FARLEY WILLIAM SOUZA SILVA

EFFECTS OF TEMPERATURE AND POPULATION DENSITY ON IMMUNE DEFENCES IN *ANTICARSIA GEMMATALIS* (LEPIDOPTERA: NOCTUIDAE)

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

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SUMÁRIO

RESUMO

SILVA, Farley William Souza. D.Sc., Universidade Federal de Viçosa, fevereiro de 2014. **Efeitos da temperatura e densidade populacional sobre defesas imunológicas em *Anticarsia gemmatalis* (Lepidoptera: Noctuidae).** Orientador: Simon Luke Elliot. Coorientadores: Eraldo Rodrigues de Lima e Ângelo Pallini Filho.

Todos os animais e plantas interagem com patógenos. Como conseqüência, patógenos representam uma importante força de seleção a seus hospedeiros, afetando direta e indiretamente, não só o indivíduo, mas também a população hospedeira. Para lidar com essas ameaças, organismos evoluíram uma série de barreiras (físicas e imunológicas) para prevenir a infecção ou combatê-las depois do seu estabelecimento. No entanto, existem elevados custos de energia e de nutrientes associadas à manutenção de um sistema imune. Alguns organismos desenvolveram estratégias para investir plasticamente nos sistemas imunológico em momentos de maior risco de infecção, por exemplo, em altas densidades populacionais. Aqui, nós verificamos se as barreiras, não só imunológicas, mas também físicas, variavam com fatores ambientais (temperatura e densidade populacional). Primeiro, descobrimos que uma barreira física (ou seja, matriz peritrófica e epitélio do intestino médio) de *Anticarsia gemmatalis* (Lepidoptera: Noctuidae), muda plasticamente de acordo com o fenótipo, o que é em si uma resposta a mudanças na densidade populacional. Além de mudar morfologicamente com o fenótipo larval, essas barreiras também variaram entre regiões do intestino médio. Em segundo lugar, verificou-se que as variáveis ambientais, principalmente a temperatura, afeta tanto o curso da doença na população larval como as suas defesas contra patógenos.

ABSTRACT

SILVA, Farley William Souza. D.Sc., Universidade Federal de Viçosa, February, 2014. **Effects of temperature and population density on immune defences in *Anticarsia gemmatalis* (Lepidoptera: Noctuidae)**. Adviser: Simon Luke Elliot. Co-advisers: Eraldo Rodrigues de Lima and Ângelo Pallini Filho.

All animals and plants interact with pathogens. As a consequence, pathogens represent an important selective force to their hosts, affecting directly and indirectly not only the individual host but also the host population. To deal with this threat, organisms have evolved a range of barriers (physical and immunological) to prevent infection or fight it after it is established. However, there are high energy and nutrients costs associated with the maintenance of an immune system. Some organisms have evolved strategies to invest plastically in immune systems at moments of increased risk of infection, e.g. at high population density. Here, we looked at whether barriers, not only immunological but also physical, varied with environmental factors (i.e. temperature and population density). First, we found that a physical barrier (i.e. peritrophic matrix and midgut epithelium) of the velvetbean caterpillar *Anticarsia gemmatalis* (Lepidoptera: Noctuidae), changed plastically according to phenotype, which is itself a response to changes in population density. Beyond changing morphologically with the velvetbean caterpillar's phenotype, these barriers also varied between regions of the midgut. Second, we found that environmental variables, mainly temperature, strongly affected both the course of disease in velvetbean caterpillar population and its defenses against pathogens.

INTRODUCTION

All animals and plants interact with parasites and pathogens (we henceforth use the term pathogens). As a consequence, pathogens represent an important selective force to their hosts (Wilson and 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. To deal with this threat, organisms have evolved a range of barriers (physical and immunological) to prevent infection or fight it after it is established (Webb and Green, 1945; Mercer and Day, 1952; Forster, 1953; Briggs, 1958; Stephens, 1959; Stephens and Marshall, 1962; Salt, 1963; Zhuzhikov, 1964; Salt, 1973; Brandt et al., 1978).

For insects, physical barriers are the cuticle and peritrophic matrix. While the first provides protection against pathogens such as fungi which attach to insect body prior to infection (Gabriel, 1968; Vincent and Wegst, 2004), the peritrophic matrix protects the haemocoel from pathogens such as bacteria and viruses acquired during feeding (Terra, 1990). Whereas the immunological barriers consist of two components: cellular and humoral responses. They are employed in phagocytosis and encapsulation response by hemocytes, induction of antibacterial peptides (AMP) and the pro-phenoloxidase (proPO) cascade that produces melanin (Little et al., 2005). PO has been isolated from haemoplymph and cuticle of insects (Wilson et al., 2001; Cotter and Wilson, 2002; Moret and Schmid-Hempel, 2009), being also responsible for melanization in the midgut in response to pathogens orally acquired (Wilson et al., 2001).

However, there are high energy and nutrients costs associated with the maintenance of the immune system. For example, Povey et al. (2009) showed that the survival of virus-infected *Spodoptera exempta* larvae increases with a high-protein diet,

suggesting a high proteic cost associated with its resistance. If the costs of mounting an immune response outweigh the benefits of its employment, other nutrient-demanding traits may be impacted and fitness components negatively affected when resources are finite. At such circumstances, organisms can rarely optimize traits simultaneously, and the result will be a trade-off between immune investment and other life-history traits such as longevity and reproduction (Ahmed et al., 2002; Stahlschmidt et al., 2013).

However, some organisms have evolved strategies to invest plastically in immune systems at moments of increased risk of infection, e.g. at high population density [“density-dependent prophylaxis” hypothesis, (Wilson and Reeson, 1998)]. The DDP hypothesis’s predictions are that, while the mortality rate decreases, the investment in mechanisms of resistance increases proportionally to the increase in population density (Wilson and Cotter, 2009). This, because the assumption in most parasite-host models is that transmission risk is density-dependent, or increases linearly with the population density (Anderson and May, 1981; Wilson and Reeson, 1998). Thus, insects should be selected to invest more in resistance at high population densities than at low densities (Reeson et al., 1998; Wilson and Reeson, 1998). However, high population density may be stressful to an insect, compromising its immune system and rendering it more vulnerable to pathogens (Steinhaus, 1958).

The phenomenon of DDP seems to occur in a wide range of animals, mainly in those presenting fluctuations in population density through generations, such as insects (Wilson and 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 and Agrawal, 2009; Wilson and Cotter, 2009). 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 and Cotter, 2009), with the "phase" which individuals live in crowding, known as "gregaria", being characterized by individuals with darker or melanized cuticle than individuals of "solitary" phase (Cotter et al., 2004). Examples of insects which have different phases are *Spodoptera littoralis, S. exempta* and *Anticarsia gemmatalis* (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; Silva et al., 2013).

Subject of study

The velvetbean caterpillar *Anticarsia gemmatalis*

Anticarsia gemmatalis Hübner is one of the main pests of soybean (*Glycine max*) in Brazil. Insecticides are commonly used to control this pest. 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 types of biological control agents are used, such as parasitoids, predators and entomopathogens. Currently, entomopathogens have an important role in the control programs, either by direct use (as bioinsecticides) or indirectly (through genetic engineering, e.g. transgenic soybean expressing genes that produce toxins against this pest). As direct use, 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), presenting thus a potential control agent in soybean integrated pest management programs (Moscardi, 1989).

Aims

The aim here is look at whether barriers, not only immunological but also physical, vary with environmental factors (i.e. temperature and population density).

In chapter one, we test whether the midgut primary defences of the velvetbean caterpillar present phase-dependent plasticity. We analyze whether its morphometry and structure change in accordance to colour transition in caterpillars, and whether those changes may provide to caterpillars a more protective barrier against invasion by *Baculovirus anticarsia*.

In chapter two, we test the hypothesis that environmental variables such as temperature and population density affect either directly or indirectly immune parameters in the velvetbean caterpillar. First, temperature may affect directly the parameters by changing the caterpillar's body condition, and second by changing caterpillars' movement rates, which presumably affects contact between conspecifics, and thus the likelihood of becoming infected.

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CHAPTER 1

Midgut structural plastic responses in the velvetbean caterpillar as signal of increased risk of parasite invasion

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ABSTRACT

Organisms use a set of primary barriers to prevent invaders and secondary responses to fight those that breach the primary barriers. However, maintaining both sets of defences active and effective may be costly. Thus, some organisms up-regulate their immune defences when high population density indicates an increased risk of being infected. Here, we found that a primary barrier (i.e. peritrophic matrix and midgut epithelium) of the velvetbean caterpillar *Anticarsia gemmatalis* also change plastically according to phenotype, which is itself a response to changes in population density. We found that beyond changing morphologically with the velvetbean caterpillar's phenotype, these barriers also vary between regions of the midgut. Whereas measurements of green caterpillars decrease from the anterior to the posterior region, black caterpillars show the opposite pattern with measurements increasing along the midgut lumen, such as thickness of the midgut epithelium, and thickness and chitin in the peritrophic matrix. Furthermore, black caterpillars presented both midgut epithelium and peritrophic matrix thicker than green caterpillars, and also more chitin in the peritrophic matrix. We hypothesize that in polyphenic insects, including the velvetbean caterpillar both primary and secondary defences may arise as a positive result of pleiotropy, where the biochemical pathways responsible for the up-regulation of the immune system are also involved in midgut properties.

