DANIEL LUIS VIOL

IS DENSITY-DEPENDENT PROPHYLAXIS TRANSMITTED TO OFFSPRING? EXPERIMENTS WITH *Anticarsia gemmatalis* (LEPIDOPTERA: NOCTUIDAE)

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

VIÇOSA
MINAS GERAIS – BRASIL
2015

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
V795i
2015

Viol, Daniel Luis, 1987-
Is density-dependent prophylaxis transmitted to offspring? : experiments with *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) / Daniel Luis Viol. – Viçosa, MG, 2015.
vii, 27f. : il. (algumas color.) ; 29 cm.

Orientador: Simon Luke Elliot.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Referências bibliográficas: f.24-27.

1. *Anticarsia gemmatalis. 2.* Inseto - Imunologia. 3. Defesas animais. I. Universidade Federal de Viçosa. Departamento de Entomologia. Programa de Pós-graduação em Entomologia. II. Título.

CDD 22. ed. 595.78

DANIEL LUIS VIOL

IS DENSITY-DEPENDENT PROPHYLAXIS TRANSMITTED TO OFFSPRING? EXPERIMENTS WITH *Anticarsia gemmatalis* (LEPIDOPTERA: NOCTUIDAE)

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

APROVADA: 28 de Julho de 2015.

Farley William Souza Silva
(Coorientador)

Eliseu José Guedes Pereira

Carla Cristina Marques Arce

Simon Luke Elliot
(Orientador)

AGRADECIMENTOS

Ao meu pai Miguel Viol e à minha mãe Célia Cristina da Silveira Viol por todo amor e amizade, pela educação, pelo exemplo, pelas oportunidades, por todo incentivo e apoio, desde sempre. Ao meu irmão Miguel, pela amizade e companheirismo. A toda minha família e amigos pela torcida e incentivo.

À Ana, pela profunda amizade e pelo amor. Por todo apoio e carinho, especialmente nas adversidades. Pela companhia. Por tornar minha vida mais feliz.

Aos colegas do Laboratório de Interações Inseto-Microrganismo pela boa convivência e pelo espírito cooperativo.

À Fernanda, Débora e Lorena pela amizade, por toda ajuda, dedicação e boa vontade com a execução dos experimentos, inclusive nos finais de semana, feriados e fora do horário normal de trabalho.

À Verônica pela amizade, ajuda nos experimentos, por sua dedicação e competência ao cuidar do laboratório e garantir um bom ambiente de trabalho a todos.

Ao Prof. Sam pela orientação desde a graduação, pelas oportunidades, pelo exemplo e pela formação crítica em ciência.

Ao Farley pela amizade, pela coorientação desde a graduação, pela ajuda em todas as etapas da produção desta dissertação, por todas as oportunidades de aprender ciência na prática.

Ao Prof. Eliseu, à Carla e ao Farley por aceitarem compor a banca de defesa, pelas sugestões e críticas.

Ao Prof. Eraldo, por esclarecimentos sobre Lepidopteros.

À Universidade Federal de Viçosa, através de seus professores e servidores, pela formação acadêmica e oportunidades. Em especial, ao Departamento de Entomologia.

A CAPES pela concessão da bolsa de mestrado.

A todos que contribuíram para finalização desta etapa. Muito obrigado!

SUMÁRIO

RESUMO

VIOL, Daniel Luis, M.Sc., Universidade Federal de Viçosa, julho de 2015. **Is Density-Dependent Prophylaxis transmitted to offspring? Experiments with *Anticarsia gemmatalis* (Lepidoptera: Noctuidae)**. Orientador: Simon Luke Elliot. Coorientador: Farley William Souza Silva.

Animais e microrganismos causadores de doenças estão em constante confronto. Enquanto patógenos evoluem de forma a superar barreiras dos hospedeiros, as defesas imunes do hospedeiro são aprimoradas para impedir o estabelecimento de doenças. Não só indivíduos, mas populações podem ser afetadas pela ação de patógenos. Assim, organismos possuem mecanismos que evitam o processo infeccioso através da percepção de sinais ambientais. O aumento da densidade de co-específicos é um destes sinais e indivíduos capazes de percebê-los poderiam aumentar plasticamente o investimento em defesas imunes; hipótese conhecida como “Profilaxia Densidade-Dependente” (PDD). Outra forma de proteção em nível de população é a transferência de defesas imunes para a prole, especialmente via herança epigenética. Defesas imunes tem um alto custo metabólico e a combinação de ambas as estratégias, PDD e transferência vertical, poderia otimizar a prevenção de doenças em organismos que estão sujeitos a surtos populacionais e possivelmente apresentam sobreposição de gerações, como insetos-praga. *Anticarsia gemmatalis* é conhecida por ter hábito solitário, porém apresenta plasticidade fenotípica dependente da densidade como algumas espécies de insetos gregários. Por isso, *A. gemmatalis* foi escolhida como modelo para testar se há transferência transgeracional de PDD. Para isso, avaliamos a atividade antimicrobiana (provavelmente uma lisozima), através da zona de inibição em placas contendo a bactéria *Micrococcus lysodeikticus*, em ovos de mariposas de diferentes fenótipos (i.e. lagartas foram criadas solitárias ou gregárias e expressaram o fenótipo verde ou preto, respectivamente). Casais foram acasalados em gaiolas nas quatro combinações possíveis entre fêmeas e machos exibindo os fenótipos “verde” ou “preto”. Dessa forma, seria possível identificar qual dos parceiros estaria contribuindo mais para a transferência de defesas à prole. Além disso, foram avaliados o número de ovos por fêmea como forma de investigar possíveis *trade-offs* entre a defesa imune e reprodução. Não houve diferenças significativas na atividade antimicrobiana nem no número de ovos de mariposas expressando o fenótipo verde ou preto na fase larval. Os

resultados indicam que, pelo menos para essa defesa imunológica, não há transferência transgeracional de PDD à prole de *A. gemmatalis*. Como essa espécie é migratória, talvez a prole não experimente as mesmas condições ambientais dos pais, não precisando assim transferir defesas imunológicas à prole mediante estímulos sofridos pelos pais. Propomos investigar a transferência de DDP em outros ínstares larvais da prole, através da avaliação de diferentes parâmetros imunes, para melhor subsidiar nossa conclusão.

ABSTRACT

VIOL, Daniel Luis, M.Sc., Universidade Federal de Viçosa, July, 2015. **Is Density-Dependent Prophylaxis transmitted to offspring? Experiments with *Anticarsia gemmatalis* (Lepidoptera: Noctuidae)**. Adviser: Simon Luke Elliot. Co-adviser: Farley William Souza Silva.

Animals and disease-causing microorganisms are in a constant fight. While pathogens evolve ways to breach the host defences, hosts enhance defences to prevent the establishment of diseases. Not only individuals themselves, but populations may be affected by pathogens. Thus, organisms have mechanisms to avoid infectious processes through perception of environmental clues. The increase in population density of conspecifics may be such a clue, and individuals able to perceiving it could increase plastically the investment in immune defences; hypothesis known as 'Density-dependent prophylaxis' (DDP). Furthermore, organisms may invest plastically in defences of offspring via epigenetic. It is known that immune defences pose a high cost to hosts, and the use of the two strategies – DDP and transgenerational transfer – could optimize the prevention of disease in organisms subject to population fluctuation and likely overlap of generations, such as insect pests. *Anticarsia gemmatalis* is known to be a lone-living insect, but presents density-dependent plasticity in phenotype as such group-living insects. Thus, *A. gemmatalis* was chosen as model to test if there is transgenerational transfer of DDP. It was assessed the antimicrobial activity (lysozyme-like activity), through inhibition zone in plates with the bacterium *Micrococcus lysodeikticus*, in eggs of moths from different phenotypes (i.e. caterpillars were raised alone or in groups and expressed green or black phenotype, respectively). Moths were paired in cages in four possible combinations between females and males expressing "green" or "black" phenotypes. Thus, it was possible to identify the contribution of each partner in the provision of immune defence to offspring. Furthermore, it was assessed the number of eggs per female as a way to find out likely trade offs between immunity and reproduction. There were no significant differences in antimicrobial activity neither in the number of eggs from moths of green or black phenotype. These results indicate that, at least for the immune defence assessed here, there is no transgenerational transfer of DDP to offspring. As this is a migratory species, maybe the offspring do not experience the same environmental conditions of the parents; hence they do not transfer

immune defences to offspring by stimuli experienced by parents. It is proposed to investigate the transfer of DDP in other larval instars of offspring, through the assessing of different immune parameters, to better support our conclusion.

