TALITTA GUIMARÃES SIMÕES

DENSITY-DEPENDENT PROPHYLACTIC IMMUNITY IN SOCIAL INSECTS: behavioural and physiological components

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

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Eraldo Rodrigues de Lima

José Henrique Schoereder

Tathiana Guerra Sobrinho

Paulo Fellipe Cristaldo

Simon Luke Elliot
(Orientador)

“Se pensarmos pequeno, coisas pequenas teremos.
Mas se desejarmos fortemente o melhor e,
principalmente, lutarmos pelo melhor,
o melhor vai se instalar em nossa vida.
Porque sou do tamanho daquilo que vejo,
e não do tamanho da minha altura.”

Carlos Drummond de Andrade

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ABSTRACT

SIMÕES, Talitta Guimarães, D.Sc., Universidade Federal de Viçosa, November, 2014. **Density-dependent prophylactic immunity in social insects: behavioural and physiological components.** Adviser: Simon Luke Elliot.

The Density-Dependent Prophylaxis (DDP) hypothesis predicts that organisms can invest more in immunological defence when in high densities. In insects, this hypothesis has been confirmed for many groups. However, most of these studies did not show an overall increase in analysed defence mechanisms and they did not analysed behavioural defences. On the first chapter, we discuss how DDP could have emerged and fixed in different groups, in accord with the degree of sociality. We then propose the hypothesis that there should be changes on DDP investment along a continuum that ranges from strictly solitary insects to eusocial insects. Thus, strictly solitary insects did not develop any plastic response of immune system (neither physiological nor behavioural) to an artificial density increase; insects that eventually undergo natural density variations can show plastic response of immune system, however, manifested only in physiological responses to protect themselves; insects that necessarily experiment natural changes in density (i.e. gregarious insects) must show plastic responses, manifested in physiological defence, but occasionally in behavioural also; and insects with high density as a predominant character along its life (i.e. eusocial insects) must show plastic response, manifested in behavioural responses, to protect the colony as a hole. Chapter 2 tests this hypothesis in an eusocial bee, *Melipona quadrifasciata*, analysing if DDP response to artificial density manipulation and if that is expressed on investment in physiological or behavioural defence. For this, workers recently emerged were kept alone, with one other nestmate or in high density for 10 days. Per capita interactions were analysed to estimate the connectivity between individuals, showing that connectivity was related to density. Two parameters in physiological immune system were tested, encapsulation response and haemocyte density in haemolymph, not finding differences between individuals kept in different density/connectivity treatments for any of these parameters. To analyse DDP investment related to behavioural defence we exposed the bees kept in different densities to another nestmate contaminated with *Beauveria bassiana* spores. Bees kept in high density/connectivity were extremely aggressive with the contaminated bee, many times killing it. This proved to be an important defence mechanism at colony level. On the other hand, bees kept alone or with only another nestmate did not show effective behavioural defence. We conclude that *Melipona quadrifasciata* shows DDP, investing in behavioural defence, in accord with our hypothesis exposed on the first chapter.

RESUMO

SIMÕES, Talitta Guimarães, D.Sc., Universidade Federal de Viçosa, novembro de 2014. **Imunidade profilática dependente da densidade em insetos sociais: componentes comportamentais e fisiológicos.** Orientador: Simon Luke Elliot.

A hipótese da profilaxia dependente da densidade (DDP, por suas iniciais em inglês) prediz que organismos devem investir mais em defesa imunológica quando se encontram em altas densidades. Em insetos esta hipótese tem sido confirmada. No entanto, a maioria desses estudos não mostra um aumento geral em todos os mecanismos de defesa do sistema imunológico analisados e não analisam quaisquer mecanismos de defesa comportamentais. No primeiro capitulo deste trabalho nós discutimos como a DDP poderia ter surgido e se fixado em diferentes grupos, de acordo com o grau de socialidade, e propomos a hipótese de que devem ocorrer mudanças no investimento em DDP ao longo de um continnuum que vai desde insetos estritamente solitários até insetos eussociais. Assim, insetos estritamente solitários, não devem desenvolver nenhuma resposta plástica do sistema imune (nem fisiológica nem comportamental) a um aumento artificial de densidade; insetos que, eventualmente, passam por variações naturais na densidadade podem mostrar resposta plástica do sistema imune, no entanto, manifestando-se apenas em respostas fisiológicas para proteger a si mesmo; insetos que necessariamente experimentam variações naturais na densidade (como insetos gregários) devem mostrar resposta plástica, manifestando em respostas fisiológicas, mas, ocasionalmente, em resposta comportamental também; insetos em que a alta densidade é uma característica predominante ao longo da sua vida (como insetos eussocais) devem mostrar resposta plástica, manifestando-se em respostas comportamentais, para proteger uma colônia como um todo. No segundo capítulo, nós testamos esta hipótese com a abelha eussocial *Melipona quadrifasciata*, analisando se há DDP em resposta à manipulação artificial da densidade e se esta é expressa no investimento em defesa fisiológica ou comportamental. Para isto, operárias recém-emergidas foram deixadas sozinhas, com uma única companheira de colônia ou em uma elevada densidade, por 10 dias. As interações *per capita* durante este período foram analisadas para estimar a conectividade entre os indivíduos, mostrando que a conectividade estava relacionada com a densidade. Foram testados dois parâmetros do sistema fisiológico, resposta da encapsulação e densidade de hemócitos na hemolinfa, não encontrando diferenças entre os indivíduos sujeitos a diferentes densidades/conectividades em nenhum destes parâmetros. Para analisar o investimento em DDP relacionado à defesa comportamental, nós expomos individualmente estas abelhas deixadas em diferentes densidades à uma abelha da mesma colônia contaminada com esporos do fungo *Beauveria bassiana*. Abelhas que foram sujeitadas a alta densidade/conectividade mostraram-se extremamente agressivas com a abelha contaminada, muitas vezes matando-a, o que se mostrou ser uma importante forma de defesa em nível de colônia. Por outro lado, abelhas que foram deixadas por 10 dias sozinhas ou em contato com somente uma outra não apresentaram comportamentos de defesa eficazes. Concluímos que *Melipona quadrifasciata* apresenta DDP, investindo em defesa comportamental. Isto é consistente com nossa hipótese proposta no primeiro capítulo.

SUMMARY

CHAPTER 1 – DENSITY-DEPENDENT PROPHYLACTIC IMMUNITY DISPLAYED IN DIFFERENT COMPONENTS IN ACCORD WITH SOCIALITY DEGREE

INTRODUCTION

In immunology, the immune defence investment is connected with host density. It is expected that hosts that live at high densities will be at greater risk of pathogen infection, due to density-dependent pathogen transmission (Reeson et al. 2000), in which the proximity to other conspecifics facilitates the transmission of pathogens and infection in a larger number of individuals. Likewise, it is expected that hosts living at high densities, should be selected to invest more in immune defence than hosts living at low densities, in order to minimize the problems caused by higher chances of pathogen transmission and ensure the other benefits of that lifestyle. Thus, insects should have developed strategies to invest, to the extent necessary, in immunity to minimize the damages of high densities or density fluctuation, without wasting energy.

In the area of immune investments related with organisms' density, Wilson and Reeson (1998) proposed the Density-Dependent Prophylaxis (DDP) hypothesis. The DDP hypothesis predicts that insects that could experience large density fluctuations should invest more in immune defence when at high densities, with plastic responses of the immune system. Some studies already confirm this hypothesis, although literature on this subject is still modest. Many research gaps remain, as some organisms developed more plasticity in physiological immune responses and others in behavioural immune responses. Curiously, this has not been tested in any organism studied for DDP, as existing research has not analysed both physiological and behavioural components in the same organism.