KEY WORDS: *Anticarsia gemmatalis*, Baculovirus, Peritrophic matrix, Defences, Chitin

INTRODUCTION

As a rule, multicellular organisms use a set of primary barriers (such as shell, bark, skin and cuticle) to limit and counter infection by parasites and pathogens (Brown et al., 1965; St Leger et al., 1986; McDowell et al., 1988; Wainhouse et al., 1990; Hotez et al., 1992; St Leger, 1995; Frohm et al., 1997; Rae et al., 2008). These barriers are not infallible, however, so once they are overcome, the triggering of other mechanisms in response, such as the immune system, is the next step. Ideally for an organism, it would be possible to actively maintain both constitutive and induced defences. However, investment in these defences may be constrained by resource availability, the likelihood of parasite encounter (and the virulence of these parasites) and genetic constraints (Rolff et al., 2005; Hamilton et al., 2008; Povey et al., 2009). Thus, if the risk of invasion by parasites correlates positively with host population density, the potential host should up-regulate its immune defences to counter the increased risk of being infected. This hypothesis [known as DDP - Density-Dependent Prophylaxis (Wilson and Reeson, 1998)] has been broadly supported by studies on both solitary and gregarious organisms (Barnes and Siva-Jothy, 2000; Wilson et al., 2002; Mills, 2012).

Most work on the DDP hypothesis has focused on the plasticity of host immune responses (i.e. secondary defences) to face parasite invasion. While no studies have examined whether primary defences present plastic physical responses (i.e. changes in morphology and morphometry) to fluctuations in population density, a few have looked at whether chemical properties of such barriers as cuticle and midgut vary plastically with population density (Wilson et al., 2001; Cotter et al., 2004). Parasites and pathogens use different routes to invade the host; whilst some of them, such as fungi, must firstly to overcome the cuticle (St Leger, 1995), other invaders that are orally

acquired during the host's feeding, such as baculoviruses, bacteria, fungi and algae, have to interact with the host midgut epithelium prior to initiate infection (Mewis et al., 2003; Blaske-Lietze and Boucias, 2005; McNeil et al., 2010; Crava et al., 2013).

The midgut is the major segment of the gut where digestion and assimilation of nutrients occurs, and is also the site where orally acquired pathogens initiate action. In lepidopteran, the alkaline midgut environment is essential to food assimilation (Terra, 1990), but also important for either virus or bacteria pathogenesis. For example, the virions of baculoviruses fuse with the cell membrane and initiate replication just after the virus occlusion bodies dissolve in the alkaline environment of the midgut (Passarelli, 2011). Meanwhile, the bacterium *Bacillus thuringiensis* (Bt) forms crystal-like inclusions during sporulation which are readily solubilized and activated in the insect midgut, leading to its destabilization and consequent insect death (Vachon et al., 2012).

But even with a conduciveness to pathogenesis occurs, the midgut possesses layers with protective function, preventing the entry of pathogens and parasites from food and infecting tissues (Passarelli, 2011). In insects, the first line of defense against invaders orally acquired is the peritrophic matrix, which is formed of a network of chitin fibrils attached to a proteins, glycoprotein and proteoglycans matrix (Lehane, 1997). This sieve-like membrane provides mechanical protection to the midgut epithelium against food abrasion, and parasites and pathogens that enter with food intake, beyond compartmentalizing phases of digestion and secreting products that contribute to digestion (Terra, 1990).

Here we test whether the midgut primary defences of the velvetbean caterpillar *Anticarsia gemmatalis* Hübner present phase-dependent plasticity. Our aim was to look

at whether its morphometry and structure change in accordance to colour transition in caterpillars, and whether those changes may provide to caterpillars a more protective barrier against invasion by *Baculovirus anticarsia* (Silva et al., 2013). We first analyzed the morphometry of the midgut epithelium of caterpillars of the green or black phenotype, and after this, analyzed the structure and morphometry of the peritrophic matrix of caterpillars of the two phenotypes, either by exposing them to that pathogen or not. To our knowledge, no density-dependent prophylaxis studies have assessed other parameters not related to immunological defences.

Anticarsia gemmatalis presents phase polyphenism, i.e. a suite of traits changing in response to local density of conspecifics. One of these is the melanization of the cuticule, which is indicative of up-regulation of immune function, and consequently, pathogen resistance (Barnes and Siva-Jothy, 2000). According to previous results it seems that there is a difference in the peritrophic matrix of melanic and non-melanic caterpillars, i.e. black and green caterpillars, respectively (Silva et al., 2013). Recent findings on melanic species such as the greater wax moth *Galleria mellonella* show that a set of cuticular-related defences enhances its capacity to survive pathogen infection (Dubovskiy et al., 2013). However, it is still unknown whether the primary defences like peritrophic matrix features are also plastically changed in polyphenic species. We hypothesized that changes in cuticle colour of the velvetbean caterpillar are linked not only to secondary (i.e. immunological defences), but also to its primary ones (i.e. peritrophic matrix and midgut epithelium).

RESULTS

Midgut epithelium morphometric analysis

The morphometric measurements of the caterpillars' midgut varied significantly in the two regions along this organ. Thickness of the midgut epithelium was directly linked to the phenotype. Black caterpillars had a thicker midgut epithelium than green caterpillars in both regions of the midgut, with a greater significant difference in the posterior ($F_{1,58}$ = 23.099, P < 0.001) than in the anterior region ($F_{1,58}$ = 7.883, P < 0.01) (Fig. 1A). In this first region, black caterpillars had the midgut epithelium 17% thicker (172 ± 7 µm, mean ± s.e.m.) than green ones (143 ± 8 µm). Whereas in the posterior region, black caterpillars had the midgut epithelium 26% thicker (179 ± 8 µm, mean ± s.e.m.) than green ones (132 ± 6 µm) (Fig. 1B,C). Despite Fig. 1 does not show the midgut epithelium in details, it is possible to observe that black caterpillars present the midgut with a more overlapping cell layers (or folded epithelial tissue) than green caterpillars.

Structural and morphometric peritrophic matrix analysis

The measurements of the caterpillars' peritrophic matrix varied significantly in the two regions along the lumen, either in caterpillars exposed to *B. anticarsia* or not. Both thickness and chitin in the peritrophic matrix were directly linked to the phenotype. Black caterpillars presented the peritrophic matrix thicker than green caterpillars in both anterior ($F_{1,37}$ = 16.636, P < 0.001) and posterior region ($F_{1,33}$ = 13.740, P < 0.001) of the midgut (Fig. 2A). In the first region, black catepillars had the peritrophic matrix 55% thicker (55 ± 5 µm, mean ± s.e.m.) than green ones (25 ± 6 µm). Whereas in the posterior region the difference was still greater, i.e. black caterpillars had the peritrophic

matrix 68% thicker (59 ± 11 µm, mean ± s.e.m.) than green ones (19 ± 3 µm) (green layer in Fig. 2B,C).

Baculovirus anticarsia treatment affected significantly the thickness of the peritrophic matrix in the posterior region ($F_{1,32}$ = 5.0617, $P < 0.05$), but not in the anterior region of the midgut ($F_{1,36}$ = 3.7438, $P = 0.060$). Post-hoc Tukey's HSD test tests show significant differences in thickness of peritrophic membrane between black caterpillars exposed (81 ± 20 µm, mean ± s.e.m.) to *B. anticarsia vs* black caterpillars non-exposed (41 ± 8 µm) ($P < 0.05$), black caterpillars exposed (81 ± 20 µm) *vs* green caterpillars non-exposed (16 ± 3 µm) ($P < 0.001$), and between black (81 ± 20 µm) and green caterpillars (23 ± 5 µm) both exposed to pathogen ($P < 0.001$). It was observed that the peritrophic matrix's structure changed more in caterpillars exposed to pathogen, regardless the phenotype, than in non-exposed caterpillars. While the peritrophic matrix of caterpillars non-exposed to pathogens presents a more integral and homogeneous layer than exposed ones (shown in green in Fig 2B,C; lower panels). Despite caterpillars exposed to pathogens present a thicker peritrophic matrix than non-exposed ones, it presents some disintegration with formation of vacuoles (green layer in Fig 2B,C; upper panels). Regarding the midgut epithelium, both caterpillars exposed and non-exposed to pathogens present no apparent disintegration or formation of vacuoles (yellow layer in Fig 2B,C). Whereas the endoperitrophic spaces (i.e. spaces between peritrophic matrix and epithelium) increased more in black caterpillars, regardless of the exposition or not to pathogens, than in greens caterpillars (Fig. 2B,C).

Black caterpillars presented 33% more chitin in the peritrophic matrix (27 ± 3, mean ± s.e.m.; measured as fluorescence intensity in the pictures) than green caterpillars (18 ± 2) only in the posterior ($F_{1,33}$ = 8.591, $P < 0.05$), but not in the anterior region of the midgut (black caterpillar: 27 ± 2 vs green caterpillar: 23 ± 2) ($F_{1,36}$ = 1.652, p =

0.207) (Fig. 3). *Baculovirus anticarsia* treatment affected significantly the chitin in the peritrophic matrix in both anterior ($F_{1,35}$ = 4.945, $P < 0.05$) and posterior ($F_{1,32}$ = 13.225, $P < 0.001$) regions of the midgut. Post-hoc Tukey's HSD tests show significant differences between black caterpillars exposed (35 ± 5, mean ± s.e.m.; fluorescence intensity) *vs* black caterpillars non-exposed (21 ± 2) to *B. anticarsia* ($P < 0.05$), black caterpillars exposed (35 ± 5) *vs* green caterpillars non-exposed (14 ± 1) ($P < 0.001$), and between black and green caterpillars exposed both exposed to pathogen (35 ± 5 and 23 ± 4, respectively) ($P < 0.05$).

DISCUSSION

The theory of density-dependent prophylaxis proposes that organisms should up-regulate their immunological responses in moments of high population density when the likelihood of being infected by pathogens is higher (Wilson and Reeson, 1998; Reynolds et al., 2011). Supporting this hypothesis, we showed that the velvetbean caterpillars reared at a higher population density were darker and more resistant to an entomopathogenic virus than green ones (Silva et al., 2013). However, to date the underlying mechanisms of such resistance have not been fully elucidated. Some studies in this species suggested that inherent characteristics of the caterpillars' midgut, such as the thickness of both midgut epithelium and peritrophic matrix, beyond the chitin content in this, could give to caterpillars a more effective barrier against *B. anticarsia* (Levy et al., 2007). Lehane (Lehane, 1997) suggests that insects only produce the peritrophic matrix as prevention or barrier against pathogens ingested during feeding. Here, we have shown that these mechanisms may be plastically changed when caterpillars face a greater threat of disease.