INTRODUCTION

Microrganisms capable of causing disease in animals are present throughout the environment, and antagonistic results of such interactions may often arise. The ecology of these interactions can present itself in many possible ways in which parasites can overcome environmental limitations, such as the seasonal time interval, (BONSALL, 2004) and therefore parasites can cause significant interference in host populations ecology (HUDSON; DOBSON; NEWBORN, 1998). The host and pathogens live in a continuous arms race, and as a result, hosts employ a range of immune defences to fight pathogen invaders, but on the other hand, pathogens use different strategies to breach the host's defences (GHOSH et al., 2011). Environmental variables may affect the result of the host-pathogen interaction, with host population density being one that deserves attention. It may affect the transmission rate of contact-transmitted pathogens within the host population, but it may also be a cue for hosts invest plastically in defences against these pathogens.

In a study by Steinhaus (1958) the risk of disease transmission increased with population density of four species of Lepidoptera. In general, group-living animals (e.g. social and gregarious insects) are more prone to contact-transmitted pathogens than solitary animals. In many host-parasite models, the the risk of pathogen transmission increases linearly with the host density (ANDERSON; MAY, 1981; REESON et al., 1998). However, in alternative models the pathogen transmission not always correlate in a positive and linear manner with the host density (BOOTS; MEALOR, 2007; WHITE; WILSON, 1999). D'Amico et al. (1996), for example, have shown that pathogen transmission decreased with the increase of population density in the gypsy moth *Lymantria dispar* (Lepidoptera: Lymantriidae). This finding and others have led a new hypothesis to explain the phenomenon, the Density-Dependent Prophylaxis hypothesis

(DDP) (WILSON; REESON, 1998).

According to the DDP hypothesis, animals subjected to fluctuations in population density have evolved plastic immune responses to to cope with the increased risk of pathogen transmission (REESON et al., 1998; WILSON; REESON, 1998). Energy resources are a limiting factor to organisms, thus investing plastically in immune defences would be a way of defend themselves when the risk of pathogen is higher or drive the energy to other life history traits, such as development and reproduction, when the risk of pathogen decreases (BOOTS; BEGON, 1993; HOSKEN, 2001).

Invertebrates have a set of innate immune defences and have no acquired immunity as known for vertebrates (STRAND, 2008), although a similar system called "immune-priming" have been discovered and investigated since the last decade (SCHMID-HEMPEL, 2005). In insects, cuticle and peritrophic matrix in the midgut are part of the immune defences working as a physical (first) barrier against pathogens (CHAPMAN, 1998). If the first barriers fail, the secondary (physiological) barriers come in action. These comprise the cellular and humoral defences, and are found inhemolymph, such as: hemocytes -cellular defences involved in the recognition of foreign bodies and microorganisms, and encapsulating these via phagocytosis (Ribeiro 2006); proteins -humoral defense with enzymatic activity (LOWENBERGER, 2001), such as pro-phenoloxidase enzyme associated melanization in tissues and foreign bodies; antimicrobial peptides, and lysozymes (CHAPMAN, 1998; NAKATSUJI; GALLO, 2012).

Lysozymes are proteins capable of reacting with high molecular weight carbohydrates present in the cell wall of Gram positive bacteria, leading to the disruption and digestion of this protective structure. These proteins are present in various types of secretions, fluids and mucus from many animals, such as in human

milk and tears. They are also found in the hemolymph and eggs of insects (MATSUURA et al., 2007). As lysozyme is easily accessed through lytic activity in bacteria-agar plates, it has being more employed in ecoimmunology studies (COTTER; KILNER, 2010).

Offsprings can be exposed to the same environmental of individuals living in high densities and this subject to the some ricks of pathogens. Bouaichi and Simpson (2003) observed in the desert locust *Schistocerca gregaria* that color, behaviour and morphology related to density-dependent phase polyphenism are passed to the offspring. Other works also indicate the passage of these features through the generations (SIMPSON; MILLER, 2007). Immune defences, however, are expensive to the organism and their transfer could cause loss of adaptive fitness in the offspring (WILSON; GRAHAM, 2015). Thus, would be DDP transfer to the offspring possible?

On one level, maternal exposure to pathogens contributes to greater immune defences for the offspring (BADYAEV; ULLER, 2009; MORET, 2006; SADD et al., 2005). This is done by stimulating the maternal immune system, antibody production and transfer to offspring via the milk and placenta in mammals. The transfer of maternal immune defences have been increasingly observed in invertebrates (BOOTS; ROBERTS, 2012). Contact with entomopathogen in the parental generation results in more resistant offsprings (FREITAK; HECKEL; VOGEL, 2009; MORET, 2006). Elthough there are situations where this cannot be done. For example, the offsprings of vector insects apparently are impacted in their fitness when the mother is stimulated by symbionts or artificially stimulated with inert particles (PIGEAULT et al., 2015; VOORDOUW; LAMBRECHTS; KOELLA, 2008).

Although the transfer mechanisms are little known, they are often associated with epigenetic (SEONG et al., 2011). The mechanism of epigenetic inheritance occurs

mainly by activation/deactivation of gene expression, but not by changes in the ADN sequence. At a greater level, the mother stimulates the defences of the offspring by compounds of accessory glands, by nutritional contribution during embryogenesis (GROSSNIKLAUS et al., 2013) or even by transferring microbial fragments (FREITAK; HECKEL; VOGEL, 2009).

Few studies have been devoted to understanding the transfer of immune defences in the context of DDP. The assumption for the occurrence of immune defences transfer is that the offspring will likely face the same environmental challenges that their parents facied, i. e. the same risk of pathogens infection due to high population density. A study of cochineal brown scale *Saissetia coffeae* showed greater resistance to pathogens in the offspring of parents which experienced high population densities (SPITZER, 2004), although not related to DDP. Other works with the locust desert *Schistocerca gregaria* and the caterpillar *Spodoptera littoralis*, which display DDP, showed an unexpected effect: offsprings of insects reared at high densities and exposed to pathogens had lower immune defences than children of lone parents (MILLER; PELL; SIMPSON, 2009; WILSON; GRAHAM, 2015).

While trying to organize the different living habits related to population density, Silva et al., (2013) proposed a continuum where one end has species of strictly solitary habit of bugs and insects as opposed to strictly gregarious. Most of the known species are on the first end and at the other end is the social insects. Between the two extremes we find species subject to fluctuations of density to high-density exposure variables levels. Extreme close strictly gregarious insects, we find e.g. the desert locusts *Schistocerca gregaria* displaying phases of high aggregation and PDD (WILSON et al., 2002). This species shows density-dependent phase polyphenism which means that according to the stage of lies, solitary or gregarious, individuals present morphological,

physiological and behavioral changes wide, an extreme form of phenotypic plasticity (SIMPSON; MCCAFFERY; HÄGELE, 1999). Miller et al. (2009) when submitting the offspring of *S. gregaria* solitary or gregarious phase to an immune challenge by applying entomopathogen spores of *Metarhizium anisopliae*, not observed more offspring of resistance gregarious individuals, contrary expected according to DDP. Nothing is known about the vertical transfer of immune defences *Anticarsia gemmatalis*, a very characteristic solitary insect, but passing by high population density outbreaks occasionally. Caterpillars have phenotypic plasticity related to density, but does not show density-dependent phase polyphenism as *S. gregaria*, which places it at the closest end to strictly solitary insects (SILVA et al., 2013).

Anticarsia gemmatalis larvae presents two phenotypes related to density and, in turn, relate to levels of immune defences. Solitary caterpillars have maintained green color while caterpillars kept with one or more conspecifics develop a black color from green. The darkening of the phenotype is explained by the greater deposition of melanin in the larval cuticle in response to the higher density. Moreover, we verified in the laboratory a gradient of intermediate stains that are observed, to a lesser extent, in larvae kept in solitary (SILVA et al., 2013). The existence of intermediate phenotypes and its unexpected manifestation for the solitary creation density, leads us to assume that the stimulus for phenotypic change might influence their parents' heritage and not only the perception of the local density. Thus, we believe that research on the occurrence of maternal effects in *A. gemmatalis* can contribute to a better understanding of density effects on the ecology study of immune defences.