In our work, we argue that the degree of host connectivity is related to the component in which the organism will invest more (e.g. physiological or behavioural). With that, we try to understand the evolution of these immune defence mechanisms in accord with the gains and losses of each strategy in each organism. We use this approach to recast some empirical studies in the light of our hypothesis, that there is a trade-off between the physiological and behavioural components as density increases. In this, our aim is not to convince the reader that our explanation is universally applicable. It is rather to provide a hypothesis, which at times is inevitably speculative in nature, to those normally explored. We have avoided to specify relationships between particular pathogens or hosts, and sought after the general paradigm. Our hypothesis should stimulate critical thought about these physiological and behavioural immune mechanisms and their implications in the organisms' life history, and be thus tested in different insects by future research.

INSECT-PATHOGEN INTERACTIONS

Insects, as all living organisms, are subject to interactions with pathogens. This can be costly to maintain because pathogens may generate problems, and can reduce host's survival, decreasing its fitness. Immune defence investments are necessary in order to prevent or minimize these damages and organisms with good investment strategies will leave more offspring, which should inherit the characteristic to invest in an specific strategy. In this manner, it is expected that the defence strategies against pathogens found in modern organisms have been the result of years of selective pressure, modulated according to different characteristics; i.e. organism's lifestyle and its natural enemies.

The pathogens transmission can occur in various ways, like contact with contaminated objects; contact with contaminated organisms; contact with contaminated conspecifics; per os; transovarially; among others. However, the transmission through contact with infected conspecifics is the currency at light of DDP. Therefore, high-density hosts will be at greater risk of pathogen infection. Thus, from the point of view of the transmission of pathogens, group life could not be a good strategy. Nonetheless, there are other factors interacting, for example, group life should be very advantageous for protection, food gathering and reproduction (in some cases). Likewise, it is expected that hosts living at high densities should be selected to invest more in immune defence than hosts living at low densities, minimizing problems caused by higher chances of pathogen transmission and ensuring the other benefits of this lifestyle; like the DDP hypothesis predicts.

However, high density alone could not have a true influence in pathogens transmission by contact with infected conspecific and, consequently, in defence against these pathogens. Connectivity seems to be the main cause of the risk of infections, because insects can be in high density but not have contact with each other (Elliot and Hart 2010). Additionally, some insects also tend to maintain a stable density and optimum contact rate to try minimize damages (eg. DeSouza et al. 2001, Altizer et al. 2003, DeSouza and Miramontes 2004, Elliot and Hart 2010). This has empirical evidence in Otterstatter and Thomson's (2007) study of disease diffusion in bumble bees, in which the insects' interaction rates were found to be the only determinant factor of infection risk. In the same vein, Elliot and Hart (2010) propose the connectivity-density dependent (CDP) hypothesis, where insects could have a plastic immune response for changes in connectivity.

SOCIAL INSECTS’ PATHOGENS

Social insects' colonies as referred as a superorganism by its similarity with multicellular organisms. This analogy is very parsimonious in the light of immunity because both seems to have faced the same organizational problems and the same intense selection pressure of pathogens (Holldobler & Wilson 2008) leading up to a series of other analogies (Cremer & Sixt 2009). Social insects can show physiological, behavioural and organizational immunity at colony-level, beyond the individual immunity, collectively avoiding and eliminating pathogens (Schmid-Hempel 2005, Cremer et al. 2007). The term social immunity (Cremer et al. 2007) refers to these collective defences, and predicts that individuals have a better defence mechanism at colony level.

The social insects’ biology is controversial because this is a very diverse group. Regardless, there are some defence strategies that are broadly present in social insects in general. Caste and tasks division is very important from the immune defence point of view, once it protects reproductive individuals and offspring of higher chances of contamination. Despite social insects living at high densities and conectivities, which leads us to expect higher chances of contamination, little is known about its pathogens. That happens because social insects’ pathogens uncommonly act as an enemy that limits the colony’s live, even when this can happen what shows that colony level’s defence seems to be effective.

The main social insects’ pathogens are virus, bacteria, fungi, protozoa, nematodes, helminths, mites and parasitic insects (Schmid-Hempel 1998). Viruses are commonly transmitted by contact with contaminated conspecific, per os, transovarially or per reproductive tract, and by vectors; with eggs and larvae usually targetted. Bacteria can be spread by vectors or by contact with contaminated conspecific or substrate, and commonly attack larvae. Fungi are passed on by contact with contaminated conspecific or substrate, and can attack larvae and adults. Protozoa are frequently transmitted per os and transovarially, to booth larvae and adults. Nematodes and helminths can be spread per os or by contact with contaminated conspecific or substrate. Mites are diffused by contact with contaminated conspecific or substrate, and can attack larvae. Parasitic insects of social insects can be cleptoparasites, commensalists or parasitoids, and parasitize either brood or adults. What all these pathogens and its relations with social insects have in common is that they are almost understudied. This probably occurs because they do not represent high risks for the colony and therefore do not cause an economical problem.

It is important to reinforce that colony's high density and individuals' high connectivity can increase the transmission chance of many social insects' pathogens. Moreover, even some social behaviours can increase it, once nurses can transmit pathogens for larvae, or workers can be exposes to pathogens when it makes grooming or trophalaxis with a contaminated conspecific. However, caste system and age-related labour division affects the epidemiological dynamics of pathogens transmission, i.e. may impede a successful transmission. Nesting habits and architecture also affect the spread of parasites.

Resistance in social insects can involve a number of behavioural and physiological defences, and pathogens would be involved in many hypothesis about the evolution of social behaviour and eusociality. Examples of social insects' characteristics that seems to have evolved by pathogens' pressure are reproductive protection, sex ratio (reducing the problem of transmission via one particular sex), close contact between individuals (behaviour that can be induced by parasites), subfertility or infertility in workers (that can be caused by parasites) and nestmate recognition (to prevent that new parasites come in the colony). The future research should validate these questions.

EMPIRICAL STUDIES ON DDP

The DDP hypothesis (Wilson and Reeson 1998), which predicts that insects that experience large density fluctuations should invest more in immune defence only when at high densities, with plastic responses of the immune system, was validate for various species of insects (Table 1).

On the other hand, some studies show that there may be specific mechanisms of the immune system that do not change or even decrease as density increases, in some insects (e.g. Wilson and Reeson 1998, Wilson et al. 2002, Cotter et al. 2004, Wilson and Cotter 2008). For example, some insects showed higher phenoloxidade activity with density increase but lower antibacterial activity, or the opposite (e.g. Cotter et al. 2004). This may occur because each organism has a specific defence strategy, investing in different mechanisms (Siva-Jothy et al. 2005).

Table 1: Insect species tested for DDP

Order	Species	Livestyle	Phase polyphenic	Density-dependent prophylactic response
Lepidoptera	*Mythimna separata*	Gregarious	Y	Virus resistance increases with density (Kinimi and Yamada 1990).
Lepidoptera	*Mamestra brassicae*	Gregarious	Y	Virus resistance increases with density, but only up to a point (intermediate density), after that, the resistance decreases (Goulson and Cory 1995).
Lepidoptera	*Spodoptera exempta*	Gregarious	Y	Virus resistance increases with density (Reeson *et al.* 1998). Phenoloxidase activity in the cuticle, haemolymph and midgut increases with density (Wilson *et al.* 2001).
Lepidoptera	*Spodoptera littoralis*	Gregarious	Y	Fungus resistance increases with density (Wilson *et al.* 2001). Phenoloxidase activity in the cuticle and encapsulation response to an artificial parasite increases with density (Cotter *et al.* 2004).
Lepidoptera	*Anticarsia gemmatalis*	Solitary aspects, but subject to density fluctuations	Y	Hemocyte densities, encapsulation response and fungus resistance increases with density (Silva *et al.* 2013).
Coleoptera	*Tenebrio molitor*	Solitary aspects, but subject to density fluctuations	Y	Fungus resistance increases with density (Barnes and Siva-Jothy 2000).
Orthoptera	*Schistocerca gregaria*	Gregarious	Y	Fungus resistance and antimicrobial activity increases with density (Wilson *et al.* 2002).
Orthoptera	*Anabrus simplex*	Gregarious	Y	Lysozyme activity and encapsulation response increases with density (Bailey *et al.* 2008).
Orthoptera	*Locusta migratoria*	Gregarious	Y	Fungus resistance increases with density (Wilson *et al.* 2002).
Isoptera	*Zootermopsis Angusticollis*	Social	N	Fungus resistance increases with density (Rosengaus *et al.* 1998)
Hymenoptera	*Acromyrmex echinatior*	Social	N	Fungus resistance increases with density (Hughes *et al.* 2002).
Hymenoptera	*Bombus terrestris*	Social	N	Phenoloxidase increases with density (Ruiz-González *et al.* 2009)