Cuticule-related defences enhance the greater wax moth's capacity to survive pathogen attacks (Dubovskiy et al., 2013), while chemical properties, such as phenoloxidase activity in the midgut of *Spodoptera* sp. vary plastically with population density (Wilson et al., 2001; Cotter et al., 2004). To our knowledge this is the first time that both structure and chemical components of primary barriers are analyzed in conjunction. We have shown that beyond changing morphologically with the velvetbean caterpillar's phenotype, the midgut barriers also vary spatially. Whereas measurements of green caterpillars decrease from the anterior to the posterior region, black caterpillars show the opposite pattern with measurements increasing along the midgut lumen, such as thickness of the midgut epithelium, and thickness and chitin in the peritrophic matrix.

Furthermore, black caterpillars presented both midgut epithelium and peritrophic matrix thicker than green caterpillars, and also chitin content in the peritrophic matrix.

These results are in accordance to Levy et al. (Levy et al., 2007) that the chitin content in the resistant velvetbean caterpillar's peritrophic matrix increases gradually from the anterior to posterior region along the midgut lumen. It is argued that chitin is related to a greater integrity in the peritrophic matrix of the velvetbean caterpillar, and consequently a higher resistance against viral infection (Levy et al., 2011). Usually, the chitin microfibrils are secreted in the anterior region of the midgut, with a sequential peritrophic matrix thickening in the middle and posterior region (Hopkins and Harper, 2001), and with the pores between the protein fibril layers being too small in diameter [*ca.* 7.8 nm in *S. frugiperda* (Ferreira et al., 1994)] that would prevent pathogens passage [e.g. baculoviruses nucleocapsids are approximately 60 x 300 nm in size (Derksen and Granados, 1988)].

Thus, if exposed to similar pathogen inocula, it is likely that black phenotype will be less infected than the green owing to its thicker and more integral peritrophic matrix. For example, honey bees infected by *Paenibacillus larvae*, showed a total disruption of the peritrophic matrix allowing bacteria to access the midgut epithelial cells (Garcia-Gonzalez and Genersch, 2013). However, whether pathogens are successful in overcoming the peritrophic matrix, other midgut properties such as epithelial thickness (or integrity) may impair the pathogen's ability to invade and cause systemic infection (Levy et al., 2009; Buchon et al., 2013).

In polyphenic insects in general, including the velvetbean caterpillar, the two sets of defences (i.e. primary and secondary) may arise as a positive result of pleiotropy, where the biochemical pathways responsible for the up-regulation of the immune system are also involved in melanization of the cuticle [as suggested by Fedorka et al.

(Fedorka et al., 2013)]. In polymorphic species, e.g. *G. mellonella*, constitutive barriers may work in conjunction with the secondary responses, where the first barrier is employed in both prevention and delaying of invaders attack, whilst the second responses may be activated (Dubovskiy et al., 2013). Thus, if that assumption is right, we might expect to find darker lepidopteran, coleopteran and locust phenotypes, which are more resistant to virus and fungi via immune responses (Wilson and Reeson, 1998; Barnes and Siva-Jothy, 2000; Wilson et al., 2002), changing aspects of their primary defence as well, such as thickening and increasing the chitin content of the peritrophic matrix of lepidopteran larve; and cuticle thickness for locusts.

MATERIALS AND METHODS

Subjects

The velvetbean caterpillar colony was maintained at the Universidade Federal de Viçosa-Brazil on artificial diet at the following conditions: 20±1 °C, 60±3 % relative humidity and 12h photophase (Hoffmann-Campo et al., 1985). Moths were allowed to oviposit on sheets of sulphite paper, and their eggs were collected daily. After hatching, caterpillars were promptly placed in 100 ml opaque plastic pots with artificial diet and kept at two population densities (i.e. 1 or 8 caterpillars per pot) until use in the respective experiments. Caterpillars were kept at these population densities as a way to stimulate their colour phenotypic changes (Silva et al., 2013). Thus, lone-reared caterpillars usually keep the green phenotype, whilst changes in colour (like black) are more often in group-reared caterpillars.

Midgut epithelium morphometric analysis

Fourth-instar caterpillars expressing either green or black phenotypes were dissected in 125 mM NaCl and midgut transferred to Zamboni's fixative solution for 24h, dehydrated in a graded ethanol series and embedded in glycol methacrylate. For the morphometric analysis, midgut from 30 green or black caterpillars were separated into two regions (anterior and posterior), and 3.5 µm thicker slices were stained with hematoxylin and eosin. After staining, midgut sections were photographed under microscope and pictures analyzed with IMAGEJ software (Bethesda, Maryland, USA, software of public domain available at: http://imagej.nih.gov/ij/) to take the thickness of

the midgut epithelium. The final value of thickness is the mean of these fifteen measurements taken along the midgut perimeter.

Structural and morphometric peritrophic matrix analysis

Twenty-four hours before oral inoculation with *B. anticarsia*, fourth-instar caterpillars expressing either green or black phenotypes were kept in isolation and starved. Afterwards, 10 caterpillars/phenotype were fed for a 24 h period on square soybean leaf pieces inoculated with 20 µL of virus suspension (6 x 10^4 polyhedra/caterpillar) obtained from the formulated product plus surfactant. In the control group, 10 caterpillars/phenotype were fed on soybean leaf pieces inoculated with 20 µL of distilled water plus surfactant. The pathogen used here was chosen because it occurs naturally in populations of velvetbean caterpillar, thus the caterpillar's midgut may have evolved barriers to cope this pathogen invasion. Three days after inoculation, caterpillars were dissected as above, and midguts were fixed and embedded as described early. The caterpillars' midguts were separated into two regions (anterior and posterior), and 3.5 µm thicker slices were stained to detect the presence of chitin. Midgut sections were washed in PBS and incubated in WGA-FITC lectin with the presence of 0.1 mM N-acetilglicosamine (1:5) [adapted from Levy et al. (Levy et al., 2011)]. After labeling, midgut sections were photographed under Olympus BX-60 microscope (Olympus Optical Co. Ltd, Tokyo, Japan) and pictures analyzed with IMAGEJ software to take the following measurements: (i) thickness and (ii) chitin in the peritrophic matrix. The final value of both thickness and chitin (which is analyzed by fluorescence in the pictures) is the mean of these ten measurements taken along the peritrophic matrix.

Data analysis

Data on the structure and morphometric of velvetbean caterpillars' midgut were verified using generalized linear models (GLM with normal distribution). Following the analysis, residual analyses were used to check suitability of models and distribution. All statistical analyses were performed in R (R Development Core Team, 2008). The models were performed by including phenotype and pathogens treatment as factors and morphometry of the midgut epithelium and the structure and morphometry of the peritrophic matrix of caterpillars as responses. Finally, we carried out post-hoc Tukey's HSD multiple comparisons tests of means with a 95% family-wise confidence level in order to contrast the factor levels, i.e. inoculation status or caterpillars' phenotypes, in all experiments.

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AUTHOR CONTRIBUTIONS

FWSS and SLE originally formulated the idea. All authors conceived and designed the experiments. FWSS and JES performed the interpretation of morphometric data. FWSS analyzed the data. All authors wrote the manuscript, reviewed the early versions and provided editorial advice.

COMPETING INTERESTS

No competing or conflict of interests.

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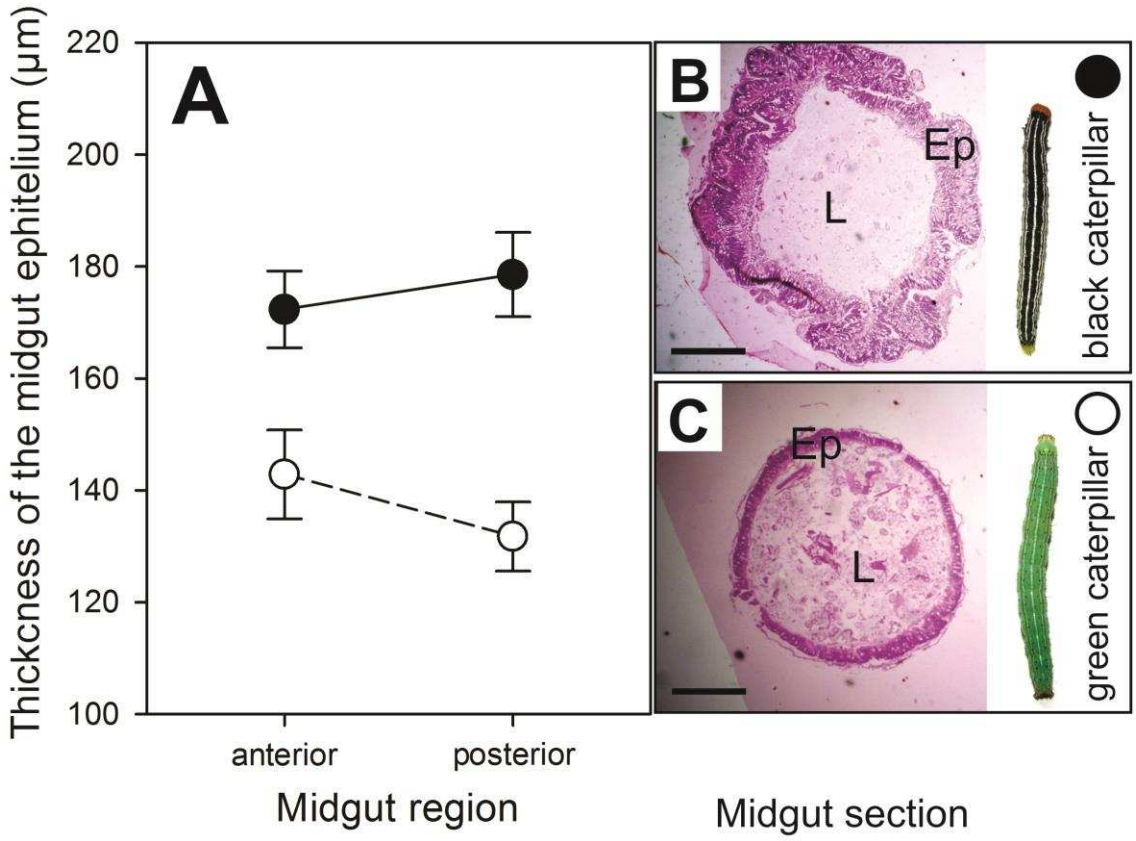

Fig. 1. Morphometry and structure of the velvetbean caterpillars' midgut changing according to phenotypes. (A) Mean (± s.e.m.) thickness of the midgut epithelium in both anterior and posterior region of the midgut of black (black circles and solid lines) or green caterpillars (white circles and dash lines). Cross-sections of midgut of black (B) and green (C) caterpillars were stained with hematoxylin and eosin and analyzed in IMAGEJ software. Ep, epithelium; L, lumen. Scale bars = 250 µm.

