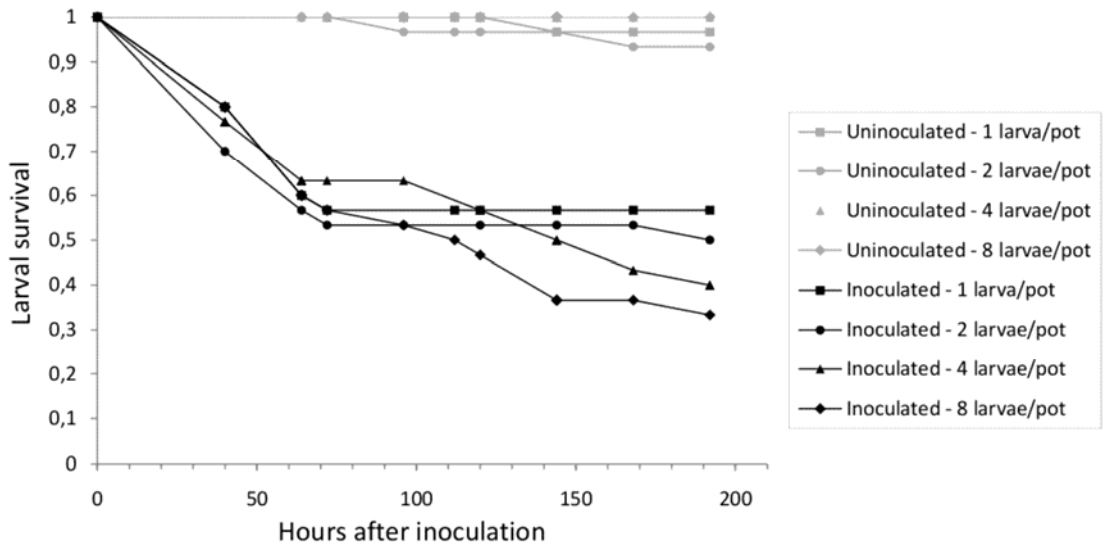

Figure 6. Survival curves of *Anticarsia gemmatalis* larvae uninoculated and inoculated with *Bacillus thuringiensis*. Larvae were reared at four densities – 1, 2, 4 or 8 per cup. The group of pathogen treatment were inoculated with 20 µl of Bt suspension (3 x 10^4 spores/larvae), while those of control treatment were inoculated with 20 µl of distilled water. The larval mortality was assessed daily, and the larvae that pupate before the experiment end were included in analysis as censored data. Survival analyses are presented in the text.

5. DISCUSSION

The relation between pathogen risk and resistance varying with host density has been investigated in previous work (Steinhaus 1958; Wilson & Reeson 1998; Barnes & Siva-Jothy 2000). This has mainly been done with gregarious insects: *Spodoptera exempta* and *S. littoralis* (Reeson *et al.* 1998; Wilson *et al.* 2001), and *Schistocerca gregaria* (Wilson *et al.* 2002); and eusocial insects: *Zootermopsis angusticollis* (Pie *et al.* 2005) and *Bombus terrestris* (Ruiz-Gonzalez *et al.* 2009). However, none of these have shown these relations in solitary species living in crowded conditions. Hence the importance of this study with *A. gemmatalis*, since the insect lives solitarily, but presents features of a gregarious insect that exhibits density-dependent phase polyphenism. In species that present this polyphenism, the individuals change not only the color phase, but also morphology, physiology and behavior (e.g. Leonard 1968; Fescemyer & Hammond 1986; Wilson *et al.* 2001; Sword 2002; Elliot *et al.* 2003; Lee *et al.* 2004; Gray *et al.* 2009; Whitman & Agrawal 2009). To date, it is not known what occurs with species that exhibit an intermediate pattern between density-dependent phase polyphenism (gregarious) and a completely solitary existence without such phenotypic changes. It seems that this plastic phenotypic adjustment is more common than previously thought.