This study aimed to investigate whether there are different forms of transfer of immune defences to the offspring of *A. gemmatalis* in the context of DDP. According to this hypothesis, the offspring individuals of high density, when young, have immune

defences larger than the offspring individuals of low density. As lysozyme is an important parameter for immune organisms in the early stage of development, we hope to find more lizosimas activity on egg individuals created in high density, when larvae, when comparing with eggs of individuals raised in low density. If our hypothesis is correct, a trade-off between immune investment and reproduction may exist. Thus, we infer about the reproduction in investment through the measurement of eggs produced for comparison with investment in immune defences, expressed by lizosimas activity.

MATERIALS AND METHODS

Study system: Anticarsia gemmatalis

Anticarsia gemmatalis Hübner (Lepidoptera: Noctuidae) is a moth whose larvae are agricultural pests, especially for soybean cultivation in Brazil. Their eggs have a bright green coloration, becoming darker with time, and last up to 3 days until the emergence of the larvae. During stage larvae have from 5 to 7 instars and ca. 18 days under laboratory conditions reach the pupal stage (MOSCARDI et al., 2012). Larvae have phenotypic differences related to color, which may vary from green to black with a number of intermediate phenotypes. Another observed phenotypic difference is how the immune defences, and darker color phenotypes have higher immune defences in relation to green color phenotypes. Pupae have initially green color after ecdise and soon become brown. Nine days are needed until the emergence of the moths.

Silva et al., (2013) investigated the hypothesis DDP in the *A. gemmatalis*. The larvae may occasionally found at high density, though not strictly live gregarious. The authors noted that changes in color phenotype occurs even in larvae in contact with only one conspecific. This phenotypic change is usually evident from the third instar. The green phenotype is more frequent in solitary caterpillars while the black phenotype is more frequent in larvae reared at higher densities. Between the extremes of green and black coloring, several intermediate phenotypes occur. In addition, unexpected phenotypes are found less frequently (i.e. green at high densities, black at low densities and intermediate at both densities).

Insect colony

Anticarsia gemmatalis colony was established in the Laboratory of Insect-Microbe Interactions at the Universidade Federal de Viçosa in 2013 with insects from the Laboratory of Biological Control at the EMBRAPA/CNPMS. These insects are kept in the laboratory at controlled conditions of 25±5 °C temperature, 60±5% relative humidity and 12:12h light cycle – methodology adapted from Hoffmann-Campo et al. (1985). The moths were housed in groups of ca. 80 pairs in wooden cages (measuring 30 x 30 x 30 cm) lined with paper sheets (where oviposition occurs) and fed *ad. libitum* with an artificial diet consisting of honey, beer, saccharose, ascorbic acid and nipagin. Adults live in average 15 days and present colour varying from gray to brown, with low level of sexual dimorphism (ANDRADE; NEGREIRO; FALLEIROS, 2004). Mating behaviours and oviposition occur in scotophase, with females mating ca. 5 times more than males during their lifetime (HOFFMANN-CAMPO et al., 2000; LEPPLA, 1976). The eggs were collected every 48h and kept in plastic pots (1L) containing plugs of artificial diet (mainly composed of textured soy protein, bean, wheat germ, beer yeast, casein and vitamin solution) until they hatched. Upon hatching, larvae were transferred to plastic pots (100 mL) in densities of 1 or 4 individuals per pot. These population densities are known to trigger colour phenotypic changes, i.e. lone-reared caterpillars are more prone to exhibit the green phenotype while group-reared caterpillars are more likely to exhibit the black phenotype (see Silva et al., 2013).

Treatments

The antimicrobial activity in *A. gemmatalis* eggs was assessed in order to test the hypothesis of immune defences transfer from parents displaying the DDP. First, newly-hatched larvae were kept in opaque plastic pots (100 mL) at densities of 1 or 4 individuals per pot, and fed *ad libitum* with artificial diet until pupation. At the 5th

instar, larvae were classified visually according to colour phenotype (i.e. green or black). As it was a subjective process, only one person classified the larvae according to their colours. To check the reliability of the visual selection, 50 larvae of the two phenotypes were randomly selected in the colony, and had their dorsal region photographed and analysed on the ImageJ software for colour differences (Fig. 1); average colour of green 130,97 ± 1,95 and black 179,57 ± 0,74 phenotypes ($F_{1,98}$=544,38; p<0,001).

Larvae were kept at these density conditions so that we could get moths with green or black “phenotypes”. After pupating, the insects were sexed and combined in 25 moths pairs in plastic cages (25 X 18 cm, height / diameter) following the phenotype treatments: female green x male green, female green x male black, female black x male green and female black x male black. By combining moths in this way we could get information whether they transfer immune factors to the eggs, and hence to the offspring also what was the contribution of each partner in the cooperation. Moths remained in the cages for 5 days so that they could mate. After this, moths were individualized in 100 mL opaque plastic pots containing artificial diet. Eggs deposited on the pot walls were collected with the aid of a fine brush every 36 hours and stored at -10 °C in the freezer until samples of 100 eggs per female (minimum amount in which was possible to assess the lytic activity) were obtained. Here, we assessed the following parameters in moths regarding their reproductive and immune investment: total number of eggs, lytic activity in the eggs and female size (body length/width mesothorax). After death, moths were promptly stored in the fridge at 5 °C for later abdomen dissection and counting of the number of spermatophores. The number of spermatophores is associated with the mating numbers that each female had; males use only one spermatophore per mating and spend ca. 24 hours to fill another spermatophore (CHAPMAN, 1998).

Lytic activity in eggs

Lytic activity in eggs was assessed by measuring the inhibition zones formed in agar plates containing the bacteria *Micrococcus lysodeikticus* ATCC 4698 (COTTER; KILNER, 2010). Plates were prepared with 1,5% agar, 0,75g of *M. lysodeikticus* in lyophilized form, 100 mL distilled water and 50 mL of potassium phosphate buffer 2M (pH 6,4). Holes were punched with the aid of glass capillary tubes (1,5 mm diameter) after hardening of the agar. Plates were incubated overnight at 33 °C, and the contaminated ones discarded. Egg samples per female were macerated with 1,2 µL PBS buffer in Eppendorf tubes (1,5 mL) with the aid of pistils. The tubes were then centrifuged for 10 seconds so that the solid material could sit at the bottom. Two aliquots of 1,2 µL per sample were pipetted into the holes of agar plates. Plates were incubated at 33 °C for 24 hours and then photographed. The pictures were adjusted with 50% more contrast for better accuracy in measurements of inhibition zones. The areas of clear zones formed in the vicinity of the holes were measured with the aid of ImageJ software. In this method, the clear area formed is positively correlated to the lysozyme concentration in the egg samples.

Image analysis

Clear zones were not completely translucent and the edges were not perfectly regular as a circle (Fig. 1-a). This could have led to loss of information it only the diameter had been measured. To ensure higher quality data, we chose to transform the data by calculating the relative area in accordance to the original clear zone area, but with the equivalent total translucency.