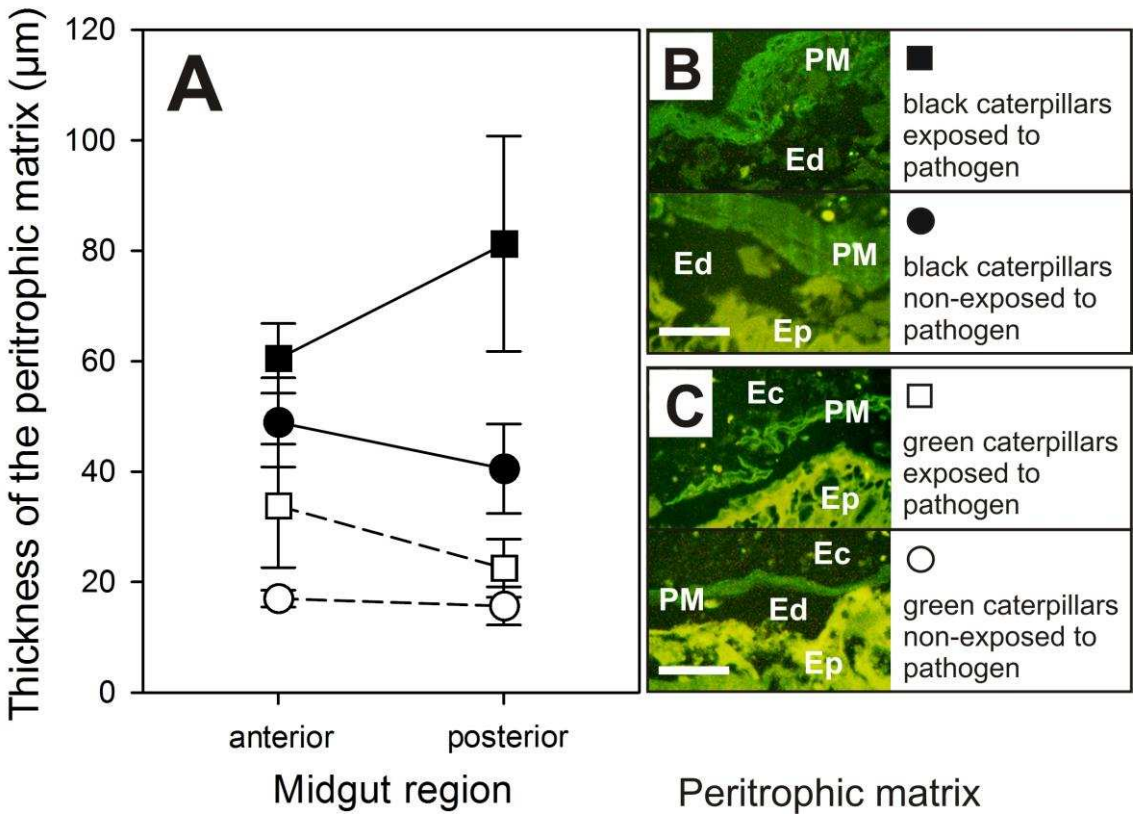

Fig. 2. Morphometry and structure of the velvetbean caterpillars' peritrophic matrix changing according to phenotypes. (A) Mean (± s.e.m.) thickness of the peritrophic matrix in both anterior and posterior region of the midgut of black (black symbols and solid lines) or green caterpillars (white symbols and dash lines), either by being exposed (squares) or not to *B. anticarsia* (circles). Midgut transverse sections of black (B) and green (C) caterpillars were labeled with WGA-FITC lectin (green) and thickness measurements of the peritrophic matrix taken in IMAGEJ software. PM, peritrophic matrix; Ec, ectoperitrophic space; Ed, endoperitrophic space; Ep, epithelium. Scale bars = 200 µm.

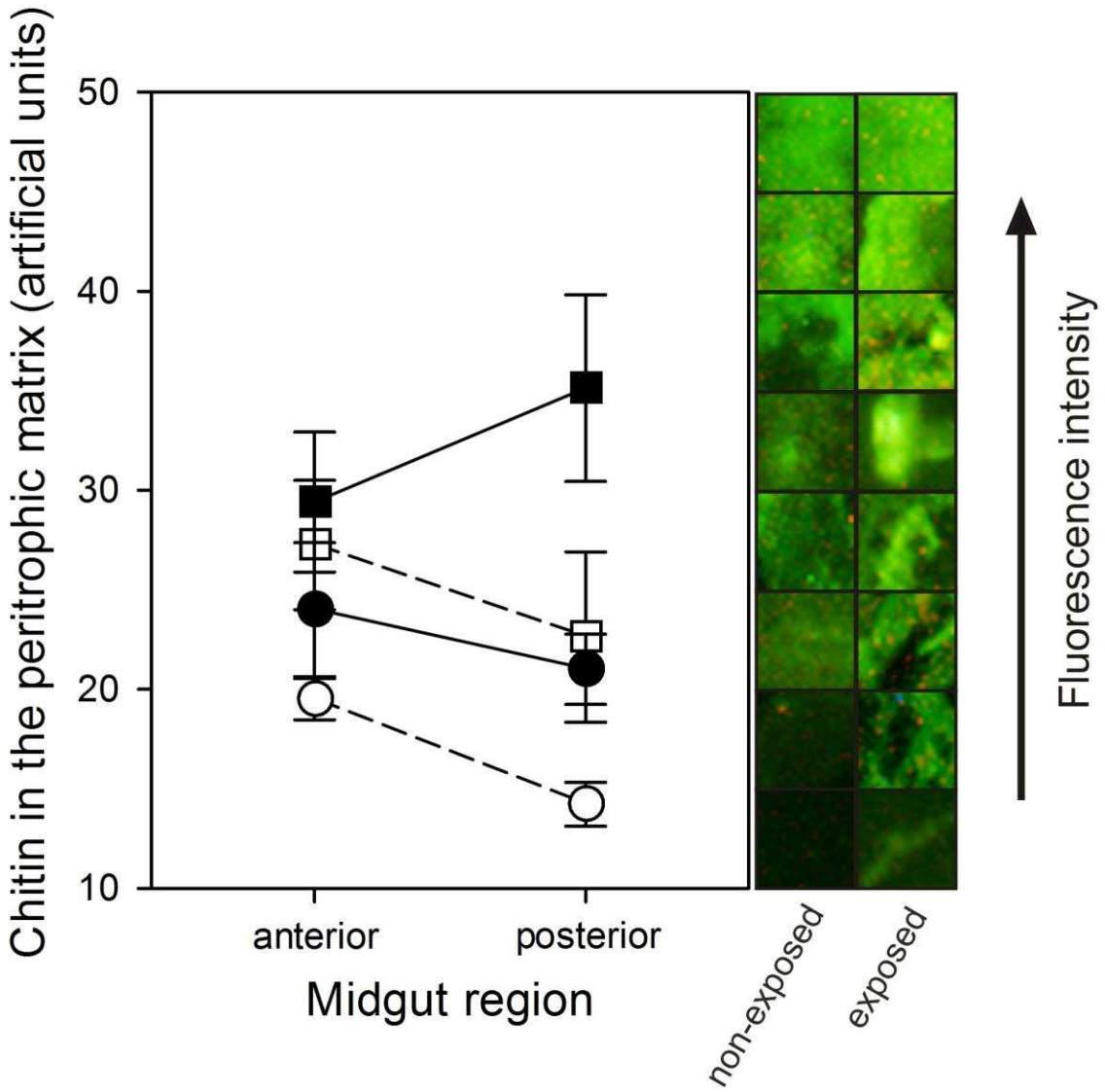

Fig. 3. Chitin in the velvetbean caterpillars' peritrophic matrix analyzed by fluorescence in the pictures. Mean (± s.e.m.) chitin in the peritrophic matrix in both anterior and posterior region of the midgut of black (black symbols and solid lines) or green caterpillars (white symbols and dash lines), either by being exposed (squares) or not to *B. anticarsia* (circles).

