MARCOS VINÍCIUS VIEIRA MATTOS

ABSENCE OF DENSITY-DEPENDENT PROPHYLAXIS AND DENSITY-DEPENDENT PHASE POLYPHENISM IN A CANNIBALISTIC CATERPILLAR, *Helicoverpa armigera* (HÜBNER) (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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APROVADA: 04 de novembro de 2016.

Farley William Souza Silva

Kalina Miranda Perkins

Og Francisco Fonseca de Souza

Simon Luke Elliot
(Orientador)

“A ciência consiste em substituir o saber
que parecia seguro por uma teoria, ou seja,
por algo problemático.”

José Ortega y Gasset

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ABSTRACT

MATTOS, Marcos Vinícius Vieira, M.Sc., Universidade Federal de Viçosa, October, 2016. **Absence of density-dependent prophylaxis and density-dependent phase polyphenism in a cannibalistic caterpillar, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)**. Adviser: Simon Luke Elliot. Co-advisers: Farley William Souza Silva and Silma Leite Rocha

Phenotypic plasticity contributes to an organism's ability to respond to changes in its environment. Population density is an environmental factor for which variation may lead to changes in investment in defences against diseases. Density-dependent prophylaxis hypothesis (DDP) predicts that organisms invest more in defences against diseases when at high population densities, as the risk of disease transmission tends to increase. Density-dependent phase polyphenism (DDPP) predicts that the morphology, physiology and behaviour of organisms may vary according to their population density. These changes may be related to differences in investment in immune defences or protective behaviours. Most studies have considered physical contact as a key stimulus for the phenotypic plasticity process, however other stimuli such as substrate vibration, volatiles and pheromones may allow this process in organisms according to their density. Under these circumstances, the hypothesis of this study is that the immune system and body colour change according to population density, even without physical contact between conspecific. Thus, was tested whether DDP and DDPP can occur even without physical contact between *Helicoverpa armigera* caterpillars, through artificial manipulation of their population density. For this, post-eclosion larvae were placed for 10 days, solitarily or in groups, in arenas designed to avoid physical contact, but allow the perception of conspecific through other density clues. After this period, was tested whether the presence of conspecific influenced: (i) the colour of the head capsule and the body, and (ii) the immune defences (encapsulation response, haemocyte densities and lysozyme activity). The results indicate that neither body colour nor immune defences were affected by the presence of conspecific. The fact of *H. armigera* caterpillars decrease aggregation in later larval instars, due to their cannibal behaviour, may represent a lower disease risk, so that there is no change in the investment in immune defences. In addition, physical

contact may be more important to trigger phenotypic plasticity than other density clues. In this way, avoid of conspecifics can represent the lack of the component that does not allow the process of phenotypic plasticity.

RESUMO

MATTOS, Marcos Vinícius Vieira, M.Sc., Universidade Federal de Viçosa, outubro de 2016. **Ausência de profilaxia densidade-dependente e polifenismo de fase densidade-dependente na lagarta canibal, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)**. Orientador: Simon Luke Elliot. Coorientadores: Farley William Souza Silva e Silma Leite Rocha

A plasticidade fenotípica corresponde a capacidade de um organismo em mudar seu fenótipo em resposta a mudanças no seu ambiente. Estudos tem mostrado que a densidade populacional é um fator ambiental que pode estar correlacionada a mudanças no investimento em defesas contra doenças, comportamento e morfologia de organismos. A hipótese da profilaxia densidade-dependente (PDD) prediz que organismos investem mais em defesas contra doenças quando estão em altas densidades populacionais, uma vez que nesta ocasião o risco de transmissão de doenças entre coespecíficos tende a ser maior. O polifenismo de fase densidade-dependente (PFDD) prediz que a morfologia, fisiologia e comportamento de um organismo poderão variar em função da sua densidade populacional. Estas mudanças podem estar relacionadas a diferenças no investimento em defesas imunes e em comportamentos de defesa. A maioria dos estudos tem considerado o contato físico como o estimulo chave para o processo de plasticidade fenotípica, no entanto outros estímulos como vibração de substrato, voláteis e feromônios talvez possam permitir este processo em organismos de acordo com sua densidade. Sob estas circunstâncias, a hipótese deste estudo é que o sistema imune e a cor do corpo mudam em função da densidade populacional, mesmo que não haja contato físico entre coespecíficos. Assim, foi testado se a PDD e a PFDD podem ocorrer mesmo na ausência de contato físico entre lagartas de *Helicoverpa armigera*, através da manipulação artificial da sua densidade populacional. Para isto, lagartas recém-eclodidas foram deixadas por 10 dias, de forma solitária ou em grupos, em arenas com o objetivo de evitar o contato físico, mas permitir a percepção de coespecíficos através de outras pistas de densidade. Após este período, foi testado se a presença de coespecíficos influenciou: (i) a cor da cápsula cefálica e do corpo e (ii) as defesas imunes (resposta de encapsulação, densidade de hemócitos e atividade de lisozima).

Os resultados indicam que nem a coloração, nem as defesas imunes foram afetadas pela presença de coespecíficos. O fato de lagartas de *H. armigera* diminuírem a agregação em instares larvais avançados, devido ao seu comportamento canibal, pode representar um menor risco de doenças de forma que não haja mudanças no investimento em defesas imunes. Além disso, o contato físico pode ser mais importante para engatilhar a plasticidade fenotípica do que outras pistas de densidade. Desta forma, evitar coespecíficos pode representar a falta do componente que não permite o processo de plasticidade fenotípica.

SUMMARY

INTRODUCTION

Phenotypic plasticity corresponds to the capacity of an organism to change its physiology or morphology in function of its interaction with the environment (Agrawal, 2001). More specifically, it corresponds to the ability of a genotype to produce distinct phenotypes that will express differences in physiology, development, morphology and behaviour within a species, in response to different environmental conditions (Via and Lande, 1985). Population density is one of the environmental conditions that will govern changes in the physiology and morphology of organisms and has been studied in phenomena known as density-dependent prophylaxis and density-dependent phase polyphenism.

Density-dependent prophylaxis hypothesis (DDP) predict that organisms experiencing fluctuations in their population density show a phenotypically plastic investment in disease resistance mechanisms (Wilson and Reeson, 1998). Organisms tend to invest more in immune defences when infection risk is higher, i.e. at high population densities. In insects, this hypothesis has been tested in organisms that live in fluctuating densities and in some groups of organisms that do not have these marked group / lone-living phases (e.g. Barnes and Siva-Jothy, 2000, Cotter et al., 2004, Cotter et al., 2008, Bailey et al., 2008, Ruiz-González et al., 2009, Gotham and Song, 2013, Silva et al., 2013) . In general, these insects have shown high resistance to parasites at high population densities, increasing immune responses like haemocyte densities (e.g. Wilson et al., 2001, Silva et al., 2013), lysozyme activity (e.g. Sowa-Jasiłek et al., 2014, Tateishi et al., 2015),

encapsulation response (e.g. Washburn et al., 1996, Krams et al., 2013), and cuticular phenoloxidase activity (e.g. Cotter et al., 2008, Binggeli et al., 2014).

Density-dependent phase polyphenism (DDPP), predict that variation in density population is a key factor for the induction of several changes in the morphology, food selection and nutritional physiology, reproductive physiology, metabolism, neurophysiology, endocrinology, molecular biology, immune responses, longevity and pheromone production of organisms (Simpson et al., 1999). Increases in density may lead to intraspecific interactions that allows stimulus such as volatile and contact pheromones, visual and mechanical contact to contribute to this process (Sword and Simpson, 2000, Sword et al., 2010, Hägele and Simpson, 2000). Phenotypic changes in colouration may often be the most conspicuous feature, so that, organisms living in crowd tend to exhibit a *gregaria* phase with darker melanised cuticle than solitary organisms (Wilson and Cotter, 2008). In insects, it is expected that individuals in *gregaria* phase invest more in defence mechanisms against parasites than individuals in *solitaria* phase. Thus, increase in cuticular melanisation can be related with an increase in defence mechanisms (e.g. Reeson et al., 1998, Barnes and Siva-Jothy, 2000, Wilson et al., 2001, Cotter et al., 2004). However, this relationship does not occur in all species of insects (e.g. Robb et al., 2003, Pie et al., 2005, Hagen et al., 2006).