IMMUNE DEFENCE COMPONENTS

Physiological and behavioural responses are potentially costly components of the immune system (Barnes and Siva-Jothy 2000, Siva-Jothy et al. 2005, Elliot et al. 2005, Schulenburg and Ewbank 2007, Wilson and Cotter 2008), so it is expected for each organism to invest in the mechanism that suits them best. The fact is that the vast majority of studies have focused only on physiological defences, leaving aside behavioural defences.

Then, how the DDP immune response will be express should vary according to the lifestyle and the selective pressures affecting each species; the behavioural component of immune defence may be regarded as the cheaper alternative in total immune defence in social insects (Hart et al. 2002, Hughes and Cremer 2007; Yanagawa and Shimizu 2007, Elliot and Hart 2010). This should happen because social insects experience high connectivity throughout life, a permanent characteristic for this group. Other reason for this may be that it allows for more complex — effectively lower-cost — responses in eusocial insects (Elliot and Hart 2010), in addition to contributing to social cohesion and cooperative behaviour (e.g. Hughes et al. 2002, Traniello et al. 2002, Cremer et al. 2007, Cremer and Sixt 2009, Jackson and Hart 2009).

Moreover, social insects act as superorganisms and thus individuals invest in protecting the colony as a whole. Therefore, the prophylactic defence mechanisms that provide this protection are primarily behavioural (i.e. cleaning habits of the colony, of themselves and of conspecifics - grooming). Additionally, if losing a number (often large) of individuals may not be costly (Bourke and Franks 1995), there is reduced selective pressure for these workers to defend themselves with physiological mechanisms, as there are behavioural mechanisms to protect its colony and thus its offspring. In social insects, infected individuals are often allogroomed by other group members, which dramatically increases their survival after exposure to the pathogen (Hughes et al. 2002). In extreme cases, infected workers may be sacrificed in a similar fashion to infected cells within an organism (Janeway et al. 2001). Parasite transmission can also be controlled by hygiene. An example is found in ants piling and burying fungi spores (Jaccoud et al 1999), and bees that encapsulate invaders and let them starve. Such behaviours at the level of the colony are analogous to the encapsulation response of the physiological immune system of solitary individuals (Schmid-Hempel 2005).

Therefore, in high connectivity conditions, just as social insects must have been selected to invest more in plastic behavioural defence, solitary insects should have been selected to invest more in plastic physiological defence, with intermediate investments in insects with intermediate lifestyles. This model of investment in physiological immune defence for social and non-social insects makes sense when taking into account the selective pressures they experience (Figure 1). It is worth considering that there are three possible outcomes of infection for insects: recovery, temporary survival (perhaps with reproduction but also perhaps with partial parasite transmission) or rapid death. For non-social insects, recovery is the best strategy, followed by temporary survival, sufficient to leave offspring, while rapid death is the worse alternative since it decreases the individual's fitness. In social insects it is the colony that produces offspring, therefore, the best strategy may be rapid death, followed by recovery (when also rapid), while the temporary survival is the worse alternative because it increases the chances of nestmate’s contamination (Figure 1).

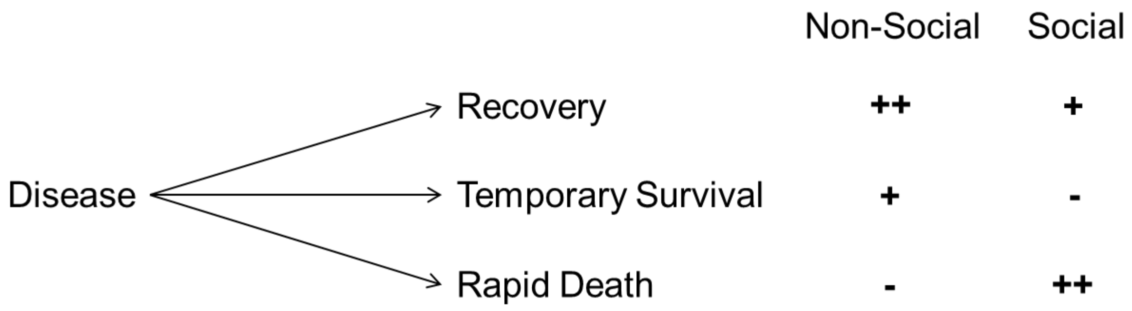

Figure 1: Roles of different alternatives for social and non-social insects. '++' symbolizes the best alternative for that organism, '+' the intermediate alternative, and '-' the worst alternative, according to its fitness.

In social insects, disease transmission is reduced by behavioural precautions (e.g. Schmid-Hempel 1998, Pontoppidan et al. 2009) with behaviours such as limiting contact with other colonies, not returning to the colony if infected with a pathogen, or restricting feeding grounds. The Connectivity-Dependent Prophylaxis (CDP) hypothesis suggests also that the selective pressure to avoid infecting kin may well be a driving force in structuring social-insect colonies (Elliot and Hart 2010).

OUR HYPOTHESIS

Based on this information, we propose the existence of a trade-off between behavioural and physiological plastic defences, as connectivity increases and becomes a permanent feature in the specie (Figure 2).

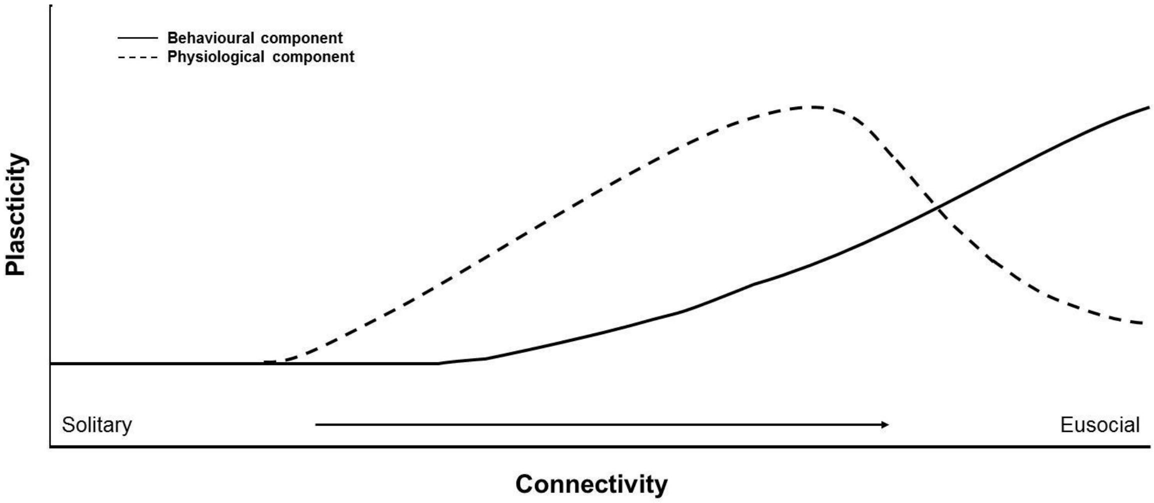

Figure 2: Plasticity in immune system of insects in accord with the connectivity prevalence over the organism life. The solid line represents the behavioural component and dotted line represents the physiological component.