CHAPTER 2

Impacts of temperature and population density on phenotypic and immune traits of the velvetbean caterpillar

ABSTRACT

While biological processes of animals, such as developmental rates and movement rates are temperature-dependent, other ones such as immune responses and pathogens resistance are density-dependent. Here, we integrate both fields of the thermal and immunological ecology by assessing whether temperature, population density and the interaction between these environmental factors affect immune traits and pathogens resistance in the velvetbean caterpillar *Anticarsia gemmatalis*. We hypothesized that environmental factors could affect either directly or indirectly the immune traits in velvetbean caterpillar. Of the traits assessed, encapsulation response was directly affected by none of the environmental factors; capsule melanization increased with temperature at both lone- and group-reared caterpillars, although the lone-read presented the most evident response; and hemocyte numbers decreased with temperature regardless of the population density. While encapsulation response and capsule melanization increased with body mass, hemocyte numbers decreased. Beyond the immune parameters, the environmental factors also dramatically affect the host-pathogen interaction. We found temperature, but not population density, to affect considerably the time from inoculation to death of velvetbean caterpillar. Thus, velvetbean caterpillars succumbed to *Baculovirus anticarsia* more quickly at higher temperatures than at lower temperatures. As hypothesized, temperature likely affected caterpillars' movement rates, and thus the contact between conspecifics, which in turn affected the phenotypic expression of group-reared caterpillars. Here, cuticle caterpillars' melanization was dependent on both temperature and population density. Our results suggest that environmental factors, mainly temperature, strongly affect both the course of disease in velvetbean caterpillar population and its defenses against pathogens. As a soybean pest, velvetbean caterpillar may increase its damage on

soybean fields under a scenario of global warming as caterpillars may reach the developmental resistance faster, and thus decrease their susceptibility to biological control by *B. anticarsia*.

Key words: *Anticarsia gemmatalis*, global warming, density-dependent prophylaxis, phenotypic plasticity, immune responses, *Ag*MNPV.

INTRODUCTION

A range of behavioural and physiological processes of animals are temperature-dependent, with metabolism, food intake, developmental rates and movement rates and pathogen resistance being, usually but not always, maximized in ectotherm organisms experiencing high temperatures (Browning, 1952; Inglis et al., 1996; Lafrance, 1968; Okasha, 1968; Schramm, 1972). Furthermore, temperature may play a critical role, not just in the behavioural and physiological processes of the organism *per se*, but also in the host-pathogen interactions (Murdock et al., 2012). For instance, thermoregulation and expression of behavioural fever in *Schistocerca gregaria* dramatically reduces its chance of succumbing to fungal disease (Elliot et al., 2002). It is likely that this response is linked to the gene expression level, as exemplified in zebrafish (Boltana et al., 2013). It presents temperature-dependent changes in the brain after expression of behavioural fever, which in turn, up-regulates some anti-viral genes related to the innate immune response, increasing thus its survival to viral attack.

However, increases in the environmental temperature experienced by ectotherm organisms may also bring them to stressful conditions. This may impact negatively other biological parameters, such as reproduction or more specifically, in the case of *Plutella xylostella*, the hatching success of eggs (Zhang et al., 2013), or interactions with bacterial endosymbiont in ants (Fan and Wernegreen, 2013). Changes in environmental temperature can also affect immune systems, which can be compromised under some circumstances, hence affecting the animal's capacity to defend itself against pathogens. Diaz et al. (2013) have shown that adult white shrimp *Litopenaeus vannamei* had some of its immunological parameters, such as the total count of hemocyte and prophenoloxidase (proPO) activity, negatively affected after exposure to thermal stress.

Whilst environmental temperature may trigger direct physiological and behavioural responses at the organism level, it may also indirectly trigger such responses through alternative pathways. The cricket *Allonemobius socius*, for example, presented a greater resistance to a bacterial challenge, after its cuticle colour changed in response to a low environmental temperature, enhancing its immune investment pleiotropically (Fedorka et al., 2013). Moreover, changes in environmental temperatures may affect the likelihood of transmission or impact of disease at both organism and population levels. It is already known that the time spent flying as well as mobility of a range of insects species increase with temperature (Cormont et al., 2011; Ruf and Fiedler, 2002). It might be thus expected that, when an organism's mobility increases with temperature, its risk of becoming infected by pathogens that are contact transmitted also increases. Nonetheless some organisms have evolved prophylactic responses to cope with the increased risk of infection under such circumstances (Density-Dependent Prophylaxis hypothesis, Wilson and Reeson, 1998).

It was shown recently that the velvetbean caterpillar *Anticarsia gemmatalis* undergoes changes in its immune repertoire (i.e. encapsulation response, capsule melanization and hemocyte numbers) when exposed to a high population density (Silva et al., 2013). As, with other polyphenic species, the velvetbean caterpillar becomes more melanized as a response to the local density of conspecifics; with this feature being indicative of up-regulation of immune function, and consequently, pathogen resistance (Barnes and Siva-Jothy, 2000). We hypothesize here that environmental factors such as temperature and population density affect either directly or indirectly those immune traits in velvetbean caterpillar. First, temperature may affect directly such traits by changing the caterpillar's body condition, and second by changing caterpillars' movement rates, which presumably affects contact between conspecifics, and thus the

likelihood of becoming infected by pathogens. We found that environmental factors, mainly temperature, strongly affect both the course of disease in velvetbean caterpillar population and its defenses against pathogens.

MATERIALS AND METHODS

Experimental design

The velvetbean caterpillar colony was maintained on artificial diet at 27 °C and 14h photophase [as described by Hoffmann-Campo et al. (1985)]. Eggs were collected daily, and after hatching caterpillars were promptly placed in 100 mL opaque plastic pots and assigned to a 2 x 4 factorial design as follow: density (1 or 8 caterpillars/pot) and temperature (20, 24, 28 or 32 °C). Temperature treatment was performed in thermostat-controlled chambers which were kept in a room with controlled conditions: 20±1 °C, 60±3 % relative humidity and 12h photophase. To avoid pseudoreplication, only one insect from a given pot was ever used.

Encapsulation and melanization

Our aim here was looking at the direct and indirect effects of the environmental factors on immune traits. Temperature may affect directly the caterpillar immune traits by changing its body mass; so caterpillars were weighed on scales (accuracy = .001 g) before each experiment. Also, temperature may affect indirectly immune traits by changing caterpillars' movement rates, which presumably affect contact between conspecifics. Two immune functions were assessed in this experiment: encapsulation response and capsule melanization. They were measured by challenging forth-instar caterpillars with a colourless nylon filament (2 mm length and 0,12mm Ø) used to mimic the presence of a parasite (Schmid-Hempel and Schmid-Hempel, 1998). Nylon filament was inserted through the first thoracic segment (dorsal region) of 30 caterpillars per treatment. Twenty-four hours after, caterpillars were dissected and the

nylon filaments were mounted on slides and photographed, thus assessing the area of cell layers formed around the nylon filament (encapsulation response) and the grayscale range (capsule melanization) with the aid of IMAGEJ 1.42q software.

Hemocyte numbers

Hemolymph was collected from 30 forth-instar caterpillars per treatment, by puncturing a small hole beside the first prolegs. A sample of 5µL of hemolymph was collected and added to eppendorf tube with 20 µL anticoagulant buffer (98mM NaOH, 186mM NaCl, 17mM Na2 EDTA and 41mM Citric acid, pH 4.5) plus 12 µL of Giemsa stain [adapted from Ibrahim and Kim (2006)]. Two aliquots of 8 µL of the suspension were added in 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 cell numbers per microliter.

Effects of temperature and population density on infection by *Baculovirus anticarsia* (AgMNPV)

Thirty forth-instar caterpillars per treatment were kept in isolation and starved for 24 hours before inoculation with *B. anticarsia.* They were infected by feeding for a 24h period on square soybean leaf pieces (15 x 15 mm) inoculated with 20 µL of virus suspension (6×10^6 polyhedra/caterpillar) (Silva et al., 2013). The leaf piece is easily consumed by one caterpillar in a single day (ensuring ingestion of a uniform number of viral particles leading to infection); caterpillars that did not consume the entire leaf

piece in this period were excluded from the experiment. Virus-inoculated caterpillars were kept as above and mortality was assessed daily until death or pupation.

Phenotypic changes under different temperatures and population density

Velvetbean caterpillar expresses different color phenotypes - green, intermediate or black - when in the presence or absence of conspecifics. The phenotypes were determined in all three previous experiments, taking into account the head capsules and body coloration (as shown in Silva et al., 2013).

Statistics

To test the effect of temperature and population density (as main factors), and phenotype and weight (used as a covariate) on immunity parameters of velvetbean caterpillar, we first fitted full models using GLM (generalized linear models) with normal distributions. Subsequent simplification was done by excluding non-significant terms. Final models were accepted when not significantly different from the previous models. Residuals of the final model were checked for suitability of the distribution. Survival data were analyzed by GLM with Weibull distribution; censoring data was used when caterpillars pupated. The models were performed including temperature, population density and caterpillars' phenotypes as factors (Crawley, 2007). Finally, we carried out contrast analysis in order to contrast the phenotypes when found significant difference among factor levels. Pearson's Chi-squared test was applied on contingency tables to test the hypothesis of independence of the frequency distribution of caterpillars expressing phenotypes under different temperatures and population density.

RESULTS

Encapsulation and melanization

The encapsulation response of the nylon filament was not affected by temperature *per se* ($F_{1,211}$ = 3.7588, p = 0.0538), however it increased via changes in body mass of caterpillars ($F_{1,207}$ = 47.5422, $p < 0.001$; Table 1; Fig. 1a, b). Thus, the velvetbean caterpillar grows heavier at higher temperatures and this in turn increases the encapsulation response. Population density did not affect the encapsulation response ($F_{1,210}$ = 2.4445, p = 0.1194). However, the encapsulation response varied with phenotype ($F_{2,208}$ = 5.4054, p = 0.0051). Caterpillars expressing the intermediate phenotype presented a higher encapsulation response than green and black phenotypes (contrast analysis: $F_{1,211}$ = 8.0765, p = 0.0049).

In this experiment we could also assess the capsule melanization formed around the nylon filament. Here, temperature did directly affect capsule melanization ($F_{1,211}$ = 9.0278, p = 0.0029; Table 1; Fig. 2a). This was also indirectly affected through changes in body mass of caterpillars ($F_{1,206}$ = 105.1891, $p < 0.001$; Table 1; Fig. 2b). Population density affected capsule melanization ($F_{1,210}$ = 7.8663, p = 0.0055), but no significant interaction was found between this factor and temperature ($F_{1,205}$ = 0.9451, p = 0.3321). Caterpillars reared in isolation had a higher capsule melanization than those reared in groups. But differently from the previous experiment, phenotype did not affected the capsule melanization ($F_{2,208}$ = 2.6796, p = 0.0709). We included encapsulation response as a covariate in the capsule melanization model as this parameter may vary with encapsulation response; and indeed capsule melanization increased with encapsulation response ($F_{1,207}$ = 4.7518, p = 0.0304).