Both DDP and DDPP assume that phenotypic plasticity occurs in function of changes in population densities and consequently in function of interactions between individuals that allows diseases transmission or changes in their phenotype. More specifically, DDP assume the density-dependent transmission, wherein disease risk can be a factor dependent of the host's population (Anderson

and May, 1981, Wilson and Reeson, 1998, McCallum et al., 2001) and that *per capita* contact rate between susceptible and infected individuals can increase linearly with population density (Begon et al., 2002). However, it has been admitted that disease risk not always is a function of host density *per se*. Elliot and Hart (2010) propose connectivity-dependent prophylaxis hypothesis (CDP), wherein the connectivity (i.e. behavioural interactions between individuals) is more predictable to disease transmission than population density alone. This may be evidenced in some organisms can stay in high density but not stablish interactions to permit disease transmission. For example, pathogens associated with vector-borne diseases and those that are transmitted during sexual encounters is more likely to depend on the proportion of infected individuals and contact between then than on their density (Wolff and Sherman, 2008). On the other hand, individuals can perceive their conspecifics through clues like substrate vibration, olfaction, acoustic perception (e.g. Burke, 1986, Downs and Ratnieks, 1999, Schaller and Nentwig, 2000, Virant-Doberlet and Cokl, 2004, Fletcher et al., 2006, Hartbauer et al., 2012) and maybe these clues can also trigger investments in immune defences when the population density increases. On this way, defence mechanisms could be improved before the physical contact between infected and susceptible individuals.

In insects, the influence of density on phenotypic plasticity has been studied especially in lepidopteran species (e.g. Wilson et al., 2003, Cotter et al., 2004, Lindsey et al., 2009, Bindu et al., 2012, Silva et al., 2013). For these insects physical contact has been considered as a factor for the perception of density, however, other insects can stay in high density but not establish physical contact for some reason. For example, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) larvae, a

recurring pest in various crops around the world (e.g. Zalucki et al., 1986, Guo, 1997, CABI and EPPO, 1996, Ahmad et al., 2005, Czepak et al., 2013, Bueno et al., 2014, Pratissoli et al., 2015), exhibit an aggressive and cannibalistic behaviour from the third instar, which can lead to avoidance of conspecifics (i.e. physical contact avoidance) and aggregation decrease (Pieters and Sterling, 1974, Kakimoto et al., 2003, Cotter and Edwards, 2006). This avoidance can represents a behavioural component of connectivity and may have a greater relevance in disease risk than population density alone.

The colour phenotypes of these caterpillars can ben expressed in variable shades of green, straw-yellow, reddish-brown and even black (CABI and EPPO, 1996). However and so far there is no work has showing the influence of population density in its colour. Regarding immunity, *H. armigera* has specifics defence mechanisms against pathogens that can make it difficult to control in crops. For example, the integument of caterpillars has leathery appearance, unlike other Heliothinae (Czepak et al., 2013) and it can be associated with a physical barrier against fungal pathogens. Moreover it was shown that humoral and cellular immune system of caterpillars can effectively respond to various pathogens (Wang et al., 2010). Knowledge about the relation between population density and immunity in *H. armigera* could be guiding to control this pest on field.

Based on this information, the hypothesis of this study is that the immune system and the body colour of an organism change in function of its population density, even without physical contact between conspecifics. Under these perspectives, the aim of this study was to test the variation of immune parameters and body colour of *H. armigera*, a cannibalistic insect that tends to prevents

physical contacts with conspecifics from the third instar, reared in different densities.

LITERATURE REVIEW

Parasite transmission

Parasites are a constant threat and represent a selective force for all living organisms, by manipulating their behaviour, survivorship, development and reproduction (Wilson and Cotter, 2008). For example, they can affect the host's behaviour, using the host to defend themselves from enemies, or make the host to move to places that benefit the parasite dispersal (Libersat et al., 2009, Houte et al., 2013). They can also promote genetic variation, changing levels of heterozygosity and allelic diversity (Whitehorn et al., 2014, Bérénos et al., 2010) or affect the partner choice for copulation, because organisms can select partners without parasites (Bonduriansky, 2001, Randerson et al., 2000, Jiggins et al., 2000).

Transmission of parasites and pathogens can occur by direct contact between infected and susceptible hosts or by indirect contact, that involves intermediate hosts or vectors (Begon et al., 2002). In insects, parasite transmission usually occurs directly between hosts or indirectly, with the environment as an intermediate (Tanada and Kaya, 1993). Transmission can be horizontal, when parasites are transmitted between individuals within a population (Andreadis, 1987, Dyson et al., 2002, Svedese et al., 2013), or vertical, when parasites are transmitted from parents to their offspring (Kukan, 1999, Itoh et al., 2014, Virto et al., 2013).

Density-dependent transmission

Many studies have assumed that transmission of diseases increases linearly with the host's population density (Anderson and May, 1981, Wilson and Reeson, 1998, Wilson et al., 2002, Dietz, 1988). This is because the parasitic threat can change with the increase in population density (Wilson and Cotter, 2008), where the *per capita* contact rate between susceptible and infected individuals increase with the population density. This transmission model is represented by the equation βSI, where S represents the population density of susceptible hosts, I represents the density of infected hosts and β represents the transmission coefficient (i. e. probability of infection because of contact between infected and susceptible host) (Dwyer, 1991, Begon et al., 2002).

Other studies, however, have shown that *per capita* contact rate between susceptible and infected individuals does not depend on the population density (D'Amico et al., 2005, Cross et al., 2013). Thus, parasite transmission is not always dependent on the host's population density. This model has been called frequency-dependent transmission (McCallum et al., 2001). Additionally, it has been proposed that parasite transmission depends on the density and frequency of cantac together (Poulsen, 1979, Ryder et al., 2007, Fenton et al., 2002) or connectivity (i. e. behavioural interactions) between individuals that can increase parasite transmission (Elliot and Hart, 2010).

Defence mechanisms against parasites

In response to the selective pressure exerted by parasites, organisms have evolved defence mechanisms against parasites that can interact to avoid or reduce the risk of infection (Wilson, 2005) and these mechanisms may be behavioural, physical or immunological. A cascade of defence components (Schmid-Hempel and Ebert, 2003) is a combination of these mechanisms and represents a global immune response of the hosts against parasites.

Behavioural defences can reduce the probability of contact and host's infection caused by parasites, such as mate selection, diet choice, social behaviour, grooming, self-medication and behavioural fever (Table 1). Physical defences represents first line of defence to prevent parasites entry into host's body through barriers such as cuticle and peritrophic membrane (Table 2). Immunological defences act to increase the chances of the host survival infected by parasite or reduce the effects caused by infection. Immunological defences comprises cellular defences (encapsulation, phagocytosis, nodulation) and humoral defences (lysozyme, attacin, cecropin and insect defensins) (Table 3).

Maintenance of any defence mechanism is costly for the organism because it demands metabolic resources. Thus, allocation of these resources to investment in immune defences may represent a trade-off against other costly life-history traits, such as reproduction, development and survival (Cotter et al., 2004, Schwenke et al., 2016, Rantala and Roff, 2005). Therefore, for the organism’s fitness be optimized, it should be able to invest plastically in defence against parasites and pathogens according to the risk of pathogen.