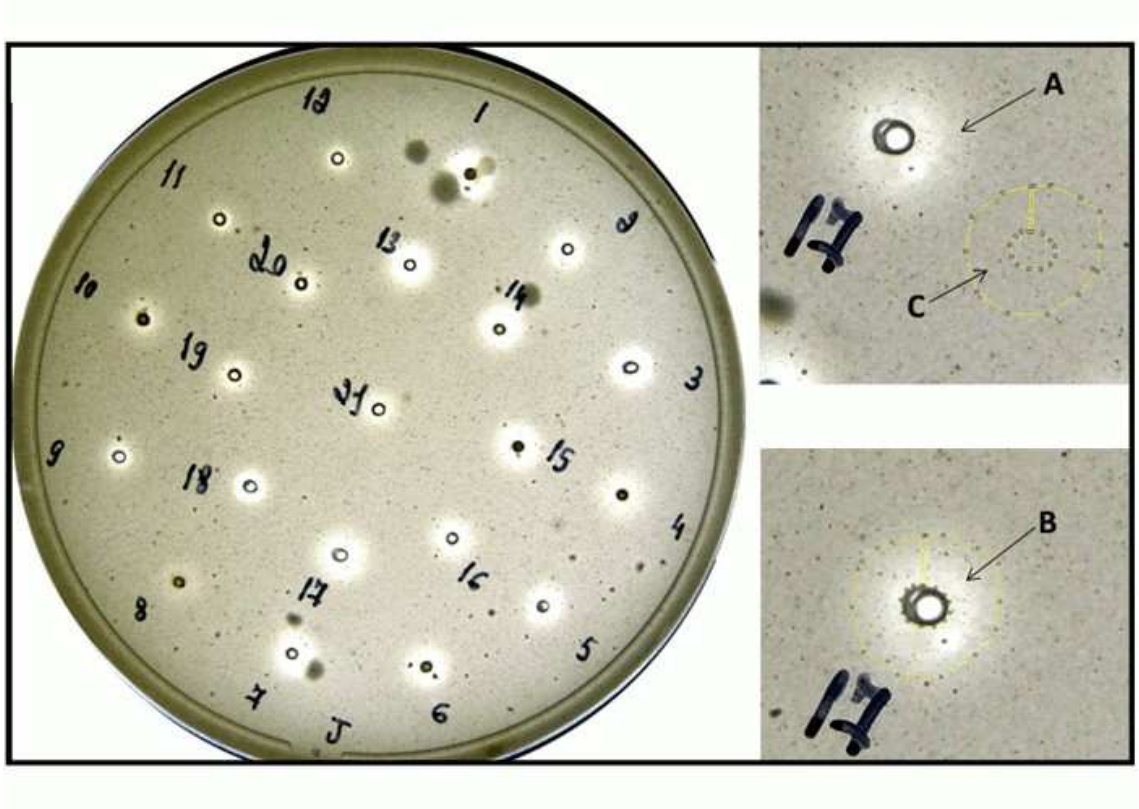

Figure 1. Clear zone by lysozyme activity in a sample of macerated eggs deposited in the Petri dish containing *Micrococcus lysodeikticus* ATCC 4698. Detail of irregular and partially translucent edges (a), border region considered positive for lysozyme activity (b) and border regions considered negative for lysozyme activity (c).

The regions near clear zones with some degree of translucency were considered positive areas of lysozyme activity (Fig. 1-b). Average gray values and the area of these regions were collected. The two opposing regions surrounding the clear area, outside the region considered positive, had their average values of gray measured and used as reference for the negative region of action of lysozyme (Fig. 1-c). ImageJ software gives gray values ranging from 0 (black) to 255 (white). Here, the gray scale would have a limit of less than average gray negative region and a threshold limit of 255 (equivalent to totally translucent region). Data were combined (Eq. 1) to generate a

translucency coefficient corresponding to the ratio between the measured average gray value in relation to the possible average value of gray. This coefficient multiplied by the area of the positive region gives the relative area with total translucency (Eq. 2). Finally, the relative area was converted to diameters to eliminate the quadratic component and allowing data to present normal distribution (Eq. 3).

Equation 1: $CT = (A - B) / (255 - B)$

Equation 2: $AR = AM * CT$

Equation 3: $DR = \sqrt{(4 * AR / \pi)}$

Where:

CT: translucency coefficient (proportion);

A: average gray value of the positive region for lysozyme activity (artificial unity);

B: average gray values of the negative region for lysozyme activity (artificial unit);

AR: relative area, with total translucency (mm^2);

AM: area as the positive region for lysozyme activity (mm^2);

DR: relative diameter converted from the relative area (mm).

Statistical procedures

Analyses were performed using the linear models in R software. Adequacy of the models was checked by residue analysis and dispersion of data. Most appropriate data distribution was normal for all data. All models built were simplified by the removal of statistically non-significant terms; the same was done with the body size

variable (ratio between body length and width of the mesothorax).

Visually assessed larval phenotype was confronted with gray scale measured on photographs of the larvae (standardized region) by Analysis of Variance (ANOVA). Variable dependent was average gray (artificial unit) and the independent variable is the larval phenotype visually labeled (green and black).

Lytic activity on eggs produced and the cumulative oviposition from the mating of four possible different couples (combination of larval phenotypes of male and female) was measured by ANOVA. Dependent variable was the relative diameter of the inhibition zones and cumulative oviposition. Independet variable is the larval phenotype of couples (green X green; green X black; black X black; black X green – female X male, respectively).

Lytic activity on eggs produced and the cumulative oviposition of females grouped according to the status of copulation (copulated and non-copulated) was measured by ANOVA.

The relationship between lytic activity of eggs (dependent variable) and total oviposition (independent variable) was verified by analysis of covariance, and the covariates were: mating status, body size and the phenotype of parent couples.

RESULTS

Parental phenotype

Lytic activity of eggs showed no significant differences in relation to larval phenotype of the parents ($F_{3,131}$=0,16; p=0,93) (Fig. 2), even when analyzed in conjunction with the female mating status ($F_{7,127}$=0,57; p=0,66) (Fig. 3). Average diameter of inhibition zones for each phenotype of parents who produced the eggs: green X green (2,76 ± 0,14), green X black (2,86 ± 0,16), black X black (2,77 ± 0,12) and black X green (2,86 ± 0,15) (female X male, respectively).

Accumulated oviposition showed no significant differences in relation to larval phenotype of the parents ($F_{3,131}$=0,88; p=0,45) (Fig. 4), even when analyzed in conjunction with the female mating status ($F_{7,127}$=1,49; p=0,24) (Fig. 5). Average number of eggs for each phenotype of parents who produced the eggs: green X green (403,11 ± 38,42), green X black (374,86 ± 27,35), black X black (347,47 ± 20,83) and black X green (385,79 ± 30,41) (female X male, respectively).

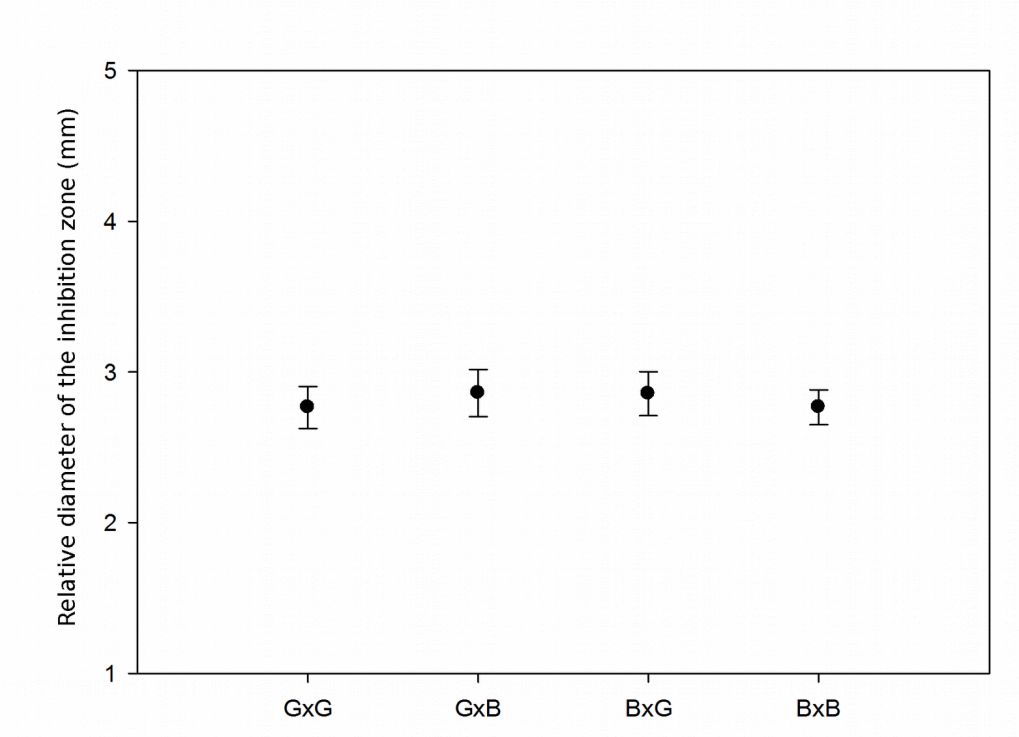

Figure 2. Lytic activity in eggs per couple parent (phenotypes green (G) and black (B); female X male, respectively).