Thus, as the best defence strategy in social insects should be the rapid death to avoid transmission to nestmates, they could invest less in physiological immune defences. On the other hand, strictly solitary insects could not have the capacity of performing plastic immune response to density variations because it is not predictable to happen. Insects that have solitary habits but may present density-dependent phase polyphenism can respond to experimental variations in density, investing only in plastic physiological immune defence. Insects that exhibit gregarious phase could invest heavily in plastic physiological immune defence when at high densities, and the plastic behavioural immune defence investment in these insects should increase as gregarious behaviour is more prevalent in their lifestyle. Social insects tend to invest more in plastic behavioural immune defence as the degree of sociality increases, due to high connectivity prevalence and pleiotropic effects.

An interesting fact that can strengthen our hypothesis is that some social insects usually present age polyethism, and tasks with a higher chance of contamination are performed by older individuals. This should confirm our hypothesis that social insects invest more in behavioural defences because they could have been selected to keep their young individuals away from contamination risks, since they have a greater working potential for the colony, leaving risky tasks only for individuals closer to their natural death, i.e. more 'expendable'. Then, as the strategy of these insects is to reduce damages caused by the losses of individuals, these losses are foreseen and expected.

CONCLUSIONS AND PERSPECTIVES

Most DDP or CDP studies do not examine both behavioural and physiological aspects for the same organism. However, considering that both are defence mechanisms and costly immunological strategies for every organism, our hypothesis is that, in case of experimental increase in connectivity: strictly solitary insects will not develop any plastic response from the immune system (neither physiological nor behavioural); while insects that should eventually experience some natural connectivity variations can show some plastic response, manifested only in physiological responses to protect themselves; insects that necessarily experience natural connectivity variation (as gregarious insects) must show plastic response, manifested in physiological responses and occasionally in behavioural responses; insects for which connectivity is a prevalent characteristic along their life (as social insects) may show plastic response, manifesting rather in behavioural responses and occasionally in physiological responses. To test this hypothesis, it is necessary to analyze insects from different lifestyles, investigating both parameters, physiological and behavioural immune defence, in response to different connectivities and densities.

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CHAPTER 2 – DENSITY-DEPENDENT PROPHYLACTIC IMMUNITY IS MORE EXPRESSED IN BEHAVIOURAL THAN IN PHYSIOLOGICAL PLASTIC DEFENCES IN A SOCIAL INSECT

INTRODUCTION

The immune system provides behavioural and physiological defences against pathogens. In general, investment in immune defences is costly, thus hosts should increase their defence investment in accord with the infection risk (Wilson and Cotter 2008). Group-living hosts are more likely to become infected by contact-transmitted pathogens than lone-living hosts. Thus, it has been proposed that the investment in defence should be greater in harmony with the crowding increase, to balance the density-dependent pathogen transmission rate and can exhibiting immune plasticity. This is known as the density-dependent prophylaxis hypothesis - DDP (Wilson and Reeson 1998).

This hypothesis has been supported in a range of studies of insects that live at fluctuating densities (Reeson et al. 1998, Reeson et al. 2000, Barnes and Siva-Jothy 2000, Wilson et al. 2002, Rolff and Siva-Jothy 2003, Cotter et al. 2004, Cotter et al. 2008, Dubovskiy et al. 2013). Moreover, the DDP hypothesis has also been confirmed in some groups of organisms that do not have these marked group / lone-living phases (Bailey et al. 2008, Ruiz-González 2009, Mills 2012, Gotham and Song 2013, Silva et al. 2013). Nevertheless, some organisms do not have an overall DDP response, showing a trade-off within the physiological immune system (Cotter et al. 2004).

Social insects live in colonies with high densities and low genetic variability, often. Therefore, they are expected to have a high risk of becoming infected by contact-transmitted pathogens. Therefore, are expected do not have significant plasticity in physiological immune system in response to changes in population density in social insects (Elliot and Hart 2010). Elliot and Hart (2010) propose that, among the components of social insects’ immune defence, behavioural immune defences would represent a more efficient and lower-cost defence when compared to the physiological components. These authors also propose that the density itself should not cause response in the immune defence because in a colony or in a cluster there could be many individuals but itd sould not come into contact with each other, so they propose connectivity-dependent prophylaxis (CDP) being more important, considering the actual individuals interaction.

Furthermore, from the immune system point of view, regarding the social insects’ colonies as a superorganism is a very parsimonious idea (Bourke and Franks 1995). Cremer et al. (2007 and 2009) make an analogy between the social insects’ colony and the immune system of mammals, where many cells can sacrifice itself during a pathogen attack in order to preserve the major organs and tissues. Thus, as the workers act altruistically to protect the colony itself, through the protection of the queen and the young offspring, it is expected to be a selective pressure for them invest more in behavioural defence than in physiological defence. In other words, individuals in social insects act as non-social organisms' defence cells, hence investing in colony defence with behavioural mechanisms seems to be more important than investing in a individual's physiological defence.

The DDP hypothesis has been tested in social insects, but without conclusive responses. Pie et al. (2005) held termites at different densities and examined their individual susceptibility to a pathogen (i.e. post-inoculation, insects were isolated from conspecifics), finding no group size effect on termite individual to the pathogen.

On the other hand, Ruiz-González et al. (2009) searched DDP in the *Bombus terrestris* bee and showed that high densities individuals had higher phenoloxidase activity than low densities individuals, but other parameters (i.e. anti-bacterial activity) showed an inverse pattern, with lower immune defence investment at higher densities.

Assuming that connectivity (here, interaction between individuals) is more important than density alone, we hypothesize that social insects with greater connectivity will invest more in plastic behavioural immune defence than in plastic physiological immune defence, for the colony protection as a whole. Our objective was to test this hypothesis.

METHODOLOGY

Experimental setup

We used a stingless bee, *Melipona quadrifasciata*, an eusocial insect that makes an interesting research candidate for two reasons. First, these organisms are kept locked individually in a cell since the laying of eggs until their emergence of adults, so they are unlikely able to detect colony density in that phase, making it easier to control the rate of conspecifics contact from the first moment of its emergence. Moreover, it is a bee of relatively large size, making possible the realization of the experiments.

Insects were taken from five *M. quadrifasciata* hives that were bought from two local honey producers. Brood combs were collected in 15 cm ø Petri dishes and maintained in an incubator at 28±2°C until the emergence of adults. Workers that were ready to emerge (identified by the colour and hair condition on head top) were removed from the brood cells and placed in another Petri dish (8.5 cm ø). They were placed according to the following distribution: alone (1st treatment); in contact with another worker, i.e. 2 *per* diah (2nd treatment); and in high density, 12 *per* dish (3rd treatment).

Bees were kept at these densities for 10 days, supplied *ad libitum* with a solution of *Apis mellifera* honey and water (1:1), and fermented pollen. Only dishes which maintained the initial density of individuals after this period were used in the trials, i.e. any individual died; the exception being the 3rd treatment (12-individuals), for which we accepted up to 2 missing individuals per dish.

In order to avoid pseudoreplication, only one individual per dish (arbitrarily chosen) was used in each experiment. We conducted the experiments in pairs, using different bees of the same dish for different experiments (Figure 1). Experiments made in pair were: encapsulation responses with haemocyte densities, and behavioural measurements, no-fungal control with fungal treatment.