Table 1: Main host behavioural defence mechanisms against parasites

Mechanism	Importance	Empirical studies
Mate selection	Avoid' potentially infected partners	Males of red flour beetle, *Tribolium castaneum*, coevolved to the parasite, *Nosema whitei* showed a reduced eagerness to mate compared to males of non-coevolved populations (Kerstes et al., 2013) Females of grain beetles, *Tenebrio molitor*, were less attracted for males infected by a tapeworm, *Hymenolepis diminuta*, than uninfected males (Worden et al., 2000)
Diet choice	Prevent parasites ingestion or enhance immunity from ingesting more nutritious diets	Larvae of Gypsy moth, *Lymantria dispar*, consume less foliage contaminated by baculovirus (from dead conspecific larvae) than uncontaminated foliage (Parker et al., 2010) African cotton leafworm, *Spodoptera littoralis*, subject to nucleopolyhedrovirus-challenge, when allowed to self-compose their diet, survived more when they chose a high protein diet than low protein diet (Lee et al., 2006)
Social behaviour	Organisms in groups use behavioural mechanisms to ensure colony survival	Honeybee (*Apis mellifera*) colonies can detect and remove adult bees infected by Deformed wing virus, probably by recognising the cuticular hydrocarbon profiles of sick individuals (Baracchi et al., 2012) Workers of the ant *Temnothorax unifasciatus* infected by the fungus *Metarhizium anisopliae*, uninfected workers whose life expectancy reduced by exposure to 95% CO2 and workers dying spontaneously exhibited the same suite of behaviour of isolating themselves from their nestmates days or hours before death (Heinze and Walter, 2010)
Grooming (Self-grooming and allogrooming)	Remove entomopathogenic fungal spores or ectoparasites	Invasive garden ant, *Lasius neglectus*, infected by fungal ectosymbiont, *Laboulbenia formicarum*, showed significantly longer self-grooming and elevated expression of immune genes relevant for wound repair and antifungal responses (β-1,3-glucan binding protein, Prophenoloxidase), in high fungal levels compared with ants carrying low fungal levels (Konrad et al., 2015) Male and female ants of *Zootermopsis angusticollis*, infected by fungus *Metarhizium anisopliae*, maintained in isolation (without allogrooming) had 1.5 times the hazard ratio of death of paired adults (with allogrooming), suggesting that mate allogrooming decreased disease susceptibility (Rosengaus et al., 2000)

Table 1: Main host behavioural defence mechanisms against parasites (continued)

Self-medication	Consumption of nutrients that enhance the physiological and immunological defence mechanisms	When allowed to choose among three host plant species, *Grammia incorrupta* caterpillars harboring early-stage parasitoids, *Chetogena edwardsi* and *Chetogena tachinomoides*, increased their consumption of a nutritious plant containing antioxidants to improve their immune system (Smilanich et al., 2011) Honeybee (*Apis mellifera*) colonies increase resin (that has fungicidal properties) foraging rates after a challenge with a fungal parasite (*Ascophaera apis*) (Simone-Finstrom and Spivak, 2012)
Behavioural fever	Reduce the parasite fitness and improve the immune function by altering body temperature	Adult desert locusts, *Schistocerca gregaria*, topically infected with the entomopathogen *Metarhizium anisopliae* var *acridum* exhibited a significant increase in a preferred environmental temperature (Bundey et al., 2003) In desert locust, *Schistocerca gregaria*, infected by fungus *Metarhizium anisopliae*, only the locusts that exhibited behavioural fever produced viable offspring before death (Elliot et al., 2002)

Table 2: Main host physical defence mechanisms against parasites

Mechanism	Importance	Empirical studies
Cuticle	Secretion of epidermis and form a rigid matrix by sclerotization of chitin and matrix of proteins. Provides mechanical protection against parasite penetration in host body	Shrimps, *Penaeus (Litopenaeus) vannamei*, are more susceptible to white spot syndrome virus infection via immersion after molting (unsclerotized cuticle) than in the period before molting (sclerotized cuticle) and wounding facilitates infection (Corteel et al., 2009) A cuticle-degrading protease of *Beauveria bassiana* enhanced fungal virulence in *Myzus persicae*, due to acceleration of conidial germination and cuticle penetration (Zhang et al., 2008)
Peritrophic membrane	Layer of insects midgut, formed by microfibers of chitin, a matrix of proteins, glycoproteins and proteoglycans. Provides mechanical protection against parasites ingested by host	Peritrophic membrane of baculovirus-exposed *Trichoplusia ni* larvae were very fragile compared with those of untreated controls, indicative of a physical/chemical change in their structure (Derksen and Granados, 1988) Peritrophic membrane of *Aedes aegypti* is digested by *Serratia marcescens* chitinase, leading to fragmentation of the membrane (Huber et al., 1991)

Table 3: Main host immunological defence mechanisms against parasites

Mechanism	Importance	Empirical studies
Melanisation	Improves the cuticle physical barrier against parasites, in addition, has antimicrobial activity	Melanic *Spodoptera exempta* larvae were found to melanise a greater proportion of eggs of the ectoparasitoid *Euplectrus laphygmae* than non-melanic larvae (Wilson et al., 2001) Melanisation response in mosquito, *Anopheles gambiae*, retards significantly *Beauveria bassiana* growth and dissemination in midgut epithelium (Yassine et al., 2012).
Encapsulation	Granulocytes (a type of haemocyte) trigger the deposition of layers of plasmocites (other type of haemocyte) on a foreign body, forming a capsule. This capsule may suffer melanisation, which is a toxic process for many parasites	Larvae of *Helicoverpa zea* infected by baculovirus (AcMNPV) showed high encapsulation and subsequent removal of contaminated cells (Washburn et al., 1996) Survival of mealworm beetle, *Tenebrio molitor*, submitted to encapsulation responses by nylon implant and subsequent fungal exposure a week later was significantly higher than survival of beetles which had been subjected to fungal infection only (Krams et al., 2013)
Phagocytosis	Engulfed and posterior digestion of bacteria and other small particles by haemocytes	Generalist herbivore *Heliothis virescens* showed higher phagocytic activity against pathogenic bacteria than specialist herbivore *Heliothis subflexa* (Barthel et al., 2014) Non-mated house cricket, *Acheta domesticus*, showed higher phagocytic activity against bacteria *Serratia marcescens* than mated individuals (Nava-Sánchez et al., 2015)
Nodulation	Engulfment of bacteria mass and other foreign bodies by aggregates of host haemocytes. The nodules formed could become melanised in response to the number of invading parasites	Tunaz et al. (2015), recorded a significantly higher number of nodules from insects associated with soil than from insects collected from plants The nodulation response against the bacterium *Serratia marcescens* in larvae of *Manduca sexta* is mediated by eicosanoids (Miller et al., 1994)

Table 3: Main host immunological defence mechanisms against parasites (continued)

Antimicrobial peptides	Produced in haemocytes, fat body and epithelial tissues and secreted into the plasma. They act against parasites directly or enhance the immunity system. They include lysozyme, attacin, cecropin and insect defensins	Antimicrobial response of *Drosophila* can discriminate between various classes of microorganisms. *Drosophila* infected by entomopathogenic fungi exhibit an adapted response by producing only peptides with antifungal activities (Lemaitre et al., 1997) Bacteria infecting *Manduca sexta* induce antimicrobial peptides responses, including cecropins, attacins and lysozymes (Hurlbert et al., 1985)
Prophenoloxidase cascade	The enzyme of prophenoloxidase cascade, phenoloxidade, participates in the tyrosine oxidation and their derivatives, yielding quinones and its polymer, melanin	In *Drosophila*, the proPO activity is important in the survival to infection by Gram-positive bacteria and fungi (Binggeli et al., 2014) *Spodoptera littoralis* with high PO activity showed slower development rates and lower pupal weights, suggesting that investment in PO is costly (Cotter et al., 2008)

Density-dependent prophylaxis hypothesis

Density-dependent prophylaxis hypothesis (DDP) (Wilson and Reeson, 1998) predicts that as way to optimize investment of metabolic resources, organisms whose density fluctuates should develop plastic responses in their immune system, depending on the density experienced. In other words, organisms tend to invest more in immune defences when infection risk is higher, i.e. in high population densities.