Hemocyte numbers

Hemocyte numbers decreased with temperature ($F_{1,228}$ = 34.2429, p < 0.001; Table 1; Fig. 3a). This is likely to be related with body mass as hemocyte numbers also decreased with caterpillars' weight ($F_{1,223}$ = 7.6326, p = 0.0062) (Table 1; Fig. 3b). Population density did not affect either hemocyte numbers ($F_{1,227}$ = 0.3396, p = 0.5606) or its interaction with temperature ($F_{1,222}$ = 3.0175, p = 0.0837). Hemocyte numbers did not change according to phenotype expressed by caterpillars ($F_{2,225}$ = 0.9306, p = 0.3958).

Effects of temperature and population density on infection by *Baculovirus anticarsia* (AgMNPV)

Survival times of velvetbean caterpillar infected by *B. anticarsia* decreased significantly with the linear increment in temperature ($\chi^2_{[5]}$ = 258.62, p < 0.001), but not with the increase in population density ($\chi^2_{[5]}$ = 258.62, p = 0. 1824) nor with the interaction between this factor and temperature ($\chi^2_{[5]}$ = 258.62, p = 0.9624; Fig. 4). The percentage mortality of velvetbean caterpillar decreased as temperature increased; as shown by the scale parameter lower than 1. The mean life times of velvetbean caterpillars reared at temperatures of 20, 24, 28 and 32 °C were 282 ± 7 (± SE), 211 ± 7, 158 ± 7 and 123 ± 5 hours, respectively. Although velvetbean caterpillars reared at 20 and 24 °C lived longer after being infected, most succumbed at the end of the experiment (97 and 85 % mortalities, respectively); whilst caterpillars reared at higher temperatures started dying earlier, the percentage mortality was lower (64 and 78 %, respectively).

The caterpillars' survival was directly linked to the phenotype ($\chi^2_{[5]} = 258.62$, $p = 0.001$). While baculovirus-infected caterpillars expressing green and intermediate phenotypes survived for mean times of 200 ± 10 (± SE) and 211 ± 9 hours, respectively, those caterpillars expressing black phenotype survived for mean times of 185 ± 9 hours.

Phenotypic changes under different temperatures and population density

The frequency distribution of color phenotypes in velvetbean caterpillars is dependent on both temperature and population density (Fig. 5). We have shown that this insect has a range of color phenotypes - green, intermediate or black, in response to contact between conspecifics (Silva et al., 2013). Here, we hypothesized that temperature affects its movement rates, and thus also affects the phenotype of caterpillars reared in group, i.e. 8 individuals per pot. As shown, temperature may play an important role in this aspect. In all experiments, the frequency distribution of phenotypes of caterpillars reared in isolation was independent of temperature (encapsulation/melanization, $\chi^2_{[6]} = 4.4536$, $p = 0.6155$; hemocyte numbers, $\chi^2_{[3]} = 1.3009$, $p = 0.7289$; and infection by *B. anticarsia*, $\chi^2_{[6]} = 4.3539$, $p = 0.6289$; Fig. 5a, c, e); whilst the frequency distribution of phenotypes was highly dependent on temperature when the caterpillars are kept in group (encapsulation/melanization, $\chi^2_{[6]} = 17.1977$, $p = 0.008$; hemocyte count, $\chi^2_{[6]} = 39.5917$, $P < 0.001$; and infection by *B. anticarsia*, $\chi^2_{[6]} = 20.9569$, $p = 0.001$). The differences are more specifically dependent on the temperature range to which caterpillars are submitted, i.e. if they develop under extreme or mid-range temperatures (contrast between extreme temperatures [20 + 32 °C] and mid-range temperatures [24 + 28 °C]: encapsulation/melanization, $\chi^2_{[2]} = 15.3621$, $p < 0.001$; hemocytes numbers, $\chi^2_{[2]} = 38.6607$, $p < 0.001$; and infection by *B. anticarsia*, $\chi^2_{[2]} = 16.4537$, $p < 0.001$; Fig. 5b,

d, f). Group-reared caterpillars expressed significantly more the black phenotype at mid-range temperatures, and less frequently at extreme ones. Meanwhile the intermediate phenotype showed an opposite pattern; it was more frequently observed at extreme temperatures, and less observed at median ones. The expression of the green phenotypes remained pretty much constant with temperatures.

DISCUSSION

In the present study, temperature was found to affect two of the velvetbean caterpillars' biological parameters, such as larval body mass (which is related to food consumption) and immune responses. Environmental factors, not just temperature but also population density, may also indirectly affect other biological parameters such as developmental time and contact between conspecifics. With changes in these parameters, immune responses may be directly up- or down-regulated. Insects such as *Locusta migratoria* nymphs are able to adjust behaviourally their food and nutrient requirements according to a specific environmental temperature (Clissold et al., 2013), while some insects can balance the food consumption to achieve a better immunological response against pathogen attack (Povey et al., 2009). With regards to velvetbean caterpillar, some of its immune functions varied according to the environmental factors. Caterpillars at both population densities presented greater capsule melanization at higher temperatures, although the most evident response was found in lone-reared caterpillars. Although this finding is not in agreement with that predicted by the 'density-dependent prophylaxis' hypothesis (see Wilson and Reeson, 1998), it matches previous results which show that lone-reared caterpillars display more of that response than group-reared ones (Silva et al., 2013).

In contrast, temperature caused an opposite effect on hemocyte numbers; it affected negatively the hemocyte numbers in both lone- and group-reared caterpillars at the same level. While some studies show this component of the immune function can be increased at high environmental temperatures (Chu and Lapeyre, 1993; Truscott and White, 1990), the decrease in hemocyte numbers found here may be related to its viability or stress-induced damage (such as apoptosis) at high temperatures (Diaz et al., 2013; Yao and Somero, 2012). As hypothesized previously, immune parameters may

change not only as a direct response to temperature, but they may change as an indirect response via changes in caterpillars' body mass. In this way, immune parameters correlated positively or negatively with velvetbean caterpillars' body mass. While encapsulation response and capsule melanization increased with body mass, hemocyte numbers decreased. Such correlations are also found in other species, for example the tropical butterfly *Bicyclus anynana* in which hemocyte numbers correlate positively with thorax mass (Karl et al., 2011).

Beyond the immune parameters, temperature and population density also dramatically affect the host-pathogen interaction. We found temperature, but not population density, to affect considerably the time from inoculation to death of velvetbean caterpillar. As suggested by theoretical work (see Johnson et al., 1982), velvetbean caterpillar succumbed to *Baculovirus anticarsia* more quickly at higher temperatures (*ca.* 5 days) than at lower temperatures (*ca.* 11 days). Nonetheless, the mortality was contrary to the temperature increasing (see Fig. 4). This may be a result of the host-pathogen interactions, as both host development time and pathogen virulence may be being affected by temperature changes.

In face of the results it seems that the *Baculovirus anticarsia* virulence is enhanced at higher temperatures. Thus, caterpillars die earlier as a function of the viral infection, but on the other hand they develop faster with increase in temperature (personal observation), as does *Tenebrio molitor* (Prokkola et al., 2013). Further, caterpillars that are not infected early by *B. anticarsia* can escape infection by shifting from the larval stage to pupae, a mechanism known as developmental resistance (Hoover et al., 2002). For example, lepidopteran larvae become more resistant to baculovirus infection as they age (Engelhard and Volkman, 1995; Grove and Hoover, 2007; Kirkpatrick et al., 1998). We hypothesize that the mechanism behind the

velvetbean caterpillars' resistance to *B. anticarsia* is similar to that found in *Lymantria dispar*, i.e. increase in the capsule melanization (McNeil et al., 2010). We thereby expect the fitness of both pathogen and host reach an "intermediate" level when environmental temperature is high. This is firstly because; as more hosts die from infection, more pathogen can disperse to new hosts. However, those caterpillars that do not die from infection can reach adulthood and reproduce (El-Sayed Hatem et al., 2011; Milks et al., 1998). On the other hand, pathogen fitness should be higher at lower temperatures as host mortality is maximum. Thus, most caterpillars die and likelihood of viral dispersal increases. Whilst the host fitness will be pretty much zero as most of them die before reaching the adulthood.

In a scenario of global warming, *B. anticarsia* should evolve to maximize its virulence or speed of action at high environmental temperatures as its host can escape infection by shifting developmental stages. In contrast, it can evolve an opposite strategy at low temperatures as the host developmental resistance will take longer to occur. Low virulence may further evolve as a result of negative genetic correlations between speed of kill and production of infective viral particles, since the longer *B. anticarsia* takes to kill its host, the more infective viral particles it can produce (see review in Cory and Myers, 2003). Here, we propose that *A. gemmatalis* uses an "all-or-nothing" strategy; it invests more in development at high temperatures, increasing thus its chance of escaping from viral infection. However, if the strategy is different at low temperatures, i.e. higher investment in immune defences, it will fail, first because the up-regulation of immune responses (in this case hemocyte numbers which is higher at low temperature) will not translate into an effective defense against *B. anticarsia*. Furthermore, the investment in immunity may impair its development (Cotter et al.,

2008; van der Most et al., 2011), thus caterpillars which do not develop faster may not benefit from developmental resistance and will succumb to viral infection.