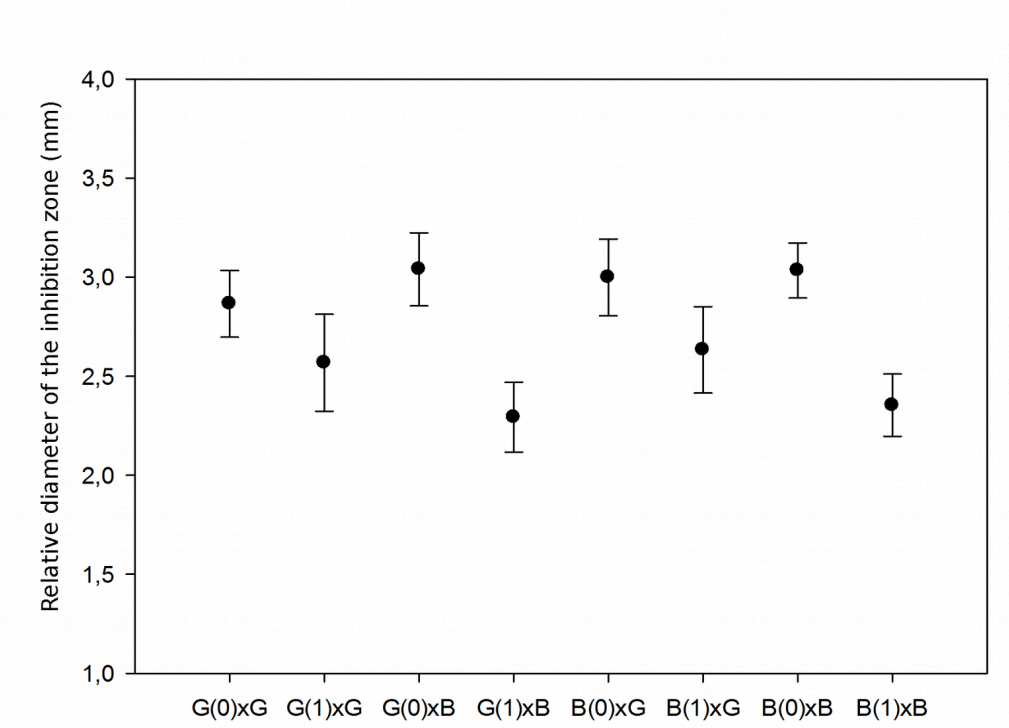

Figure 3. Lytic activity in eggs per couple parent and mating status (phenotypes green (G) and black (B); female X male, respectively; 0 no-mated, 1 mated).

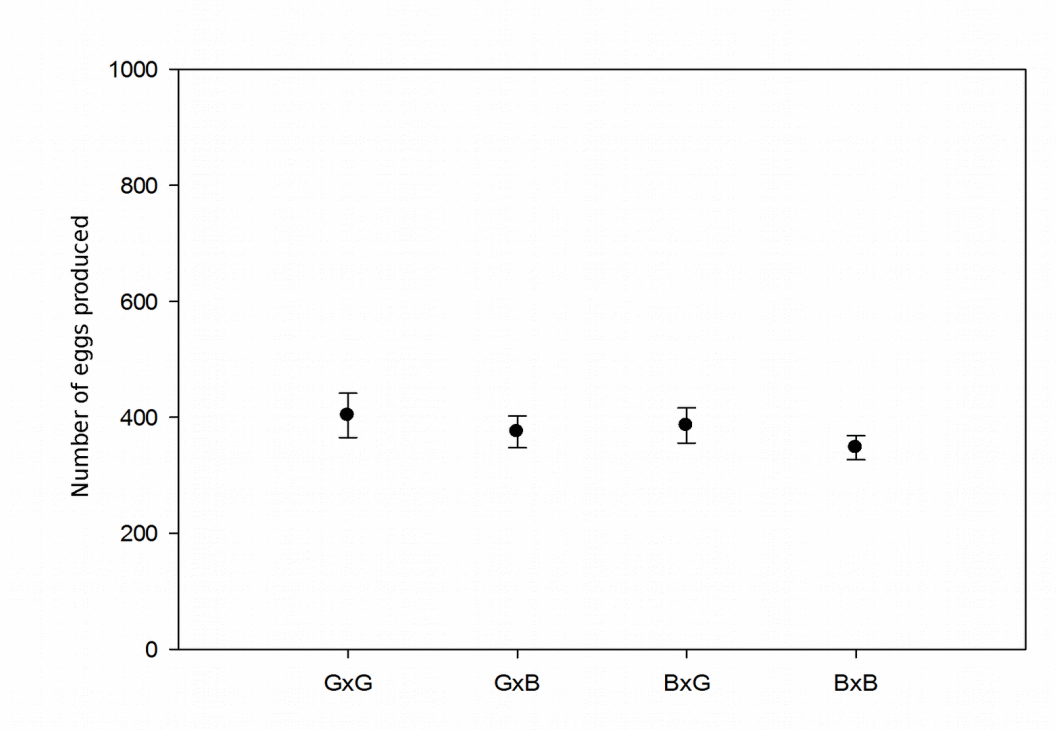

Figure 4. Eggs produced per couple parent (phenotypes green (G) and black (B); female X male, respectively).

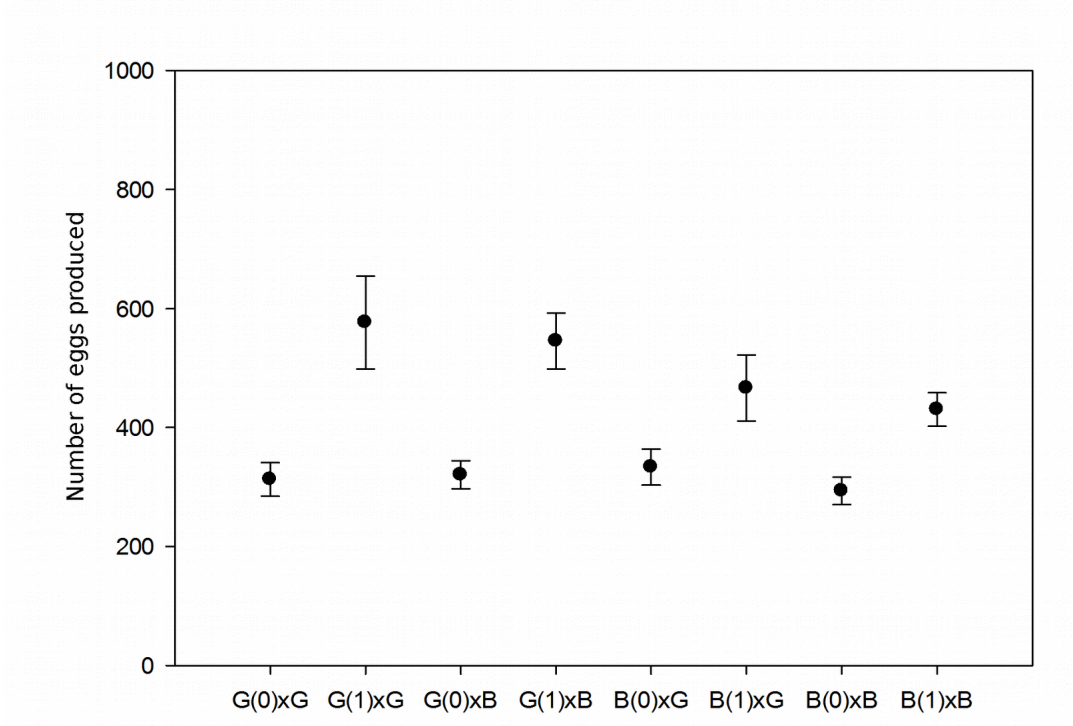

Figure 5. Eggs produced per couple parent and mating status (phenotypes green (G) and black (B); female X male, respectively; 0 no-mated, 1 mated).

Copulation status

Eggs produced by non-copulated females showed greater lytic activity than eggs produced by females copulated ($F_{1,133}$=13,55; p<0,001) (Fig. 6). Average diameter of the inhibition zones depending on the mating status: non-copulated (2,87 ± 0,09) and copulated (2,70 ± 0,11).