Many studies have tested this hypothesis, especially in invertebrates (Mills, 2012, Cotter et al., 2004, Cotter et al., 2008, Barnes and Siva-Jothy, 2000, Ruiz-González et al., 2009). As an example, Silva et al. (2013) showed that caterpillars of *Anticarsia gemmatalis* , considered to be naturally solitary, showed plastic changes in their colour phenotype when reared in crowded conditions. In addition, the crowded caterpillars showed a greater degree of encapsulation, higher haemocyte densities and greater resistance to *Baculovirus anticarsia* than caterpillars reared in solitary condition. Similarly, Srygley (2012) showed for Mormon crickets (*Anabrus simplex*) that group-reared adults exhibit higher levels of prophenoloxidase and encapsulation than solitary-reared adults.

Although this hypothesis is accepted for various organisms, some studies showed reduced immune responses when individuals are reared at higher population densities. Piesk et al. (2013) showed that larvae of *Pieris napi* were negatively affected by increased population density in all immune parameters tested (phenoloxidase and prophenoloxidase activities, haemocyte number and encapsulation rate). The authors argue that the decrease in performance of the

immune system of gregarious larvae, in this case, may have led to a decrease in energy storage, probably due to food shortages generated by the increase in population density.

Density-dependent phase polyphenism

Density-dependent phase polyphenism (PPDD) is a form of phenotypic plasticity wherein organisms change their phenotype, including morphology, anatomy, colouration, development, reproduction, physiology, biochemistry, molecular biology, behaviour, chemical ecology (pheromones) and other aspects of ecology, in response to clues perceived by the change in population density of conspecifics (Pener, 2009). Increases in density may lead to intraspecific interactions that allows stimulus such as volatile and contact pheromones, visual and mechanical contact to contribute to this process (Sword et al., 2010, Hägele & Simpson, 2000). Phenotypic changes in colouration may often be the most conspicuous feature, and generally, organisms living in crowd conditions exhibit the *gregaria* phase state, with a darker, more melanised cuticle than the solitary organisms (Wilson and Cotter, 2008). In insects, it has been reported that that the individuals in the *gregaria* phase state invest more in defences against parasites than do individuals in the *solitaria* phase state. This is because the increase in cuticular melanisation can be related to an increase in defences (Reeson et al., 1998, Barnes and Siva-Jothy, 2000, Wilson et al., 2001, Cotter et al., 2004), although this does not occur in all insects (Robb et al., 2003, Pie et al., 2005, Hagen et al., 2006).

Study system: *Helicoverpa armigera*

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is one of the main insect pests in Africa, Asia, Europe and Oceania (Zalucki et al., 1986, Guo, 1997, CABI and EPPO, 1996). This pest was also recently recorded in Brazil, in the States of Goiás, Bahia, Mato Grosso, São Paulo e Espírito Santo (Czepak et al., 2013, Bueno et al., 2014, Pratissoli et al., 2015). Larvae of this insect feed on several crops such as tomatoes, soybeans, maize and cotton, annually causing damage over US$ 2 billion around the world (Tay et al., 2013). Its success has been attributed to its great voracity, reproduction, dispersion and resistance to insecticides (Yang et al., 2013).

The life cycle of *H. armigera* lasts ca. 6 weeks at temperature of 28 ° C, but can be extended to 73 days at temperatures of 16 to 18 ° C (Zalucki et al., 1986), indicate of an optional diapause (Kurban et al., 2007, Liu et al., 2010). *Helicoverpa armigera* has 5-6 larval stages; the sixth larval stage is facultative, which its occurrence depending on genetic, environmental conditions and diet quality (Araújo, 1990). Caterpillars present extremely variable colours, with shades of green, straw-yellow, reddish-brown and even black (CABI and EPPO, 1996). Adults present sexual dimorphism, with females showing pink-brown wings with a wingspan of ca. 40mm and a more rounded abdomen than the male, while males have greenish-brown wings with ca. 35mm wingspan (Jayaraj et al., 1982).

Helicoverpa armigera may present cannibalistic behaviour in its larval stage, probably due to nutrient deficiencies (Pan et al., 2015, Xiao et al., 2010), or as way to competition when population increase (Chapman et al., 1999).

Cannibalism may be beneficial as it provides nutrients, but may also incur costs when it increases the chances of infection by parasites (Dhandapani et al., 1993). This behaviour usually begins when caterpillars are in the third instar (Kakimoto et al., 2003) and may partially explain why aggregation decreases from the third instar onwards, in this insect (Pieters and Sterling, 1974, Cotter and Edwards, 2006).

Helicoverpa armigera has several resistance mechanisms that can make it difficult to control. The integument of its caterpillars has a more leathery aspect than other Heliothinae (Czepak et al., 2013), acting as physical barrier, and this may be associated with resistance to contact insecticides. In addition, Wang et al. (2010), challenging this insect with different pathogens (*Bacillus thuringiensis*, *Klebsiella pneumoniae*, *Candida albicans*, and *Autographa californica* nucleopolyhedrovirus) showed that the these caterpillars' haemocytes were able to phagocytize both fungi and gram-positive and gram-negative bacteria. Furthermore they detected the presence of different antimicrobial peptides acting against pathogens. This shows that humoral and cellular immune system of this caterpillars can effectively respond to various pathogens.

METHODOLOGY

Experimental setup

Helicoverpa armigera eggs were obtained from BUG Agentes Biológicos (Piracicaba, São Paulo, Brazil). The eggs were maintained at 25 (± 2) ºC and relative humidity 50% for two days until eclosion of the larvae. Post-eclosion larvae were placed in arenas designed to avoid physical contact, while allowing perception of conspecifics, through other clues such feromones, volatiles and substrate vibration (see below and Figure 1). Using these arenas, three treatments were designed: *solitary* (test larva in the central area with no other conspecifics), *low density* (test larva in the central area with one conspecific larva in one of the peripheral areas) and *high density* (one larva in the central area with four larvae in four alternating peripheral areas). Note that the arenas were designed with eight surrounding compartments but insect numbers were limited. Caterpillars were fed *ad libitum* with an artificial diet adapted from Greene et al. (1976). Briefly, this diet consisted of: white bean, brewer's yeast, soybean meal, wheat germ, powdered milk, ascorbic acid, sorbic acid, NIPAGIN and vitamin solution. The experimental design employed randomized blocks to minimize effects of external conditions that could affect the results of experiments. Sixteen blocks were set up and each of these contained two plates of each treatment (Fig. 2). These two plates corresponded to different dependent variables that were measured and were made because only one caterpillar from the central area of each plate was tested. The treatments were kept in a climate-controlled room (25 ± 2ºC, 70 ± 2% relative humidity and 12h photophase), until the tenth day post-eclosion (approximately 4^{th} instar). After ten

days post-eclosion, the test caterpillars (from the central area) were used to measure immune parameters (Figure 2).