Lastly, interactive effects between temperature and population density may create an emerging property in velvetbean caterpillar's morphology and physiology. Temperature may interfere directly with host movement rates (Cormont et al., 2011; Ruf and Fiedler, 2002), which in turn can affect the likelihood of direct contact between conspecifics, and thus phenotypic changes in this species. Velvetbean caterpillar, as other polyphenic species, has evolved plastic prophylactic responses to cope with the increased risk of parasite infection at high population densities (Barnes and Siva-Jothy, 2000; Silva et al., 2013; Wilson et al., 2002), with cuticle melanization being cue of prophylactic responses. So, here we consider this feature being a visible evidence of the velvetbean caterpillar's movement rates. Indeed, cuticle melanization is dependent on both temperature and population density. As hypothesized above (see material and methods), temperature likely affected caterpillars' movement rates, and thus the contact between conspecifics, which in turn affected the phenotypic expression of group-reared caterpillars.

In all experiments, the frequency distribution of phenotypes of lone-reared caterpillars was independent of temperature; whilst the frequency distribution of phenotypes is shown to be highly dependent on temperature when velvetbean caterpillar are kept in contact with conspecifics. The differences are more specifically dependent on the temperature range to which caterpillars are submitted, i.e. whether they develop under extreme or median temperatures. Group-reared caterpillars presented significantly more the black phenotype at median temperatures and less frequently at extreme ones. This shows that extreme temperatures, whether low or high, may impair the caterpillars' movement, and thus the likelihood of contact between them. Despite some work

showing temperature affecting directly the movement and distribution (Jian et al., 2004), and even the cuticular melanization of insects (Prokkola et al., 2013), here we take a different approach to examine how temperature may affect indirectly the morphological and physiological traits via changes in contact between conspecifics.

Our results suggest that environmental factors, mainly temperature, strongly affect both the course of disease in velvetbean caterpillar population and its defenses against pathogens. Temperature affected differently the immune parameters assessed here, with no changes in encapsulation response, increasing in capsule melanization and decreasing in hemocyte numbers as temperature increases. It is likely that capsule melanization is the main mechanism to the velvetbean caterpillar's resistance against a virus, since the increase in capsule melanization at high temperatures matches up with the highest caterpillars' survival. As discussed above, this immune function may be an essential component of the velvetbean caterpillar's developmental resistance. It is also shown here how extreme temperatures may impact the population dynamics of this species, firstly by changing aspects of the host-pathogen interactions and second by changing the likelihood of contact between conspecifics. This last aspect may trigger plastic responses, and consequently the investment in immune functions towards pathogen attack. As a soybean pest, velvetbean caterpillar may increase its damage on soybean fields under a scenario of global warming as caterpillars may reach the developmental resistance faster, and thus decrease their susceptibility to biological control by *B. anticarsia*.

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Table 1. Analysis of deviance table testing the effects of environmental factors, (i) temperature (20, 24, 28 or 32 °C) and (ii) population density (1 or 8 larvae/pot) as main factors, and larval phenotype (green, intermediate or black) and weight used as covariates on (a) encapsulation response, (b) capsule melanization and (c) hemocyte densities of velvetbean caterpillar. Contrasts are model simplifications by excluding non-significant terms and/or grouping treatment factors which are not significantly different of each other. *F*-tests: **P* <0.05, ***P*<0.01, ****P*<0.001

encapsulation response	**d.f.**	**deviance**	**res. d.f.**	**F**	**Pr (>F)**
temperature	1	324.4	211	3.7588	0.0538
density	1	211.0	210	2.4445	0.1194
phenotype	2	933.1	208	5.4054	**0.0051****
weight	1	4103.6	207	47.5422	**0.0001*****
contrast					
phenotype (intermediate *vs.* green + black)	1	688.9	211	8.0765	**0.0049 ****
weight	1	4837.1	210	56.7052	**0.0001*****
capsule melanization					
temperature	1	2185.5	211	9.0278	**0.0029 ****
density	1	1904.3	210	7.8663	**0.0055 ****
phenotype	2	1297.4	208	2.6796	0.0709
encapsulation response	1	1150.4	207	4.7518	**0.0304 ***
weight	1	25465.3	206	105.1891	**0.0001 *****
hemocyte numbers					
temperature	1	227206361	228	34.2429	**0.0001*****
density	1	2253384	227	0.3396	0.5606
phenotype	2	12348703	225	0.9306	0.3958
temp^2	1	85409023	224	12.8722	**0.0004*****
weight	1	50643040	223	7.6326	**0.0062 ****

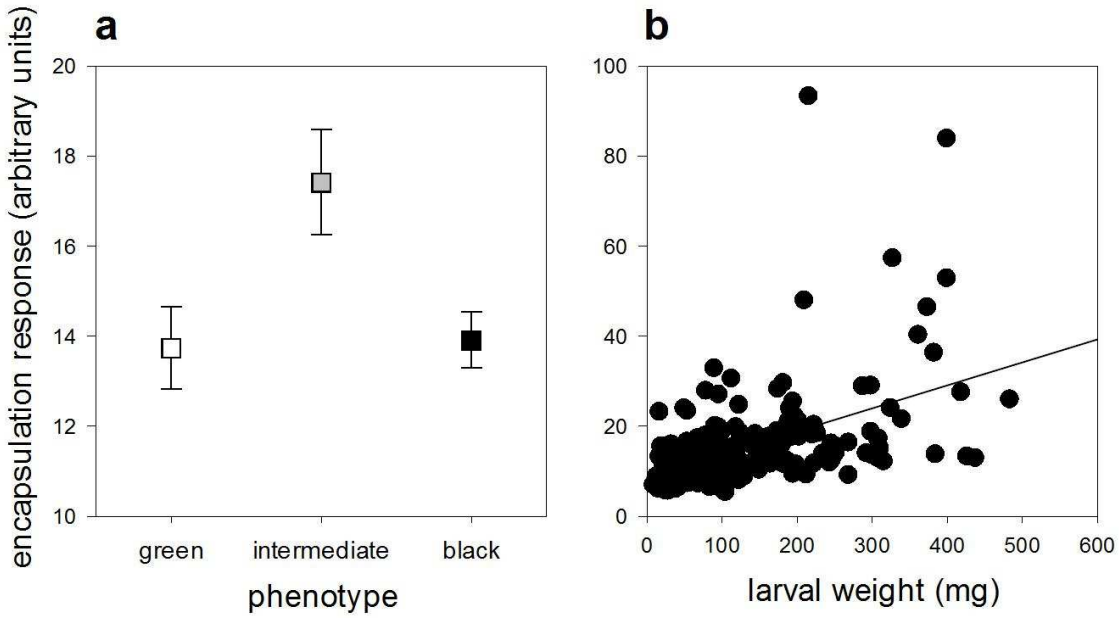

Figure 1. Encapsulation response of velvetbean caterpillar according to the covariates, phenotype and larval weight. Caterpillars were kept alone or in groups of 8 in four thermostat-controlled chambers with a gradient of temperatures (20, 24, 28 or 32 °C). As a result of the interaction between temperature and population density, caterpillars expressed the phenotypes green, intermediate or black (see Fig. 5). As temperature may affect directly the caterpillar immune traits by changing its body mass, we used larval weight as this measurement. Shown are points with means and SE, and the curve of best model fit.

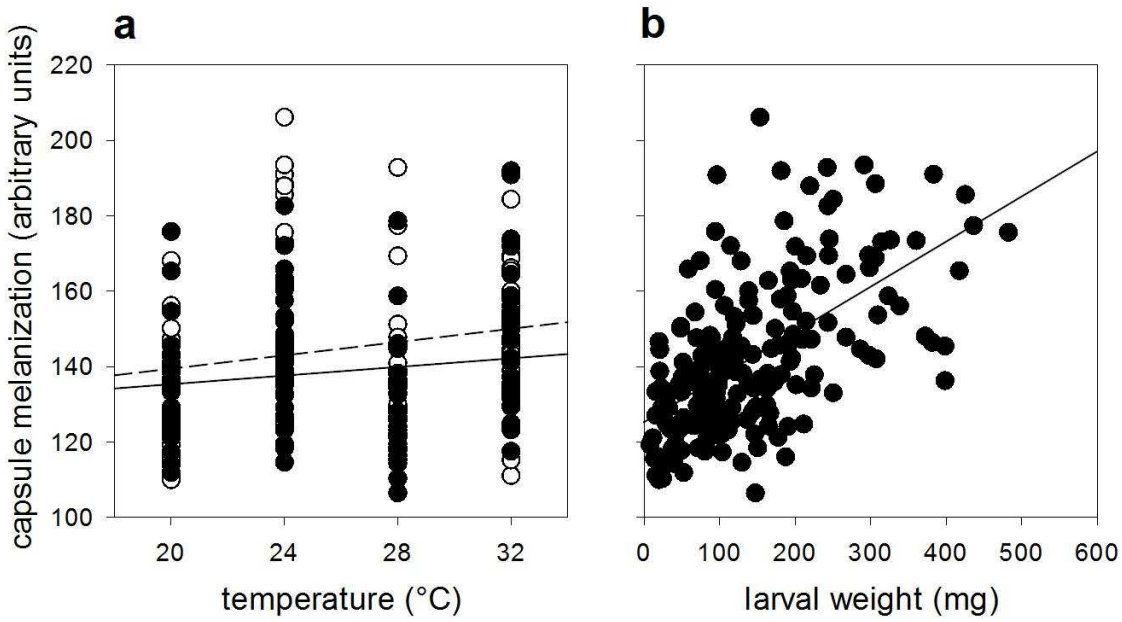

Figure 2. Capsule melanization of velvetbean caterpillar according to the environmental factors, temperature and population density; and the covariate, larval weight. Caterpillars were kept alone or in groups of 8 in four thermostat-controlled chambers with a gradient of temperatures (20, 24, 28 or 32 °C), and weighed before the experiment as a measurement of body condition. **a)** The open circles and dashed line are representing the population density of 1 caterpillar per cup; the filled circles and solid line represent the population density of 8 caterpillars per cup. **a and b)** Shown is the range of points with the curve of best model fit.