The resistance arising from phase polyphenism may be one of the strategies used by some species to deal with the higher disease risk. In some of these, the amount of tactile stimulus received during the larval stage is supposed to trigger cuticular melanization, through to enzymatic reactions, and increased individual defense to pathogens (Reeson *et al.* 1998). In *A. gemmatalis*, larvae presented a similar behavior, expressing a higher frequency of darkest phenotypes in moments of increased contact among conspecifics. In tests of independence, we showed the frequency of larval color

to be associated with rearing density. This observation corroborates that of Fescemeyer & Hammond (1986), where the proportion of black *A. gemmatalis* larvae increased with density, both in laboratory and field experiments. They argue, however, that this phenotypic change does not occur until larvae have molted to the last instar. This would not fit very well with the "density-dependent prophylaxis" (DDP) hypothesis (Wilson & Reeson 1998), since such changes would be related to evolution of defense mechanisms in individuals faced with higher infection risk during crowded periods. This risk would not be expected to affect the last instar of *A. gemmatalis* larvae, since they stop feeding (Hoffmann-Campo *et al.* 1985), so are not vulnerable to pathogens that infect orally. Thus, phenotypic changes and prophylactic investment in this species should occur in the stage of higher infection risk by viruses, i.e., in the first three instars (Moscardi 1999). In contrast to Fescemeyer & Hammond (1986), we observed (data not shown) that *A. gemmatalis* larvae start to change their phenotype around the fifth day after eclosion, approximately the 3rd instar.

Furthermore, we showed (in the tests of independence of frequency of larval color) for the first time that this change depends, more specifically, upon the presence or absence of conspecifics. This highlights, at least in part, that is necessary for only one conspecific to trigger the phenotypic and prophylactic responses in this lepidopteran species, giving a new context to DDP hypothesis. It predicts that prophylaxis would be a density-dependent factor, but it does not explicitly treat the relation with pathogen resistance being contact-dependent.

In the desert locust *S. gregaria* (one model-species used to test this DDP hypothesis, see Wilson *et al.* 2002), the amount of tactile stimuli, i.e., repeated touching with a paintbrush the outer face of nymph's hind femurs, will elicit individual phase changes and define what phenotype the individual will adopt (Simpson *et al.* 2001). In

A. gemmatalis, the stimulus that triggers the phenotypic change appears to be more sensitive, since the contact with only one conspecific is able to trigger the reactions on individual. Apparently, this change is only of phase, but there is a possibility to be affecting the behavior of larvae as well, since as noted (personal observation), after the phenotypic changes, *A. gemmatalis* larvae move to different locations of the rearing recipient.

In partial support of the DDP hypothesis, we found that larvae reared at higher densities (or simply in contact with conspecifics – 2 larvae per pot) and expressing black phenotypes presented elevated prophylactic immune defenses. Both encapsulation and hemocyte numbers increased with larval density and with this darker coloration. Hemocytes are cells responsible for immune responses in insects, including encapsulation and melanization, which act in defense against pathogens invading the hemolymph (Marmaras & Lampropoulou 2009). Encapsulation in *A. gemmatalis* increased even when we analyzed the two lowest densities (1 *vs.* 2 larvae/pot), but not when we excluded density 1 from the overall analysis. This encapsulation was also a function of the larval phenotype, with the higher encapsulation occurring in the black phenotype.

Hemocyte numbers also increased according to the larval rearing density, such that those reared in contact with conspecifics had increased hemocyte numbers compared to those reared alone. This was a response to the rearing density, whereas when analyzed with density 1 removed, the increase tendency on cells number continued. This reinforces the idea that larvae that are in constant contact with others, elevate their immunological responses to minimize the increased risk of pathogen exposure, since the increased frequency of the black phenotype is directly associated with population density.