Arenas were made from plastic Petri dishes (15cm diameter x 15mm height) with segments divided by a metal mesh (aperture 0.10mm, thread 0.06mm). This was designed to prevent physical contact between larvae yet allowing the perception of clues from conspecifics such as volatiles and substrate vibration. The arenas contains a circular central area of 19.1 cm^2 and 8 subdivisions around the central area also of 19.1 cm^2. (Figure 1)

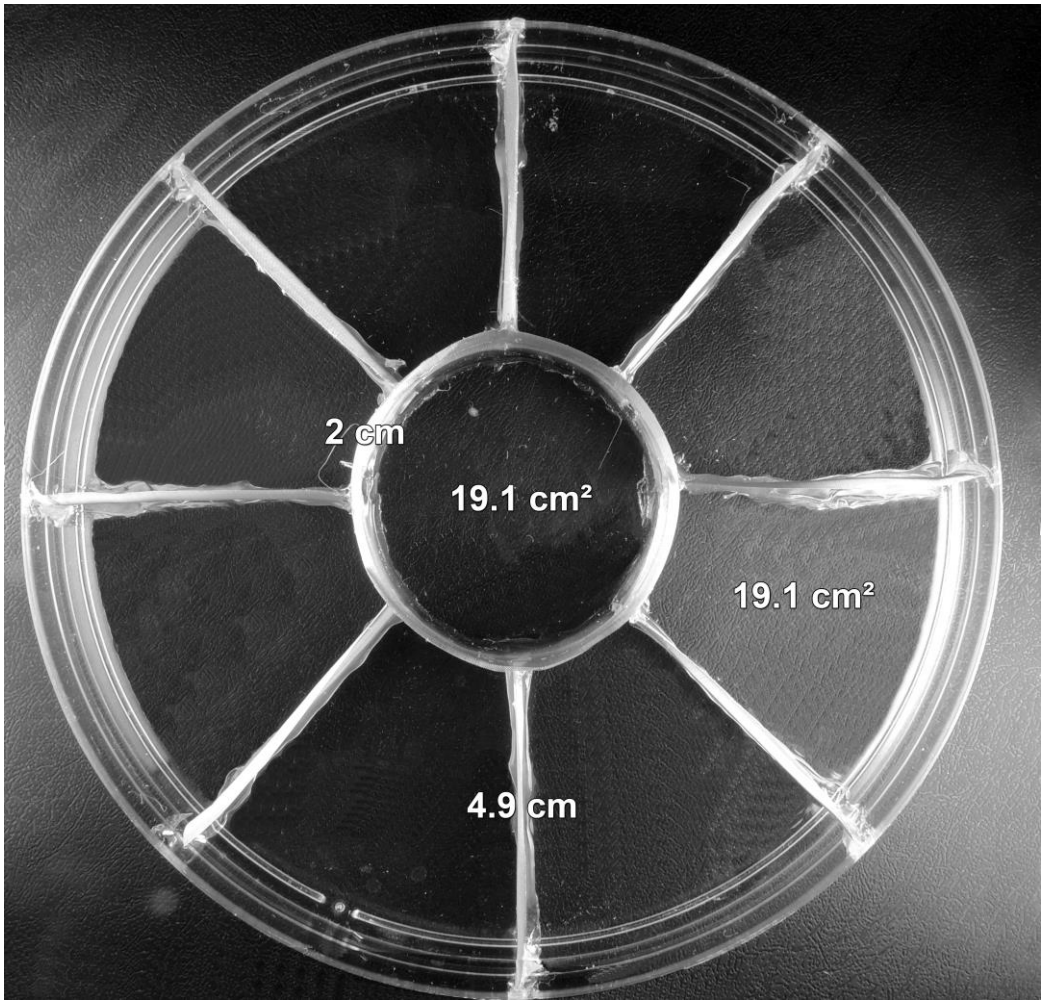

Figure 1: Arena used to rear *Helicoverpa armigera* at different population densities. Test caterpillars, subsequently used to assay immune parameters, were kept in the central area, while conspecifics serving as a stimulus to indicate local population densities were kept in the surrounding segments. All nine compartments (1 plus 8) had the same area (19.2 cm²). The central circular area was separated from the peripheral compartments using a mesh of 2 cm circumference while the peripheral compartments were separated from one another by a mesh of 4.9 cm length. Mesh size was 0.10mm of aperture and 0.06mm of thread.

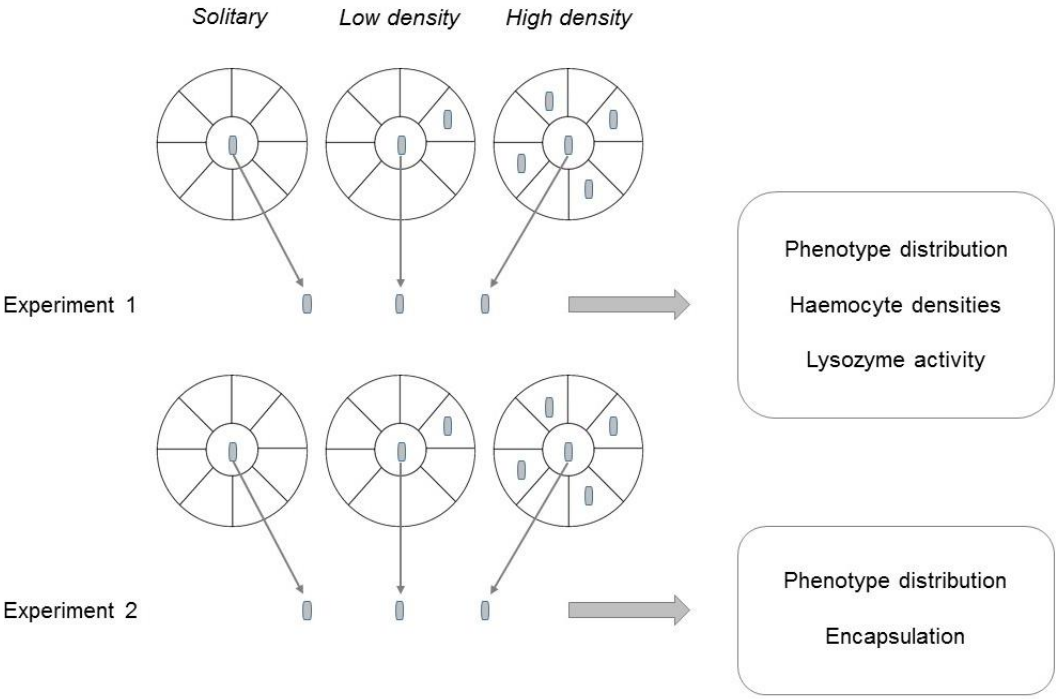

Figure 2: Diagrammatic representation of experimental design. Larvae were kept in arenas for ten days post-eclosion. Only caterpillars of the central area of each arena were used to immune parameters measurement. One caterpillar of each treatment (solitary, low density and high density) was used in haemolymph extraction to assess haemocyte densities, lysozyme activity and phenotype colour distribution (experiment 1). Similarly, one caterpillar from each treatment was used to measure encapsulation response and phenotype colour distribution (experiment 2).

Effect of population density on phenotypic colour traits of *Helicoverpa armigera*

To assess if *H. armigera* caterpillars exhibit density-dependent phase polyphenism, i.e. if their phenotype colour depend to population density, insects that were maintained on central area of arenas were photographed (n= 188). For this, a Nikon camera (Coolpix P510) was used. The software ImageJ was used to measure colour of body and head capsule of these caterpillars. Using images that

were converted to grey scale ranging from 0 to 255 (pixel values were mapped to 8-bit), and using a selection tool to mark the interest areas, the grey variation in the body and head capsule of each caterpillar was measured.

Effect of population density on immune parameters in *Helicoverpa armigera*

To assess if *H. armigera* caterpillars exhibit density-dependent prophylaxis, the following immune parameters were tested: encapsulation response, haemocyte densities and lysozyme activity.

Encapsulation response

In order to simulate a parasite invasion to activate immune response, a nylon filament (0.2 mm ø x 2 mm length) was inserted between the last two pairs of prolegs of each caterpillar. Before twenty-four hours, these caterpillars (n= 78) were dissected and the filament was placed on slides and photographed with stereomicroscope Zeiss (model Stereo Discovery V20).