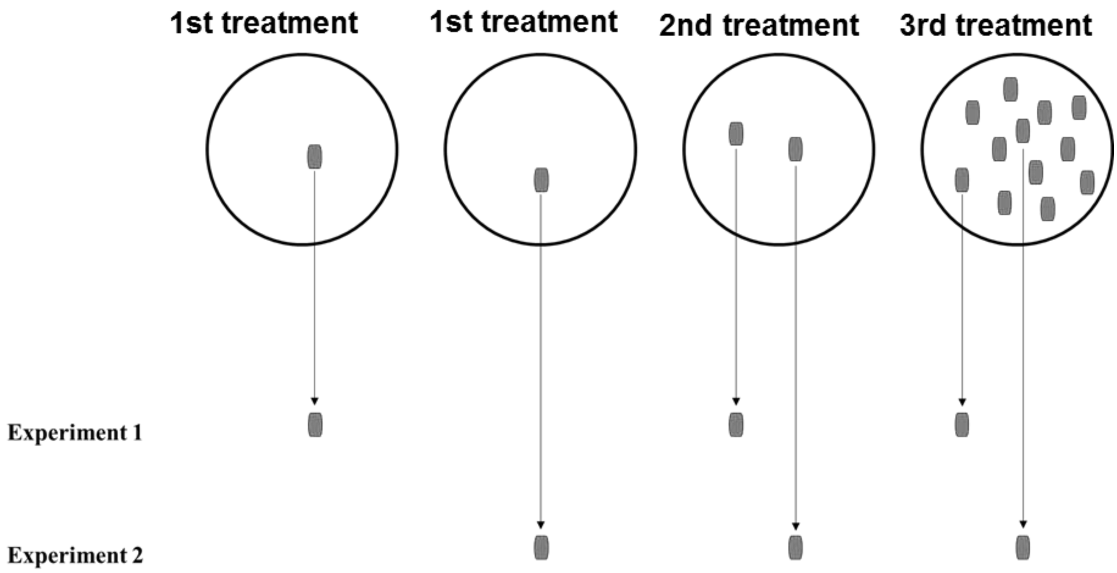

Figure 1: Use of treatment dishes in paired experiments. Was used two dishes of 1st treatment and the single bee of each one was used for each experiment. One dish of 2nd treatment was used for two experiments, using one bee for each. For 3rd treatment one dish was used for two experiments too, using two randomly choice bees, one for each experiment. The experiments running in pairs were: (i) encapsulation responses (experiment 1) with haemocyte densities (experiment 2); (ii) behavioural measurements, no-fungal control (experiment 1) with fungal treatment (experiment 2).

We analysed the *per capita* interaction among bees of our density treatments. We completed 60 observations of 10 minutes for each density, counting both intentional (antennation, trophallaxis, grooming, etc) and unintentional (encounter when walking) interactions. The total number of interactions was counted and divided by the number of individuals on the dish to determine the *per capita* interaction.

Physiological parameters

Tested workers (n=90) were challenged with a colourless nylon filament (0.2 mm ø x 2 mm length) inserted through the lateral region of the thorax (between the second and third legs pair). Seventy-two hours after the nylon challenge, we removed the nylon filaments and mounted these on to slides to be photographed under a stereomicroscope Zeiss (model Stereo Discovery V20), with all conditions standardized, i.e. same light settings, zoom etc. Two measurements were taken: (i) capsule area and (ii) capsule melanisation, using AxioVision and ImageJ software.

Capsule area was assessed by measuring the area of the cell layer formed around the filament. We cannot control with complete certainty the amount of the nylon filament that was inserted in the bee. However, after removal we could define it clearly through visualizing the hair present in the bee thorax. Thus, primarily, we measure the total area of the nylon filament inserted into the bee body, and afterwards, we measured the area formed by the cell layer, and then calculated the percentage of encapsulation. The measures were performed with AxioVision software.

To measure capsule melanisation, images were converted to greyscale with ImageJ software, allowing quantification as the mean value of grey in each image (methodology modified from Silva et al. 2013).

To count the haemocyte densities, test workers (n=81) were decapitated and 5 µl of haemolymph were collected from each with a pipette. Upon collection, haemolymph was diluted with 10 µl of 2% Giemsa stain in an Eppendorf tube. Ten µl of this solution were pipetted onto a Neubauer chamber under a microscope (Olympus model SZ61) and 5 squares (with 0.04mm² each) were counted. After that, we created an average of the five quadrants counted and multiplied by the correction factors of the Neubauer chamber and of the dilution. Here, we used the total number of cells, without taking into account the different haemocyte types (methodology modified from Silva et al. 2013).

Behavioural parameters

Test workers (n=120) were placed individually in Petri dishes (8.5 cm ø) with a paper filter disc. Meanwhile, workers from the same colony were collected, marked on their thorax with a water-based corrective marker (Toque Mágico®). For the fungal treatment, marked bees were taken to a laminar flow cabinet to be contaminated with spores of *Beauveria bassiana*. For this, 10-day old, sporulating dishes of *B. bassiana* was used. Four 5mm square pieces were cut from the plates and placed in the dish where the marked bees were held. These dishes were vigorously shaken (manually) for 5 seconds. Subsequently, one of these contaminated bees was placed on each test dish. For no-fungus controls, we performed the same procedures but without fungus. Video recordings were made during 1 hour and then analysed to verify the following previously defined behaviours: antennation, self-grooming, allogrooming and aggression in the tested bees.

We previously certify that *B. bassiana* can parasitize *M. quadrifasciata*, leading to death in the infected individuals.

Statistical Procedures

All tests were verified using generalized linear models (GLM) in R statistical software (R Core Team, 2014). Models were analysed to verify their significance, and following the analysis, data overdispersion was checked to determine whether the distribution was the most suitable. No effects of the colony of origin were found in any of the experiments and so these data are not considered further. When there was significant difference between densities, we made between-group comparisons.

RESULTS

Connectivity

For lone individuals, we did not record any interactions (naturally). *Per capita* interactions were statistically different among groups ($F_{[2,27]}$= 47.32, P<0.01; Figure 2). These results showed that there is an increment in the *per capita* interaction with density group. According to these results we can say that in *Melipona quadrifasciata* bees the density is in accordance with the connectivity, i.e., higher density leads to higher connectivity. Thus, in subsequent experiments we continue using "Individuals/dish" in *x*-Axis because it represents both density and connectivity.

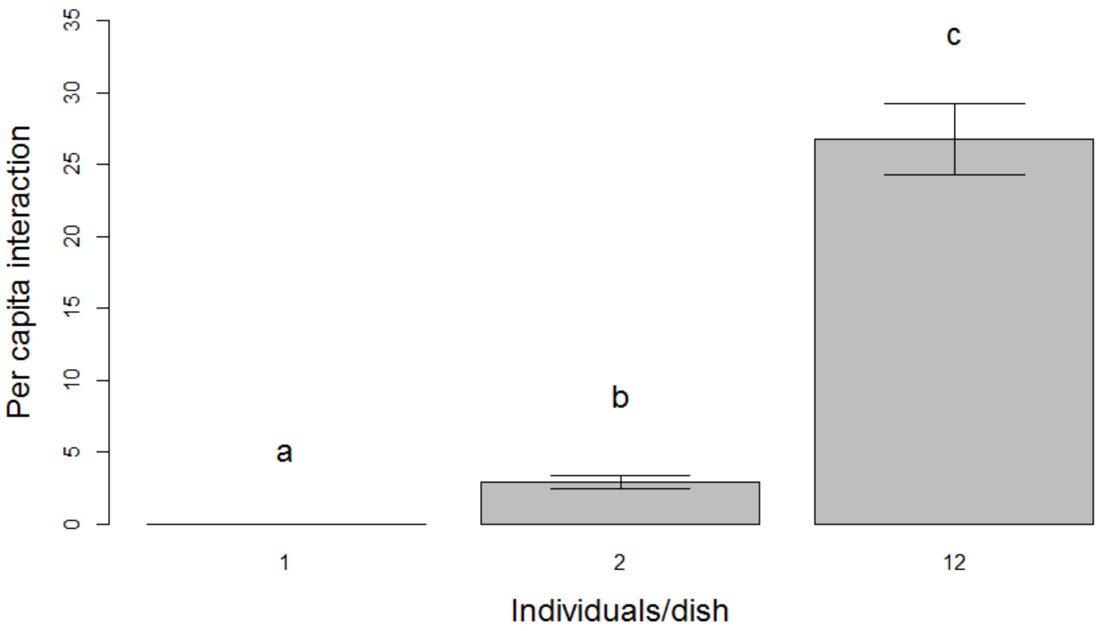

Figure 2: *Per capita* interaction for individuals held in the three density treatments (1, 2 or 12 individuals/dish). Both intentional (antennation, trophallaxis, grooming, etc) and unintentional (encounter when walking) interactions were observed for 10 minutes (n=60). The total number of interactions was counted and divided by the number of individuals on the dish to determine the per capita interaction. Different letters among treatments indicate a significant difference.