Accumulated oviposition of non-copulated females was lower than the oviposition by females copulated ($F_{1,133}$=43,89; p<0,001) (Fig. 7). Average number of eggs produced depending on the mating status: non-copulated (321,47 ± 6,80) and copulated (497,45 ± 21,56).

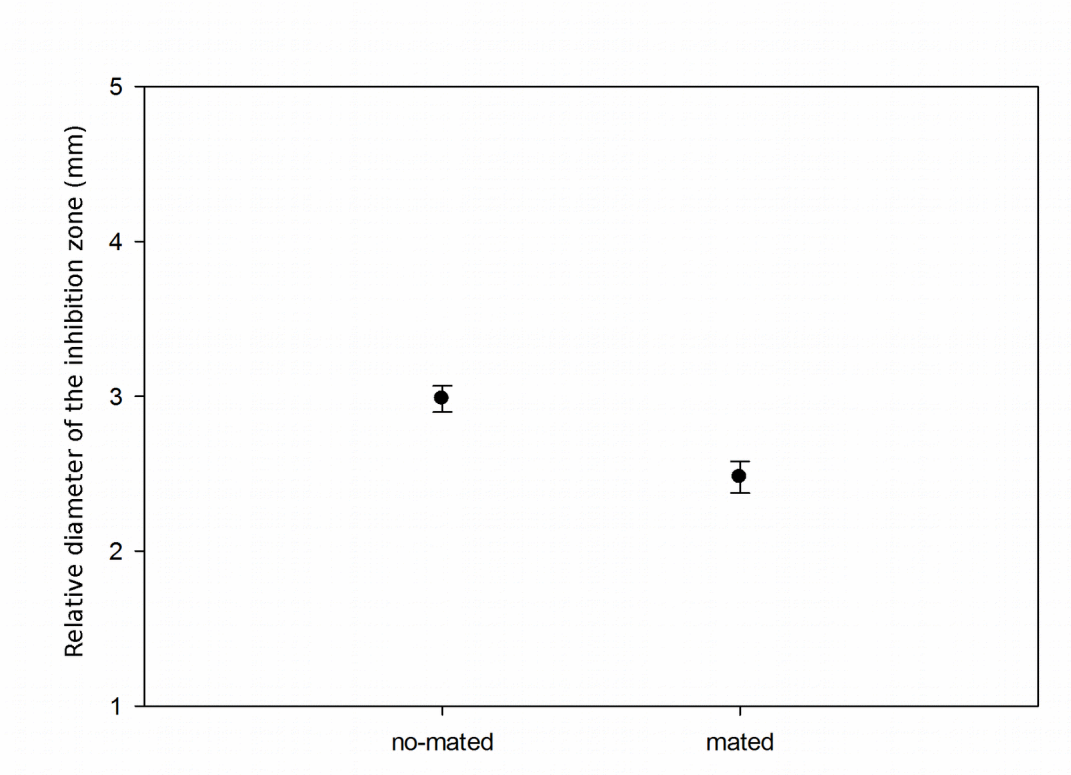

Figure 6. Lytic activity in eggs produced by females non-copulated and copulated.

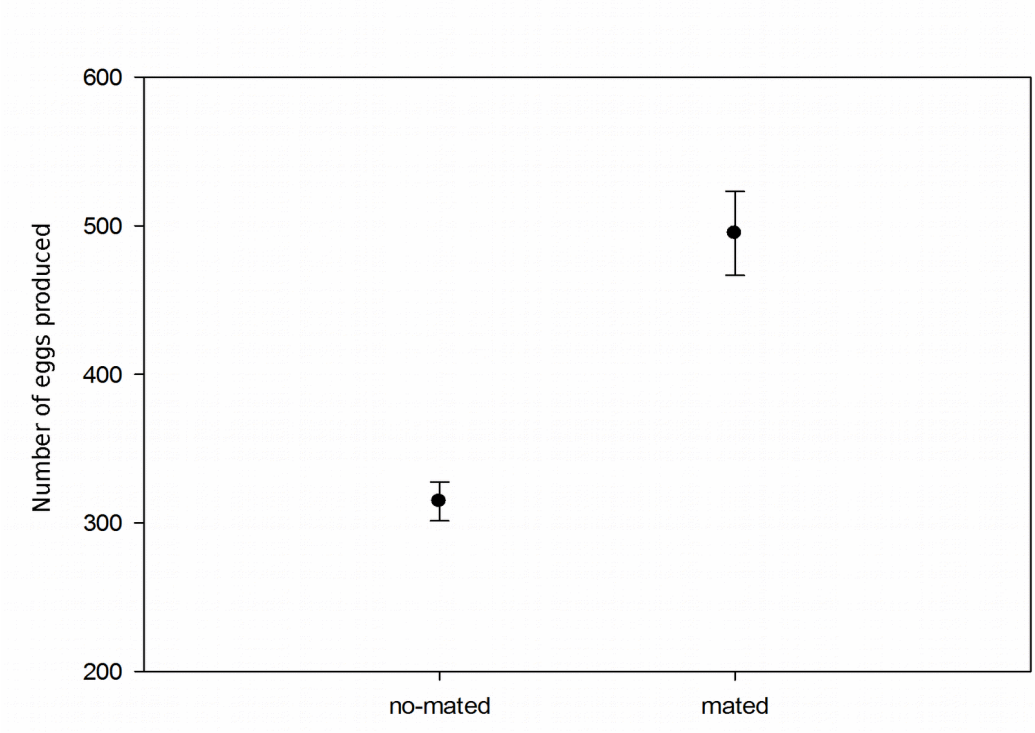

Figure 7. Eggs number produced by females non-copulated and copulated.

Lytic activity and oviposition

Lytic activity decrease with increase of number of eggs ($F_{7,131}$=7,46; p=0,007) and this was determined for female mating status ($F_{7,131}$=7,16; p=0,008). Females do non-copulated had higher rates than females copulated (Fig. 8).

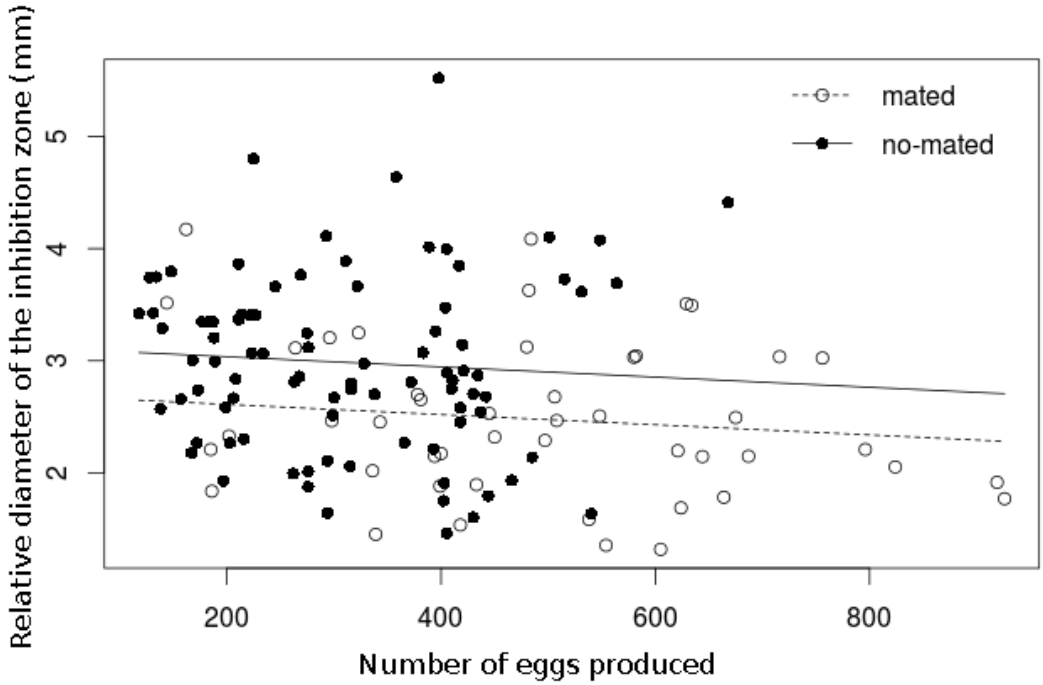

Figure 8. Lytic activity related to the number of eggs produced for females copulated and non-copulated.