Capsule area formed around the nylon and capsule melanisation were measured using ImageJ software. The total area of the nylon and the area covered by the haemocyte layer was measured, and from rule of three, the percentage of encapsulation was calculated. Capsule melanisation was measured in grey scale image from grey variation in the nylon filament (methodology modified from Silva et al., 2013).

Haemocyte densities

The haemocyte densities was quantified from the haemolymph extracted. A hole was made between the last two pairs of prolegs of each caterpillar (n= 70). Then 4 µl of haemolymph was collected and mixed with 10 µl of anticoagulant (98 mM NaOH, 186 mM NaCl, 17 mM Na_2 EDTA and 41 mM citric acid, pH 4.5) and 6µl of Giemsa. 10 µl of this solution was pipetted on one side of Neubauer chamber (Bright-Line, Precicolor HBG, Germany) and haemocytes were counted under a microscope (Olympus model SZ61). To obtain the amount of haemocytes per microliter, the total haemocyte number was multiplied by the Neubauer chamber correction factor and divided that by the number of quadrants counted multiplied by the dilution factor of the solution.

$$\text{Concentration} = \frac{\text{Number of cells X } 10.000}{\text{Number ofsquare X dilution}}$$

Detection of lysozyme activity

The lytic activity against *Micrococcus lysodeikticus* cell wall was determined using haemolymph of caterpillars from different treatments. Petri dish were filled with 10 ml of a mixture containing 1.5 g of agar diluted in 100 ml of distilled water, 0.75 g of freeze-dried *M. lysodeikticus* in 50 ml of 0.2 M potassium phosphate buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 and 1 M HCl) and 150 µl of streptomycin sulphate solution (100 mg streptomycin sulphate and 1 ml sterile distilled water). With a capillary glass, twenty holes (2 mm ø) were

made in each plate. Then the holes were filled with 1 µl of undiluted haemolymph from each sample (n= 68) (two replicates per sample). The plates were incubated at 33 ºC for twenty-four hours, after that they were photographed and the diameters of the clear lytic zones were measured from images obtained (methodology modified from Kurtz et al., 2000).

Statistical procedures

The effects of population density on immune parameters (haemolymph density, lysozyme activity and encapsulation response) and phenotypic traits of *Helicoverpa armigera* were verified using linear models in R statistical software. Models were analysed to verify their significance, and following the analysis, data overdispersion was checked to determine whether the distribution was the most suitable. Dependent variables that do not follow a normal distribution were $\log_{10}$ transformed. Simplification of the models was made eliminating non significant terms of experiments.

Analysis of Variance (ANOVA) was used to confirm the influence of population densities treatments on immune parameters. Dependent variables were nylon area covered by haemocyte layer (percentage), nylon melanisation converted in grey scale (artificial unit), haemocyte number (cells/µl) and clear zone lytic area (mm^2). Independent variable was number of caterpillars placed in arenas (1, 2 and 5 caterpillars per arena).

Similarly, Analysis of Variance (ANOVA) was used to confirm the influence of population densities treatments on phenotypic colour traits. Dependent

variables were colour of body and head capsule converted in grey scale (pixel values). Independent variable was number of caterpillars placed in arenas (1, 2 and 5 caterpillars per arena).

RESULTS

Effect of population density on phenotypic colour traits of *Helicoverpa armigera*

The phenotypic colour traits evaluated were not affected by presence (or number) of conspecifics in *H. armigera* caterpillars reared for 10 days in the treatments: *solitary* (1 larvae *per* arena), *low density* (2 larvae *per* arena) or *high density* (5 larvae *per* arena). Neither the body colour ($F_{[2;169]} = 0.156$, $P = 0.855$; Figure 3a) nor the colour of the head capsule ($F_{[2;169]} = 0.247$, $P = 0.781$; Figure 3b) were statistically different among population density treatments.

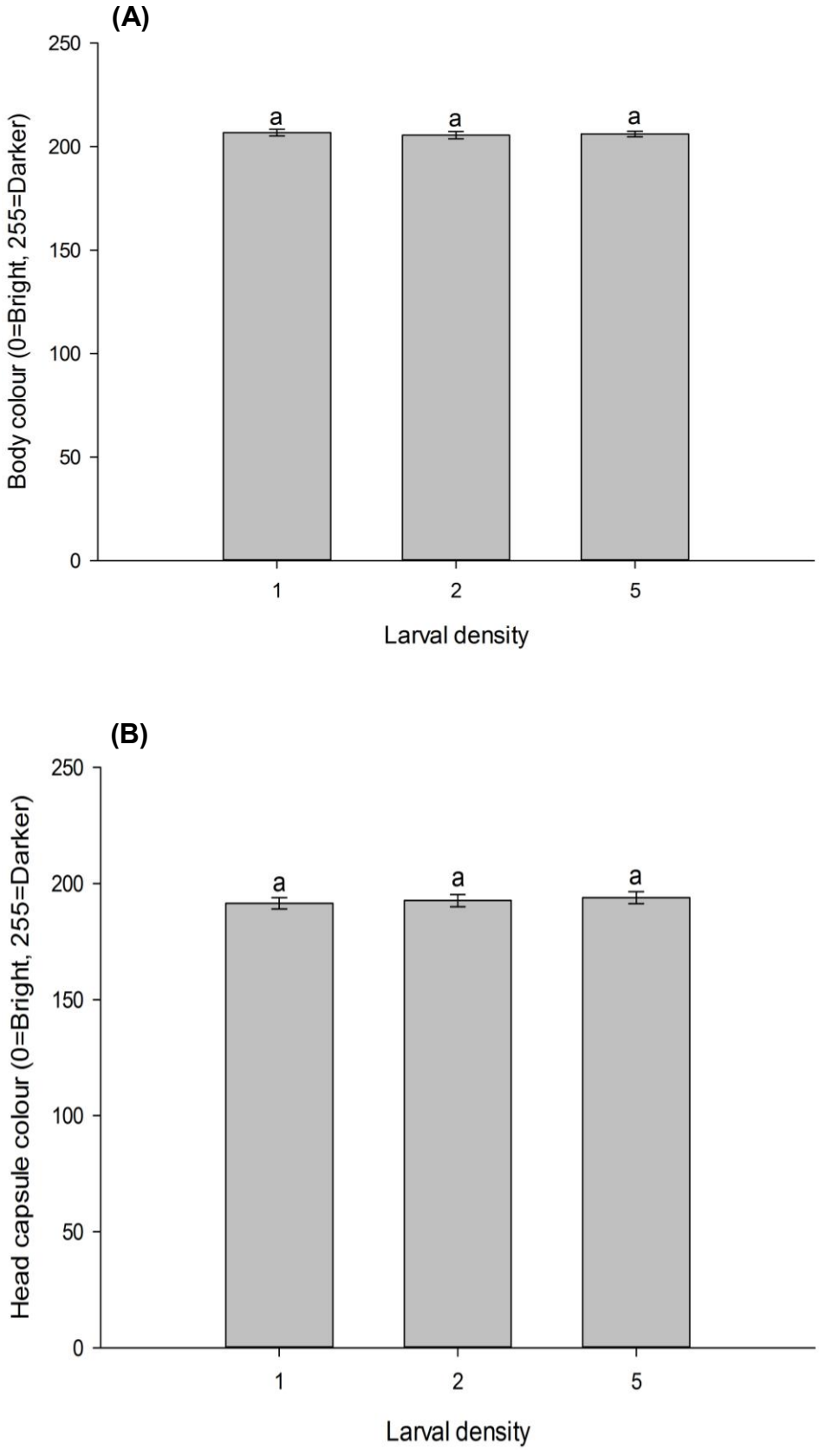

Figure 3: Body (A) and head capsule (B) colours according to population density in *Helicoverpa armigera*. Larvae (n= 188) were reared at densities of 1, 2 and 5 per arena for

10 days post-eclosion and then were photographed. The images were converted to a grey scale ranging from 0 (bright colour) to 255 (darker colour) and colour was measured by grey variation in the body and in the head capsule of each caterpillar. Same letters among treatments indicate non-significant differences.