It is expected that there are trade-offs in immune defenses, since these requires a investment to be maintained. For example, Povey *et al.* (2009) showed that some immune functions, and consequently, survival of infected *S. exempta* larvae (related specie of *A. gemmatalis*), are directly associated with the intake of protein-rich diets, suggesting a cost to defense. In a multivariate approach, Cotter *et al.* (2004) showed that some immune functions are greater in crowded and black larvae, while others are not. For instance, solitary-reared *S. littoralis* larvae presented higher melanization than crowded larvae. In the present study, the melanization of *A. gemmatalis* larvae also responded in the opposite direction to that found in other insects' prophylactics mechanisms, in that it decreased according to larval crowding. It is worth pointing out that the trigger for the change appears to be the same: higher melanization occurred in solitary larvae and intermediate and green phenotypes. Melanization is a process mediated by hemocytes and fat body (Wang *et al.* 2010), and only occurs after the formation of a capsule around the invader (in this study, the nylon filament inserted in larvae) by hemocytes (Marmaras & Lampropoulou 2009). Negreiro *et al.* (2009) suggest that the defense of *A. gemmatalis* larvae to AgMNPV may be attributed to higher hemocyte numbers in hemolymph. Thus, it is likely that solitary-reared larvae have low hemocyte numbers, yet begin the melanization process faster than larvae in high densities.

Immunological responses may have been the main factors in the defense of *A. gemmatalis* larvae to *Baculovirus anticarsia* (AgMNPV). With the increase of population density, and consequently, pathogen threat, larvae presented greater survival when they had previous contact with conspecifics. Previous works have also shown this relation between larval density and resistance to virus, both in the field and laboratory (Reeson *et al.* 2000; Wilson *et al.* 2001). Goulson & Cory (1995) found similar results

to the present study, where *Mamestra brassicae* larvae reared in 2, 4 and 10 individuals per recipient and infected with MbNPV presented greater survival than those reared at a density of one individual per recipient.

This same response may not occur for *A. gemmatalis* larvae when faced with another kind of pathogen, such as *Bacillus thuringiensis* (Bt). The increase in population density did not trigger prophylactic responses against this bacterium: larvae reared at all densities were equally susceptible. It is probable that immune responses of these larvae are linked to selective pressure that each pathogen has been exercising on its population. AgMNPV occurs naturally in *A. gemmatalis* populations (Moscardi 1999), and due to its specificity (Castrol *et al.* 1999), it likely had presented a selective pressure driving the evolution of plastic prophylactic mechanisms in this lepidopteran species. According to Wilson & Reeson (1998), DDP is a phenomenon that occurs in a specifically coevolved system, such as AgMNPV-*Anticarsia gemmatalis* larvae. However, Barnes & Siva-Jothy (2000) tested the DDP in a non-coevolved insect-pathogen system, and found that it occurs even in a host from a population not previously exposed to that pathogen. In the present study there was not this plastic prophylactic adjustment in larvae inoculated with Bt (a non-specific bacterium that infects a wide range of lepidopteran hosts) (Liu *et al.* 2010; Valicente *et al.* 2010). Bt is a pathogen not found infecting insects in the field; thus, epizootics do not occur, and there is no reason to be a density-dependent increase in infection risk. The mechanism for Bt infection is very specific, so it is unlikely that selection pressure from another pathogen (including bacteria) would affect susceptibility to it.

These results may have some implications in the control of this pest in the field. Firstly, AgMNPV is one the most extensively used control agents against *A. gemmatalis* in soybean fields (Levy *et al.* 2009), and its use in moments of high larval density could

mean its failure as a control agent. Secondly, AgMNPV production is done through inoculation *in vivo*, and density effects may disturb the large-scale virus production. It is also possible that the passage of AgMNPV through the *A. gemmatalis* larvae (i.e., horizontal transmission) living at high densities leads to negative effects on the virus, such as the production and/or release of viral particles (Gomi *et al.* 1997), and deletion of genes essential for its infectivity (Pan *et al.* 2007), whereas in higher larval densities there will be more barriers to viral replication, such as increased hemocyte numbers.

In conclusion, we have shown that DDP is a phenomenon more common that thought, occurring even in a species known such as solitary, *A. gemmatalis*. Furthermore, the prophylactic responses in this species are not only density-dependent, but are more specifically contact-dependent. This may provide a new context to this hypothesis, in which even solitary species may present a plastic prophylactic response at crowded moments. Besides, we showed that the lower *A. gemmatalis* susceptibility to pathogen occurs in the black phenotype. It will be important to determine if this phenotypic changes affects only resistance, or if it affects the behavior of larvae as well.

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