DISCUSSION

To answer the question *is DDP transmitted to offspring in Anticarsia gemmatalis?* we hypothesized that it would be transmitted from laboratory observations. We saw sporadically solitary larvae exhibiting an intermediate phenotype. We create the assumption that larvae were inherited traits gregarious through one or both parents. To test this hypothesis, we assessed an immune response (i.e. lytic activity) in eggs of parents came from caterpillars with known phenotype and larval density. We have shown that at least the immune factor assessed here is not transferred from parents to offsprings via eggs. Furthermore, the lack of immune transfer is not a matter of parental "phenotypes", as in all parental combinations the lytic activity in eggs was the same, this if females are mated or not to "black" or "green" males.

It is expected that in insects the density-dependent defences be transferred from parents to offsprings when they share a similar environment (e.g. population density and the risk of pathogens transmission) during their lifetime (BOUAÏCHI; SIMPSON, 2003). This may be more expected for insects that are subject to fluctuations in population density, such as *A. gemmatalis*. Thus, the transfer of immune defences from parentes to offsprings would be advantageous in terms of fitness, as these would be prepared to face possible pathogen treats in the environment (MAENO; TANAKA, 2009). But watching other *A. gemmatalis* biology characteristics can question whether the fact that offspring of the environment will be similar to the parents. First, because it was reported that adults have high migratory capacity (FESCEMYER; HAMMOND, 1988; SOSA-GÓMEZ, 2004) and could use density as a signal to emigrate. Thus avoid the increased risk of disease transmission and also the negative effects of the competition for resources intraspecific. Second, because it is a holometabolous insect, adults do not share in full the same ecological niche of offspring (GULLAN;

CRANSTON, 2007). In terms of lifetime, it is plausible to assume that individuals of *A. gemmatalis* spend less time in contact with juvenile conspecifics than the locust desert *Schistocerca gregaria*, a hemimetabolous whose adults share the same type of food nymphs. This lower exposure could reduce the risk of pathogen transmission. *S. gregaria* females deposit eggs next to a chemical compound that stimulates the offspring become gregarious from the contact in the first instar life (SIMPSON; MILLER, 2007), a way to transfer DDP. Two characteristics of *A. gemmatalis* highlighted, migratory capacity and fact of belonging to holometabolous, cast doubts the assumption that the offspring may experience the risks of parental environment. So our main result can provide a useful guide to a better understanding of DDP transfer to insects with characteristics similar to *A. gemmatalis*.

We also assessed a reproductive parameter, the number of eggs per female, to investigate a possible trade-off between imune investment and egg's production. For not having also found significant differences between the number of eggs produced for each treatment - combination of couples with known phenotype and larval density, it was not possible to analyze this trade-off in the context of DDP, as initially proposed. In the same way we do with lysozyme activity, better detail the treatments, separating females copulated and non-copulated, and found the same negative result.

We grouped the data on lysozyme activity and the number of eggs according to the mating status with the aim to know more about the reproductive biology of *A. gemmatalis* and try to understand our main results. It was found a lower lytic activity in eggs from mated females than in eggs from unmated females, likely due to reallocation of resources during egg production. Some organisms have the strategy of investing energy resources to produce more offsprings but with lower quality (BEGON; TOWNSEND; HARPER, 2007). Most egg production by females copulated can be

explained by energy resources and stimulating the male transfers to female during copulation (SVÄRD; WIKLUND, 1988). Oviposition non-copulated females it is due to the steady stream of maturation oocytes (PAPAJ, 2000). Despite there being a control of maturation via hormones to wait for the perfect time - after copulation and by the host plant, oviposition can not be contained permanently (GILLOTT, 2005).

We found a significant correlation between lysozyme activity and number of eggs produced for data grouped by mating status which indicates the existence of a trade-off between reproduction and immune defences, but outside the context of DDP. Immune defences require significant resources and can be better used in a numerous offspring, but less resistant, depending on the reproductive strategy adopted (STAUDACHER; MENKEN; GROOT, 2015).

Although our results indicate that there is no transfer of DDP to the offspring, this hypothesis must not be abandoned. We chose to work with eggs because we believe that its characteristic immobility and consequent lack of interaction with conspecifics, it would be appropriate to eliminate any influence on your immune defences that were not inherited by parents. However, stimulated immune defences can manifest later. In *Drosophila melanogaster*, the expression of antimicrobial peptides was observed from the third larval instar (TAPADIA; VERMA, 2012). Therefore, we propose to investigate the hypothesis during the different stages of *A. gemmatalis* development, including evaluation of other immune parameters was the hemocytes density, melanization and encapsulation of foreign bodies.

Another way to test the DDP hypothesis, in the context of vertical immune transfer, would be through challenge a pathogen challenge in offsprings from parents raised, when larvae, in different population densities, as in the study by Wilson et al. (2015). This approach is interesting for challenging offspring in a situation where all the

immune defences as a whole is put to the test.

While caution is required when interpreting negative evidence, our work indicates that the DDP is not transferred to offspring of *A. gemmatalis* and constitutes a starting point for investigations with different approaches.

REFERENCES

ANDERSON, R. M.; MAY, R. M. The population dynamics of microparasites and their invertebrate hosts. **Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences**, v. 291, n. 1054, p. 451–524, 1981.

ANDRADE, F. G.; NEGREIRO, M. C. C.; FALLEIROS, Â. M. F. Aspectos dos mecanismos de defesa da lagarta da soja *Anticarsia gemmatalis* (Hübner, 1818) relacionados ao controle biológico por baculovirus Anticarsia (AgMNPV). **Arquivos do Instituto Biológico**, v. 71, n. 3, p. 391–398, 2004.

BADYAEV, A. V; ULLER, T. Parental effects in ecology and evolution: mechanisms, processes and implications. **Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences**, v. 364, n. 1520, p. 1169–1177, 2009.

BEGON, M.; TOWNSEND, C. R.; HARPER, J. L. **Ecologia de Indivíduos a Ecossistemas**. 4. ed. Porto Alegre: Artmed, 2007.

BONSALL, M. B. The impact of diseases and pathogens on insect population dynamics. **Physiological Entomology**, v. 29, n. 3, p. 223–236, 2004.

BOOTS, M.; BEGON, M. Trade-offs with resistence to a granulosis virus in the Indian meal moth, examined by a laboratory evolution experiment. **Functional Ecology**, v. 7, n. 5, p. 528–534, 1993.

BOOTS, M.; MEALOR, M. Local interactions select for lower pathogen infectivity. **Science**, v. 315, n. 5816, p. 1284–1286, 2007.

BOOTS, M.; ROBERTS, K. E. Maternal effects in disease resistance: poor maternal environment increases offspring resistance to an insect virus. **Proceedings of the Royal Society. Series B: Biological Sciences**, v. 279, n. 1744, p. 4009–4014, 2012.

BOUAÏCHI, A.; SIMPSON, S. J. Density-dependent accumulation of phase characteristics in a natural population of the desert locust *Schistocerca gregaria*. **Physiological Entomology**, v. 28, n. 1, p. 25–31, 2003.

CHAPMAN, R. F. **The insects. Structure and function**. 4. ed. Cambridge: Cambridge University Press, 1998.

COTTER, S. C.; KILNER, R. M. Sexual division of antibacterial resource defence in breeding burying beetles, *Nicrophorus vespilloides*. **Journal of Animal Ecology**, v. 79, n. 1, p. 35–43, 2010.

D'AMICO, V.; ELKINTON, S.; DWYER, G.; BURAND, J. P. Virus transmission in gypsy moths is not a simple mass action process. **Ecology**, v. 77, n. 1, p. 201–206, 1996.

FESCEMYER, H. W.; HAMMOND, A. M. Effect of larval density and plant age on size and biochemical composition of adult migrant moths, *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae). **Environmental Entomology**, v. 17, n. 2, p. 213–219, 1988.

FREITAK, D.; HECKEL, D. G.; VOGEL, H. Dietary-dependent trans-generational immune priming in an insect herbivore. **Proceedings of the Royal Society. Series B: Biological Sciences**, v. 276, n. 1667, p. 2617–2624, 2009.

GHOSH, J.; LUN, C. M.; MAJESKE, A. J.; SACCHI, S.; SCHRANKEL, C. S.; SMITH, L. C. Invertebrate immune diversity. **Developmental and Comparative Immunology**, v. 35, n. 9, p. 959–974, 2011.