Physiological parameters

Physiological parameters were not affected by density/connectivity. Neither capsule area ($F_{[2,87]}$=1.68, P=0.19; Figure 3), capsule melanisation ($F_{[2,87]}$=1.85, P=0.16; Figure 4) nor haemocyte densities ($F_{[2,78]}$=2.95, P=0.06; Figure 5) were statistically different among density/connectivity group tested here.

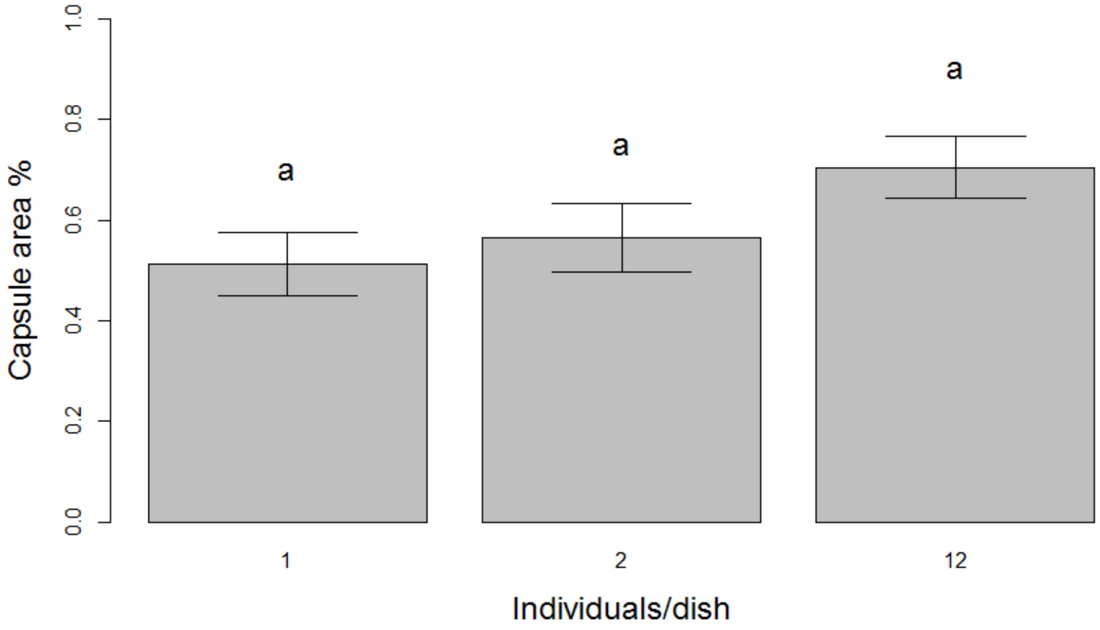

Figure 3: Capsule area (%) for individuals held in the three density/connectivity treatments for 10 days. Tested workers (n=90) were challenged with a colourless nylon filament (0.2 mm ø x 2 mm length) inserted through the lateral region of the thorax (between the second and third legs pair). Seventy-two hours after the nylon challenge, we removed the nylon filaments and mounted these on to slides to be photographed. Capsule area was assessed by measuring the area of the cell layer formed around the filament in relation with the total area of the nylon filament inserted into the bee body. The measures were performed with AxioVision software. Same letters among treatments indicate a not significant difference.

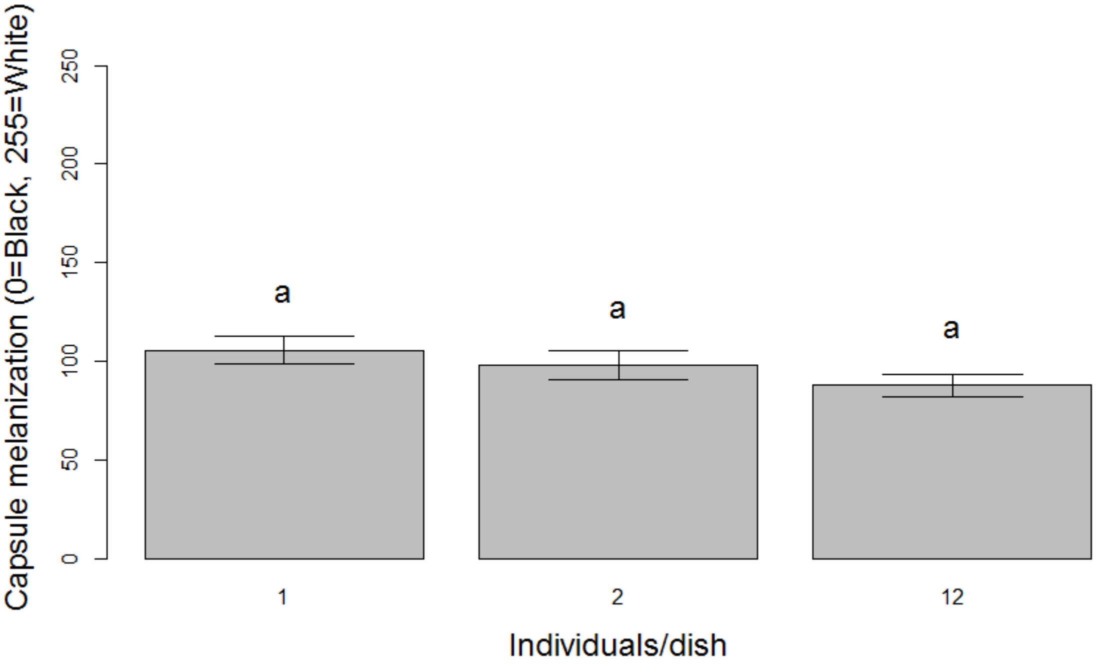

Figure 4: Capsule melanisation for individuals held in the three density/connectivity treatments for 10 days. Tested workers (n=90) were challenged with a colourless nylon filament (0.2 mm ø x 2 mm length) inserted through the lateral region of the thorax (between the second and third legs pair). Seventy-two hours after the nylon challenge, we removed the nylon filaments and mounted these on to slides to be photographed. Images were converted to greyscale with ImageJ software, allowing quantification as the mean value of grey in each image. Same letters among treatments indicate a not significant difference.