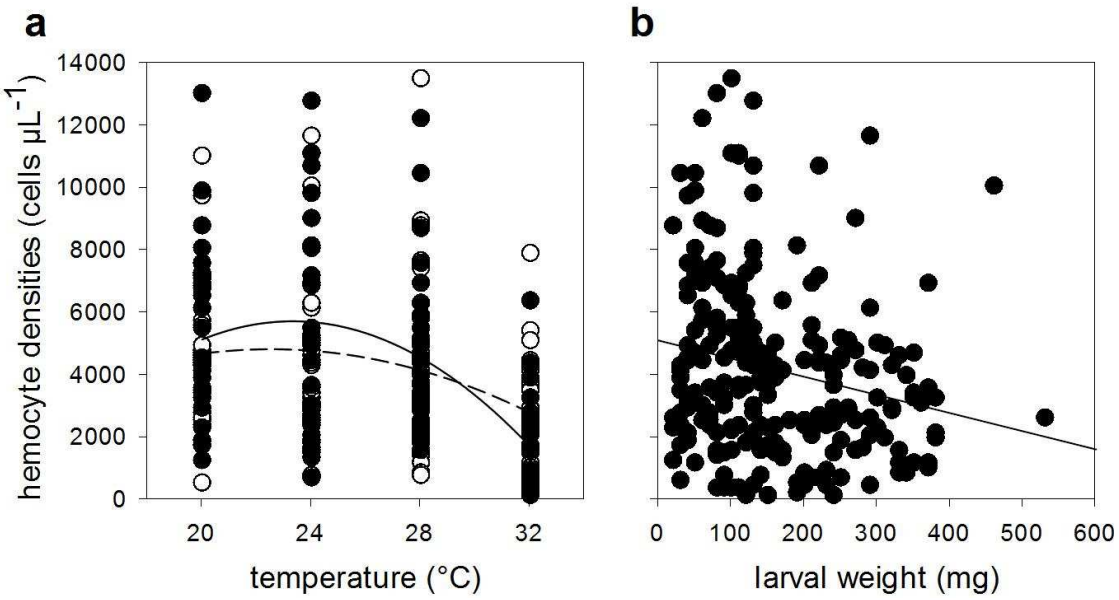

Figure 3. Hemocyte numbers of velvetbean caterpillar according to the environmental factors, temperature and population density; and the covariate, larval weight. Caterpillars were kept alone or in groups in four thermostat-controlled chambers with a gradient of temperatures (20, 24, 28 or 32 °C), and weighed before the experiment as a measurement of body condition. **a)** The open circles and dashed line are representing the population density of 1 caterpillar per cup; the filled circles and solid line represent the population density of 8 caterpillars per cup. **a and b)** Shown is the range of points with the curve of best model fit.

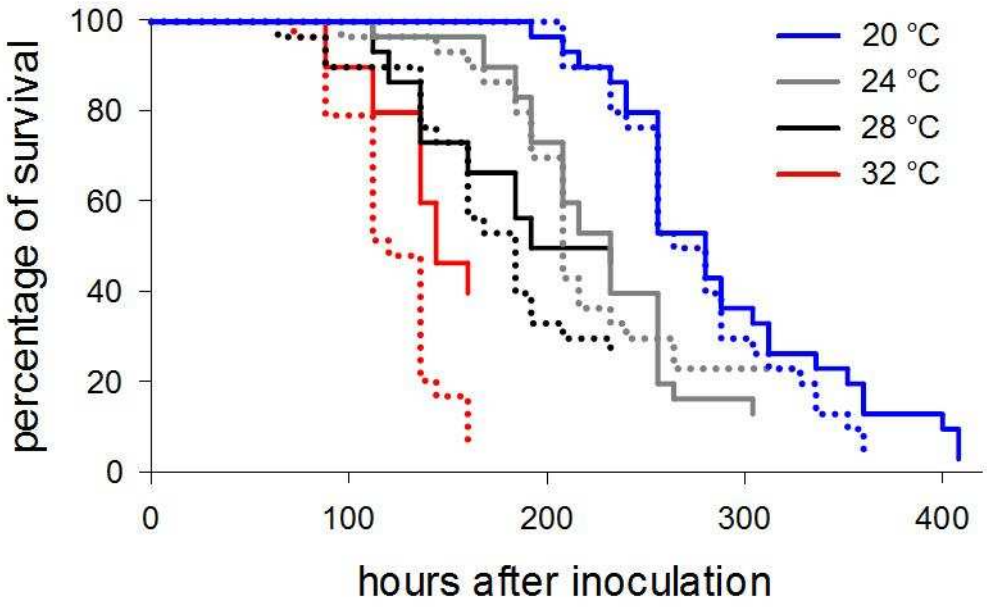

Figure 4. Survival curves of velvetbean caterpillar inoculated with *Baculovirus anticarsia*. Caterpillars were kept alone or in groups of 8 in four thermostat-controlled chambers with a gradient of temperatures. Dashed lines represent lone-reared caterpillars; solid lines represent group-reared caterpillars. Larval mortality was assessed daily, and the larvae that pupated were included in analysis as censored data. Survival analyses are presented in the text.

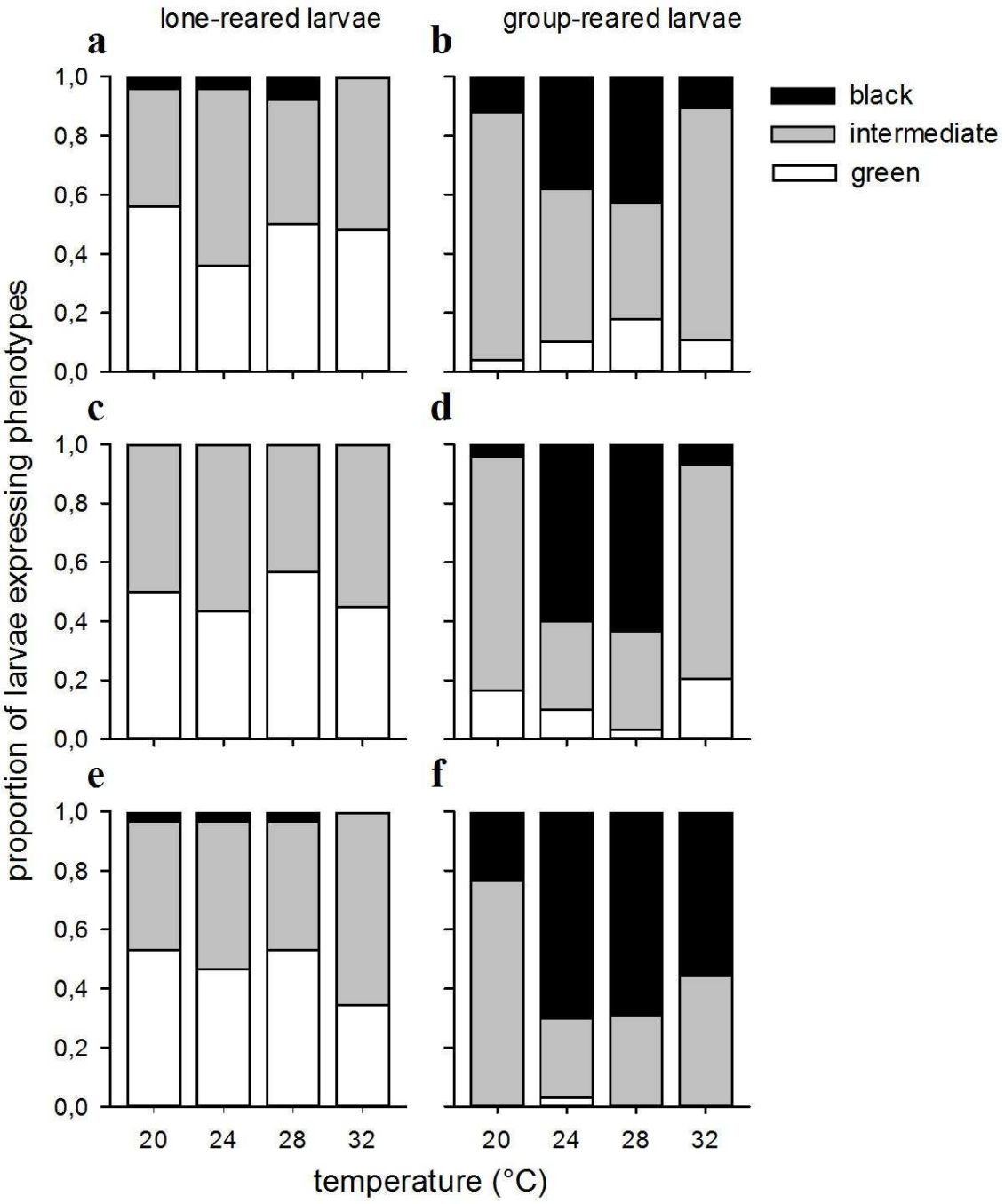

Figure 5. Frequency distribution of velvetbean caterpillars expressing color phenotypes according to the environmental factors, temperature and population density. Caterpillars were kept alone or in groups of 8 in four thermostat-controlled chambers with a gradient of temperatures (20, 24, 28 or 32 °C), and expressed the phenotypes green, intermediate or black as a result (see statistical tests in text). Frequency distributions are shown for insects used subsequently in each experiment: **a-b)** encapsulation response

and capsule melanization; **c-d)** hemocyte numbers; and **e-f)** susceptibility to *Baculovirus anticarsia*.

CONCLUDING REMARKS

1. We found that primary barriers such as peritrophic matrix and midgut epithelium of the velvetbean caterpillar changed plastically according to phenotype, which is a response to changes in population density. Beyond changing morphologically with phenotype, these barriers also varied spatially.

2. Temperature affected biological parameters of the velvetbean caterpillar, e.g. body mass (which is related to food consumption) and immune responses. Environmental variables, not just temperature but also population density, indirectly affected other of its biological parameters such as developmental time and contact between conspecifics. This will reflect dramatically in the host-pathogen interaction.