GILLOTT, C. **Entomology**. 3. ed. Netherlands: Springer, 2005.

GROSSNIKLAUS, U.; KELLY, W. G.; FERGUSON-SMITH, A. C.; PEMBREY, M.; LINDQUIST, S. Transgenerational epigenetic inheritance: how important is it? **Nature Reviews. Genetics**, v. 14, n. 3, p. 228–235, 2013.

GULLAN, P. J.; CRANSTON, P. S. **Os insetos: um resumo de entomologia**. 4. ed. São Paulo: Roca, 2012.

HOFFMANN-CAMPO, C. B.; MOSCARDI, F.; OLIVEIRA, L. J.; SOSA-GÓMEZ, D. R.; PANIZZI, A. R.; CORSO, I. C.; OLIVEIRA, E. B. **Pragas da soja no Brasil e seu manejo integradoEmbrapa Soja. Circular Técnica, 30**, Embrapa, 2000.

HOFFMANN-CAMPO, C. B.; OLIVEIRA, E. B. DE; MOSCARDI, F. **Criação massal da lagarta da soja.** Embrapa, 1985.

HOSKEN, D. J. Sex and death: microevolutionary trade-offs between reproductive and immune investment in dung flies. **Current Biology**, v. 11, n. 10, p. 379–380, 2001.

HUDSON, P. J.; DOBSON, A. P.; NEWBORN, D. Prevention of population cycles by parasite removal. **Science**, v. 282, n. 5397, p. 2256–2258, 1998.

LEPPLA, N. C. Circadian rhythms of locomotion and reproductive behaviour in adult velvetbean caterpillars. **Annals of the Entomological Society of America**, v. 69, n. 1, p. 45–48, 1976.

LOWENBERGER, C. Innate immune response of *Aedes aegypti*. **Insect Biochemistry and Molecular Biology**, v. 31, n. 3, p. 219–229, 2001.

MAENO, K.; TANAKA, S. The trans-generational phase accumulation in the desert locust: morphometric changes and extra molting. **Journal of Insect Physiology**, v. 55, n. 11, p. 1013–1020, 2009.

MATSUURA, K.; TAMURA, T.; KOBAYASHI, N.; YASHIRO, T.; TATSUMI, S. The antibacterial protein lysozyme identified as the termite egg recognition pheromone. **PloS one**, v. 2, n. 8, p. e813, 2007.

MILLER, G. A.; PELL, J. K.; SIMPSON, S. J. Crowded locusts produce hatchlings vulnerable to fungal attack. **Biology letters**, v. 5, n. 6, p. 845–848, 2009.

MORET, Y. “Trans-generational immune priming”: specific enhancement of the antimicrobial immune response in the mealworm beetle, *Tenebrio molitor*. **Proceedings of the Royal Society B: Biological Sciences**, v. 273, n. 1592, p. 1399–1405, 2006.

MOSCARDI, F. et al. Artrópodes que atacam as folhas da soja. In: HOFFMANN-CAMPO, C. B.; CORRÊA-FERREIRA, B. S.; MOSCARDI, F. (Eds.). . **Soja: Manejo Integrado de Insetos e outros Artrópodes-Praga**. 1. ed. Brasília: Embrapa, 860 p., 2012.

NAKATSUJI, T.; GALLO, R. L. Antimicrobial peptides: old molecules with new ideas. **Journal of Investigative Dermatology**, v. 132, n. 3, p. 887–895, 2012.

PAPAJ, D. R. Ovarian dynamics and host use. **Annual Review of Entomology**, v. 45, n. 1, p. 423–448, 2000.

PIGEAULT, R.; VÉZILIER, J.; NICOT, A.; GANDON, S.; RIVERO, A. Transgenerational effect of infection in *Plasmodium*-infected mosquitoes. **Biology Letters**, v. 11, n. 3, p. 1–4, 2015.

REESON, A. F.; WILSON, K.; GUNN, A.; HAILS, R. S.; GOULSON, D. Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density. **Proceedings of the Royal Society B: Biological Sciences**, v. 265, n. 1407, p. 1787–1791, 1998.

SADD, B. M.; KLEINLOGEL, Y.; SCHMID-HEMPEL, R.; SCHMID-HEMPEL, P. Trans-generational immune priming in a social insect. **Biology Letters**, v. 1, n. 4, p. 386–388, 2005.

SCHMID-HEMPEL, P. Evolutionary ecology of insect immune defenses. **Annual Review of Entomology**, v. 50, n. 22, p. 529–51, jan. 2005.

SEONG, K. H.; LI, D.; SHIMIZU, H.; NAKAMURA, R.; ISHII, S. Inheritance of stress-induced, ATF-2-dependent epigenetic change. **Cell**, v. 145, n. 7, p. 1049–1061, 2011.

SILVA, F. W. S.; VIOL, D. L.; FARIA, S. V.; LIMA, E.; VALICENTE, F. H.; ELLIOT, S. L. Two’s a crowd: phenotypic adjustments and prophylaxis in *Anticarsia gemmatalis* larvae are triggered by the presence of conspecifics. **PloS one**, v. 8, n. 4, p. e61582, jan. 2013.

SIMPSON, S. J.; MCCAFFERY, A. R.; HÄGELE, B. F. A behavioural analysis of phase change in the desert locust. **Biological Reviews**, v. 74, n. 4, p. 461–480, 1999.

SIMPSON, S. J.; MILLER, G. A. Maternal effects on phase characteristics in the desert locust, *Schistocerca gregaria*: a review of current understanding. **Journal of Insect Physiology**, v. 53, n. 9, p. 869–876, 2007.

SOSA-GÓMEZ, D. R. Intraspecific variation and population structure of the Velvetbean Caterpillar, *Anticarsia gemmatalis* Hübner, 1818 (Insecta: Lepidoptera: Noctuidae). **Genetics and Molecular Biology**, v. 27, n. 3, p. 378–384, 2004.

SPITZER, B. W. Maternal effects in the soft scale insect *Saisetia coffeae* (Hemiptera: Coccidae). **Evolution**, v. 58, n. 11, p. 2452–2461, 2004.

STAUDACHER, H.; MENKEN, S. B. J.; GROOT, A. T. Effects of immune challenge on the oviposition strategy of a noctuid moth. **Journal of Evolutionary Biology**, v. 31, n. 8, p. 1568–1577, 2015.

STEINHAUS, E. A. Crowding as a possible stress factor in insect disease. **Ecology**, v. 39, n. 3, p. 503–514, 1958.

STRAND, M. R. The insect cellular immune response. **Insect Science**, v. 15, n. 1, p. 1–14, 2008.

SVÄRD, L.; WIKLUND, C. Fecundity, egg weight and longevity in relation to multiple matings in females of the monarch butterfly. **Behavioral Ecology and Sociobiology**, v. 23, n. 1, p. 39–43, 1988.

TAPADIA, M. G.; VERMA, P. Immune response and anti-microbial peptides expression in malpighian tubules of *Drosophila melanogaster* is under developmental regulation. **PLoS ONE**, v. 7, n. 7, p. e40714, 2012.

VOORDOUW, M. J.; LAMBRECHTS, L.; KOELLA, J. No maternal effects after stimulation of the melanization response in the yellow fever mosquito *Aedes aegypti*. **Oikos**, v. 117, n. 8, p. 1269–1279, 2008.

WHITE, K. A. J.; WILSON, K. Modelling density-dependent resistance in insect-pathogen interactions. **Theoretical Population Biology**, v. 56, n. 2, p. 163–181, 1999.

WILSON, K.; THOMAS, M. B.; BLANFORD, S.; DOGGETT, M.; SIMPSON, S. J.; MOORE, S. L. Coping with crowds: density-dependent disease resistance in desert locusts. **Proceedings of the National Academy of Sciences of the United States of America**, v. 99, n. 8, p. 5471–5, 16 abr. 2002.

WILSON, K.; GRAHAM, R. I. Transgenerational effects modulate density-dependent prophylactic resistance to viral infection in a lepidopteran pest. **Biology Letters**, v. 11, n. 3, p. 1–4, 2015.

WILSON, K.; REESON, A. Density-dependent prophylaxis: evidence from Lepidoptera–baculovirus interactions? **Ecological Entomology**, p. 100–101, 1998.