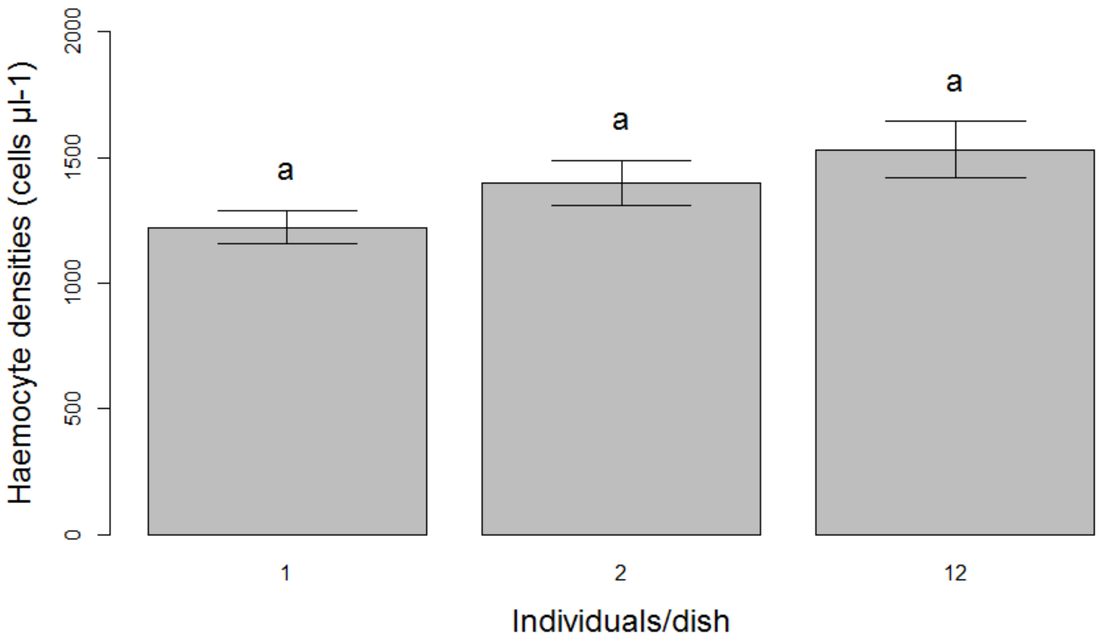

Figure 5: Haemocyte densities for individuals held in the three density/connectivity treatments for 10 days. Workers (n=81) were decapitated and 5 µl of haemolymph were collected from each with a pipette. Upon collection, haemolymph was diluted with 10 µl of 2% Giemsa stain in an Eppendorf tube. Ten µl of this solution were pipetted onto a Neubauer chamber under a microscope (Olympus model SZ61) and 5 squares (with 0.04mm² each) were counted. After that, we created an average of the five quadrants counted and multiplied by the correction factors of the Neubauer chamber and of the dilution. We used the total number of cells, without taking into account the different haemocyte types. Same letters among treatments indicate a not significant difference.

Behavioural parameters

Behavioural parameters were affected by density/connectivity and by fungi treatment. Without fungi the only behaviour observed was antennation, indicating a behaviour change when in contact with an infected bee. Without fungi antennation for marked bees by tested bees was more frequent at higher density/connectivity ($F_{[2,57]}$=26.85, P=<0.01; Figure 6).

With fungi all behaviours were observed: antennation ($F_{[2,57]}$=45.56, P=<0.01), allogrooming ($F_{[2,57]}$=201.62, P=<0.01), selfgrooming ($F_{[2,57]}$=38.38, P=<0.01) and aggression (F[2,57]=251.4, P=<0.01) were all affected by density/connectivity. With fungi, antennation showed a different pattern than without fungi (Figure 7a), with less antennation on high densities/connectivities. This seems to happen because in contact with a contaminated bee, tested bees should have other priorities, like to exterminate contaminated bees. However, this priorities were related with density/connectivity, indicating plasticity in the behavioural immune system (Figure 7).

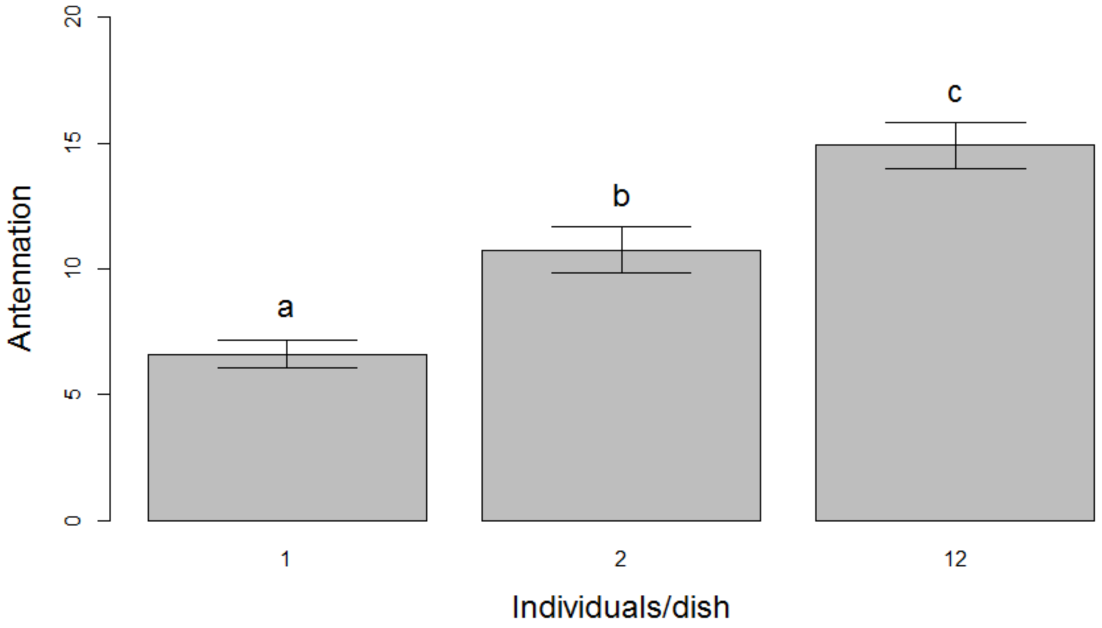

Figure 6: Antennation of individuals held in the three density/connectivity treatments for 10 days to marked bees, collected in the colony, without fungi. Treatment workers (n=60) were placed individually in Petri dishes (8.5 cm ø) with a paper filter disc. Workers from the same colony were collected, marked on their thorax with a water-based corrective marker (Toque Mágico®) and take in contact with a treatment bee. Video recordings were made during 1 hour. Different letters among treatments indicate a significant difference.