Effect of population density on immune parameters in *Helicoverpa armigera*

The immune parameters evaluated were not affected by presence (or number) of conspecifics in *H. armigera* caterpillars reared for 10 days at three density treatments: *solitary* (1 larvae *per* arena), *low density* (2 larvae *per* arena) or *high density* (5 larvae *per* arena).

The degree of encapsulation of the nylon filament inserted into the test insects, measured as the area covered by the haemocyte layer and the degree of melanisation of this filament, was not affected by the caterpillars population densities (capsule area $F_{[2;60]} = 0.405$, $P = 0.668$, Figure 4a; and melanisation of this capsule $F_{[2;60]} = 0.361$, $P = 0.698$, Figure 4b). Similarly, haemocyte densities ($F_{[2;52]} = 0.691$, $P = 0.505$, Figure 5) and lysozyme activity against cell walls of the bacterium *M. lysodeikticus* ($F_{[2;51]} = 0.680$, $P = 0.510$; Figure 6), were unaffected by population density treatments in *H. armigera.*

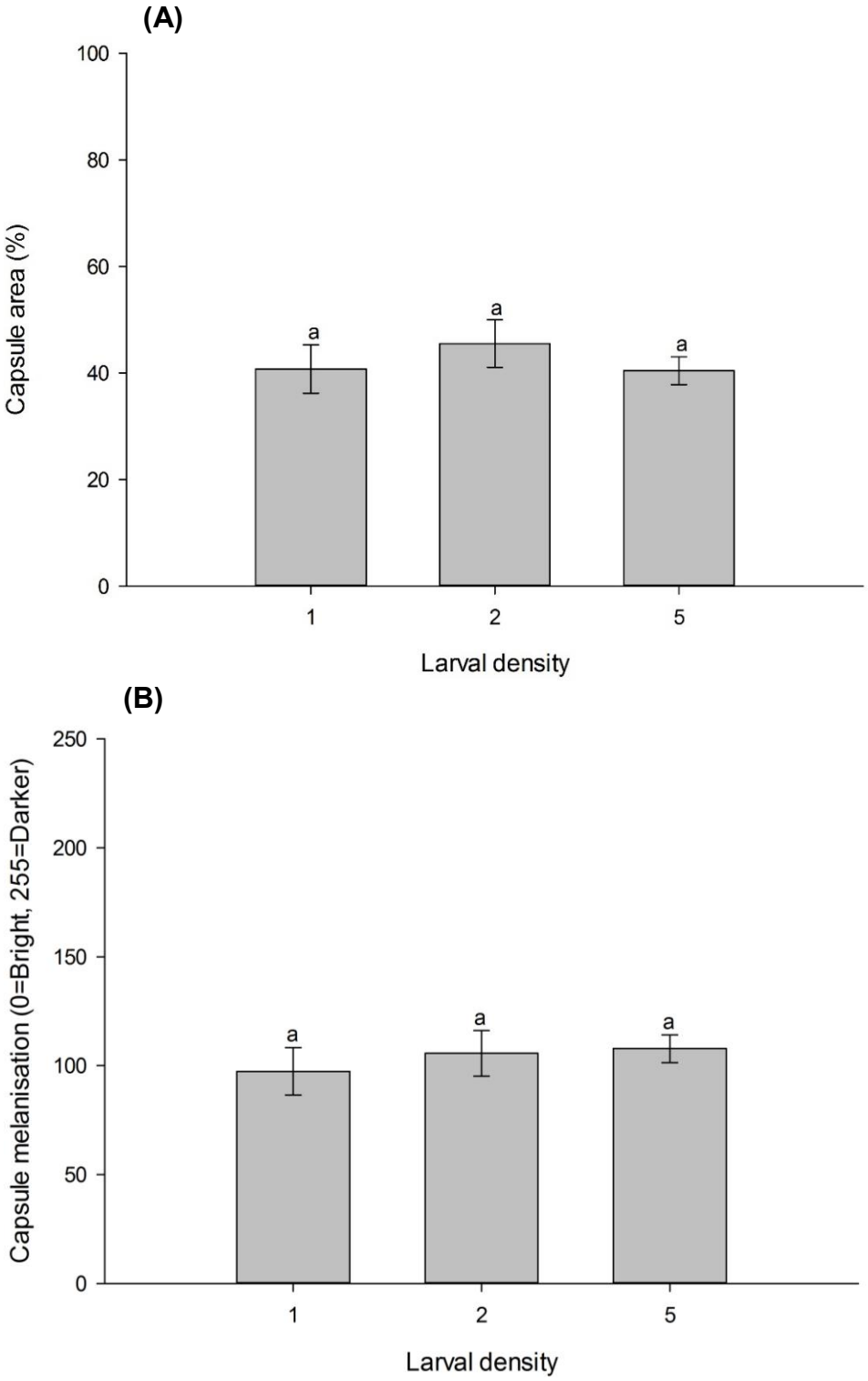

Figure 4: (A) Capsule area formed around the nylon filament inserted in caterpillars of *Helicoverpa armigera* reared in different densities for 10 days. (B) melanisation of the nylon filament. Caterpillars (n= 78) were held in an arena with none, one or four conspecifics, with perception of clues allowed, but contact prevented by a metal mesh. Then, they were challenged with a colourless nylon filament (0.2 mm ø x 2 mm length)

inserted between the last two pairs of prolegs. Twenty-four hours after the nylon challenge, the nylon filaments were removed and mounted on to slides to be photographed. The capsule area and melanisation measurement were performed with ImageJ software. Same letters among treatments indicate non-significant differences. Same letters among treatments indicate non-significant differences.

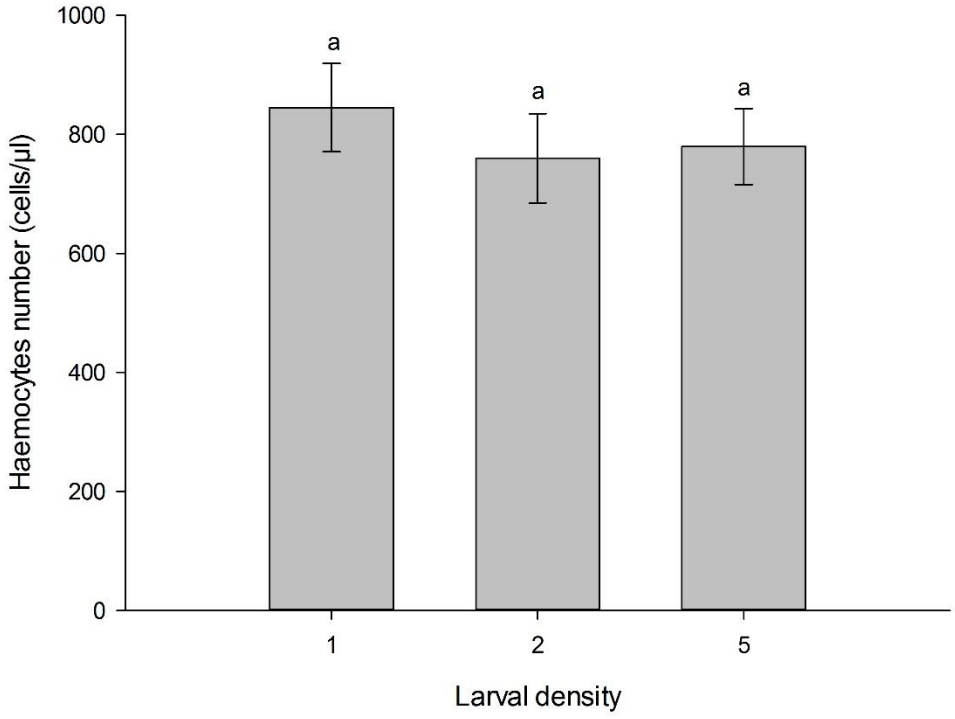

Figure 5: Haemocyte densities for *Helicoverpa armigera* reared at three different population densities for 10 days. Insects (n= 70) were held in an arena with none, one or four conspecifics, with perception of clues allowed, but contact prevented by a metal mesh. After ten days of this treatment, by which stage insects had progressed to fourth instar, haemocyte densities were measured from haemolymph taken from these insects. Same letters among treatments indicate non-significant differences.

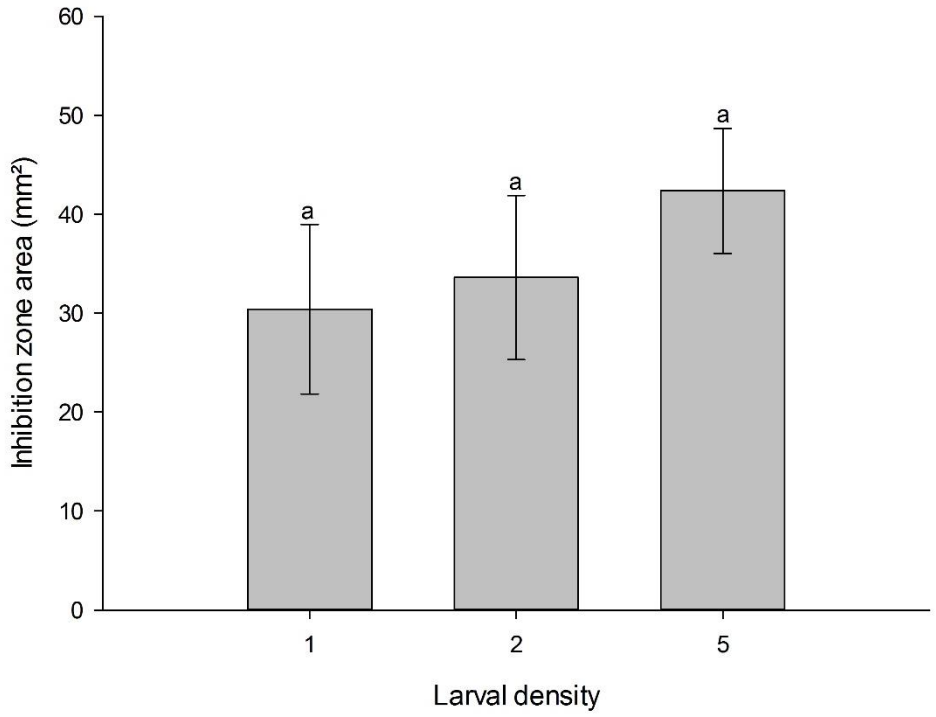

Figure 6: Lysozyme activity against *Micrococcus lysodeikticus* cell wall in *Helicoverpa armigera* reared at different population densities for 10 days. Post-eclosion larvae (n= 68) were held in an arena with zero, one or four conspecifics, with perception of clues allowed, but contact prevented by a metal mesh. After ten days of this treatment, by which stage insects had progressed to fourth instar, a lysozyme activity assay was made from haemolymph taken from these insects. Same letters among treatments indicate non-significant differences.

DISCUSSION

Considering that, beyond the physical contact, other density clues can trigger phenotypic plasticity, *Helicoverpa armigera* larvae maintained in arenas, separated by a metal mesh, for 10 days, not exhibit differences in immune defences or phase polyphenism in function of its population density. Caterpillars maintained in different densities showed similar investment in encapsulation response, haemocyte densities and lysozyme activity. Additionally, these caterpillars did not exhibit density-dependent phase polyphenism, since individuals in different densities had similar colours in their body and head capsule.

Under these perspectives, two assumption may be considered: first, even perceiving their conspecifics through density clues, beyond the physical contact, *H. armigera* does not display neither DDP nor DDPP, and second, physical contact may be more important to trigger the DDP and DDPP than other density clues.

The first assumption may be valid since that *H. armigera* caterpillars tend to live progressively less clumped with the later larval stage and therefore they not show a plastic immune response as a function of its population density. In this, case even that the organism recognize its conspecifics through vibrational or olfactory clues, is not advantageous to invest in improving of its immune system, since its stay in low density and the risk of disease transmission is low. As observed by previous studies, caterpillars of this species decrease aggregation mainly from the third instar and cannibalistic and aggressive behaviour may be a factor influencing this decline (Pieters and Sterling, 1974, Cotter and Edwards, 2006).

The second assumption can be considered if individuals not stablish interactions capable of transmitting diseases, supporting the hypothesis of connectivity-dependent prophylaxis showing that behavioural interactions, more specifically physical interactions, are more important to trigger the immune responses than population density alone (Elliot and Hart, 2010). Furthermore, Kakimoto et al. (2003) observed uniform spatial distribution in *H. armigera*, so that each individual tends to repel or eliminate its conspecific, so there is a homogenous spacing between these individuals. In this case, avoiding conspecifics due to cannibalism could represent the lack of connectivity component that does allow disease transmission.

Phase polyphenism does not seem density-dependent in *Helicoverpa armigera*, but colour variation in this specie can be explained by its feeding (Ma et al., 2008, Yamasaki et al., 2009). For example, Ramos and Morallo-Rejesus (1976) found that caterpillars fed on different plants show variations in coloration of its body. Yamasaki et al. (2009) observed that this variation in body colour may be related to part of the plant consumed by the larvae. However, they noticed that larval coloration was not the same among larvae reared on the same diet, suggesting that there is still a plastic component involved in the determination of larval coloration, requiring more studies. In addition, the lack of physical contact may be the reason for caterpillars do not exhibit density-dqependent phase polyphenism, since previous studies have shown the importance of physical contact in the induction of phase polyphenism in other insects. Simpson et al. (2001) showed that desert locusts, *Schistocerca gregaria*, in the solitarious phase were repeatedly touched in the outer face of a hind femur and this led to the transition to gregarious phase

behaviour. Hägele and Simpson (2000) investigated the influence of mechanical, visual and contact chemical stimulation on behavioural gregarization in solitary nymphs of desert locusts, *S. gregariaI*, and found that the mechanical stimulation was powerfully gregarizing factor than the others clues.

Sample size and density treatments used in this study were similar to previous studies wherein DDP and DDPP were tested in other insects. For example, Kazimirova (1992) assessed the density effects on phase polymorphism of *Mamestra brassicae* by rearing larvae at five densities: 1, 2, 4, 6 and 8 individuals *per* petri dish (100 x 15 mm). Cotter et al. (2004) in a study that tested DDP in *Spodoptera littoralis*, used 1 larva for *solitary* treatment and 3 larvae for *crowded* treatment. Silva et al. (2013), in a similar study with *Anticarsia gemmatalis*, used four densities to test DDP: 1, 2, 4 or 8 larvae *per* container. Thus, these densities can be sufficient to show density effects on phenotypic plasticity in insects. However, in previous studies, tested organisms, were allowed to be in physical contact, while in this study was tested if even without physical contact, other clues, like substrate vibration, olfaction and acoustic perception, can trigger phenotypic plasticity in individuals who exhibit cannibalistic behaviour and tend to avoid physical contact with conspecifics.

More studies are needed to see if the perception of the population density and consequently the phenotypic plasticity (i.e. density-dependent prophylaxis and density-dependent phase polyphenism) can be trigged for clues such as pheromones, volatile and vibrations. One way to test this is using species that DDP has been observed and separate those into arenas like these that were used in this study to see if even without contact these individuals exhibit phenotypic plasticity.

Thus, this work is a step for further investigations regarding the role of density clues in expression of phenotypically plastics processes.

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