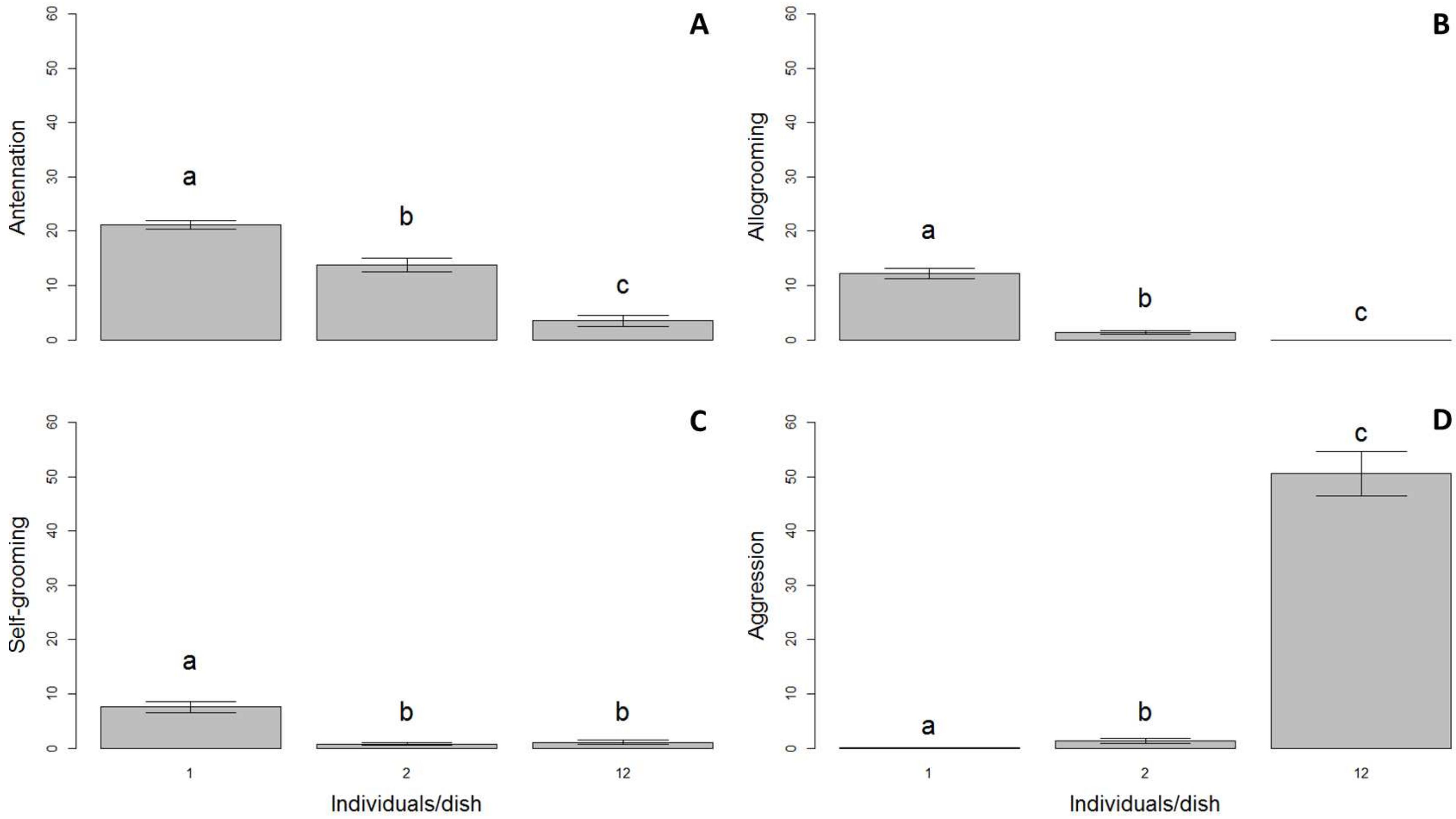

Figure 7: Behaviours exhibit of individuals held in the three density/connectivity treatments for 10 days to marked bees, colleted in the colony, with fungi. Treatment workers (n=60) were placed individually in Petri dishes (8.5 cm ø) with a paper filter disc. Meanwhile, workers from the

same colony were collected, marked on their thorax with a water-based corrective marker (Toque Mágico®) and contaminated with spores of *Beauveria bassiana*. Subsequently, one of these contaminated bees was placed on each test dish. Video recordings were made during 1 hour. Different letters among treatments indicate a significant difference. A- Antenation; B- Allogrooming; C- Self-grooming; D- Agression.

DISCUSSION

Our objective was to evaluate with different connectivity degrees between individuals should generate plastic changes in immune system (physiological or behavioural) in eusocial insects. Fisrt, we found that, in *Melipoja quadrifasciata* bee, connectivity was in accord with density, i.e. the *per capita* interaction on these bees was much higher in the 3rd treatment than in 2nd. This seems very parsimonious, but it not always happens, many organisms may live in the same place but do not make contact with each other. Since, it is very important to measure the connectivity in order to understand the real importance of density as a determinant of plastic changes.

In the physiological parameters that we examined, bees did not show significant plasticity in the immune system with different densities/connectivities. However, analysing biologically our results, there was a slight tendency (not statistically significant) for greater physiological immune investment with higher connectivity. This is in accord with the propose of a trade-off between behavioural and physiological plastic defences with connectivity increasesin which insects with higher and prevalent connectivity invest more in behavioural plastic defences but may invest (lower) in physiological plastic defence too.

There are many studies showing greater investment in physiological immune defences with higher densities in non-social insects (e.g. Barnes and Siva-Jothy 2000, Cotter et al. 2004, Cotter et al. 2008, Dubovskiy et al. 2013, Reeson et al. 1998, Reeson et al. 2000, Rolff and Siva-Jothy 2003, Wilson et al. 2002, Silva et al. 2013) but for social insects, there is only previously two tests of DDP. Our results is similar to a previous study with termites (Pie et al. 2005) and supports the hypothesis of Elliot and Hart (2010) that we do not expect greater plastic responses in physiological defences in social insects. Meanwhile, Ruiz-González et al. (2009) demonstrated density-dependent physiological prophylaxis in the *Bombus terrestris* bee. Perhaps this difference could be due to little differences in the sociality degree between *B. terrestris* and *M. quadrifasciata*. How much greater the sociality degree higher should be the prevalence if high connectivity, causing changes in the investment in different plastic responses

While we did not find a plastic effect of connectivity on physiological immune defences, we did find an effect on behavioural defences. Bees in the 3rd, high-connectivity, treatment perform lower antennation in the bee contaminated with fungus and soon vigorously attacked it, killing them in most cases. Showing a quickly recognition and an effective defence. On the other hand, the 1st treatment bees performed more antennation to contaminated bees (perhaps indicating a failure to identify contamination) and carried out allogrooming, which we expect to be a less effective defence than aggression because in social insects the best alternative when contaminated is the rapid death to avoid contaminate nestmates. In most cases, 2nd treatment bees took the strategy of moving away from the other bee. First treatment bees would often perform self-grooming after performing allogrooming in a contaminated bee. It is expected that 3rd treatment bees could perform self-grooming after killing the infected bee, but such behaviour was not widely observed within the observation period (1 hour). When in contact with uncontaminated bees (control) the only observed behaviour was antennation, occurring more often as connectivity increased, once again demonstrated a greater activity in bees held in higher connectivities.

This results are consistent with the propose to a greater investment in behavioural than in physiological plastic defences, in response to higher and prevalent connectivities. Behavioural defences may give pleiotropic benefits where they act in conjunction with other behaviours to contribute for the group cohesion, for example (Elliot and Hart 2010). Moreover, the social insect colony is considered as a superorganism from the point of view of the immune system. As with the immune system of mammals, where many cells can sacrifice themselves during attack by a pathogen, thus preserving the major organs and tissues; in a social insect colony a large number of workers are fully dispensable for the colony's defence (Bourke and Franks 1995, Cremer et al. 2007, Cremer and Sixt 2009). Thus, as the workers act altruistically to protect the colony, through the protection of the queen and the young offspring, it is expected for there to be a selective pressure for them not to invest as much in individual physiological immune defence, as they do in behavioural defence.

We consider "superorganism" a useful concept to emphasize that, similar to a human body (i.e.), only a colony as a whole can produce offspring, the relevant currency in evolution. Therefore, behavioural defence mechanisms in social insects are more important because they act at the colony level (e.g. Janeway et al. 2001), unlike physiological defence mechanisms that promotes self-defence.

Now, should be necessary to test if bees of this age (10 days) naturally not investing heavily in physiological immune defences. This could happen because *M. quadrifasciata* have age polyethism, then, only older workers conduct high-risk tasks that might lead to pathogens contamination, thus, it could be that only these are able to widely develop their immune system. To respond that it is necessary to make tests using older workers. It is probably that the result show the same pattern of a major investment in behavioural defence, but may have an overall increase in both physiological and behavioural immune defences. Maybe, in the results presents by Ruiz-Gonzalez et al. (2009), were they used older bees, it occurred this overall increase in immune defence that reflects in a statistically significant growth in physiological parameters in higher density.

Finally, we conclude that our hypothesis was confirmed for *M. quadrifasciata*, i.e., this bee develop a plastic response to higher densities/connectivities investing more in behavioural than in physiological defence. For a complex understanding of DDP in social insects other studies are necessary, always applying both physiological and behavioural parameters and taking into account the connectivity.

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