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From Isolation to Overcrowding: Effects of Population Density on Immunity and Insect-Pathogen Interactions in Lepidoptera

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Thesis submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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ABSTRACT

SOARES, Carolina de Oliveira, D.Sc., Universidade Federal de Viçosa, July, 2025. **From Isolation to Overcrowding: Effects of Population Density on Immunity and Insect-Pathogen Interactions in Lepidoptera**. Adviser: Simon Luke Elliot. Co-advisers: Sheena Catherine Cotter, Farley William Souza Silva and Eduardo Carlos Costantin.

Density-dependent phase polyphenism is a phenomenon in which insects develop distinct phenotypes in response to population density, resulting in different morphological and physiological characteristics within the same species. Meanwhile, host population density is one of several factors that can influence the dynamics of host-pathogen interactions in insect populations. In this thesis, we investigated how population density and the phenotypes expressed by *Anticarsia gemmatalis* influence the infection dynamics of the AgMNPV baculovirus, and whether two economically important species, *Spodoptera frugiperda* and *Spodoptera eridania*, show any degree of density-dependent phase polyphenism, through changes in larval coloration or immune responses. In *A. gemmatalis*, we demonstrated the occurrence of the three color phenotypes (green, intermediate, and black) at both tested densities (1 and 4 larvae per container, representing solitary and gregarious conditions, respectively), with the higher density inducing a greater frequency of dark-colored phenotypes. Black phenotypes were more susceptible to AgMNPV and showed greater viral replication, in spite of the greater cuticular melanization. Green phenotypes survived longer and produced more viral occlusion bodies. Furthermore, the viral material originating from gregarious larvae was more virulent in subsequent infections. On the other hand, density did not influence the levels of circulating hemocytes in this species. In *S. frugiperda* and *S. eridania*, we observed species-specific responses to larval density. *Spodoptera eridania* showed clear changes in coloration, while *S. frugiperda* showed limited phenotypic variation. In *S. eridania*, the encapsulation response was intensified at higher densities, and both species showed increased hemocyte counts at the highest density, partially in line with the Density-Dependent Prophylaxis (DDP) hypothesis. In *S. frugiperda*, darker phenotypes produced a greater number of viral occlusion bodies after infection with SfMNPV. Altogether, our results demonstrate that density-induced phenotypic changes can modulate host susceptibility and viral replication, and that such phenotypic adjustments may be more common than previously thought, even in species without evident gregarious behavior. Thus, we expand the understanding of the applicability of DDP in insects and highlight the importance of considering species-specific characteristics in the interactions between population

density, immunity, and pathogen susceptibility.

Keywords: density-dependent phase polyphenism; epizootics; *Anticarsia gemmatalis*; *Spodoptera spp.*; insect-pathogen interaction; immune response

RESUMO

SOARES, Carolina de Oliveira, D.Sc., Universidade Federal de Viçosa, julho de 2025. **Do Isolamento à Superlotação: Efeitos da Densidade Populacional na Imunidade e nas Interações Inseto-Patógeno em Lepidópteros**. Orientador: Simon Luke Elliot. Coorientadores: Sheena Catherine Cotter, Farley William Souza Silva e Eduardo Carlos Costantin.

O polifenismo de fase dependente da densidade é um fenômeno no qual insetos desenvolvem fenótipos distintos em resposta à densidade populacional, resultando em diferentes características morfológicas e fisiológicas dentro de uma mesma espécie. A dinâmica das interações hospedeiro-patógeno em populações de insetos pode ser influenciada por diversos fatores, entre eles a densidade populacional, que pode atuar como importante modulador do curso de epizootias. Nesta tese, investigamos como a densidade populacional e os fenótipos expressos por *Anticarsia gemmatalis* influenciam a dinâmica de infecção pelo baculovírus AgMNPV, e se duas espécies de importância econômica, *Spodoptera frugiperda* e *Spodoptera eridania*, apresentam algum grau de polifenismo de fase dependente da densidade, por meio de alterações na coloração larval ou em respostas imunes. Em *A. gemmatalis,* demonstramos a ocorrência dos três fenótipos de coloração (verde, intermediário e preto) em ambas as densidades testadas (1 e 4 larvas por pote, representando condições solitária e gregária, respectivamente), sendo que a maior densidade induziu maior frequência de fenótipos escuros. Fenótipos pretos foram mais suscetíveis ao AgMNPV e apresentaram maior replicação viral, apesar da presumida maior melanização cuticular. Já os fenótipos verdes sobreviveram por mais tempo e produziram mais corpos de oclusão do vírus. Além disso, o material viral originado de larvas gregárias foi mais virulento em infecções subsequentes. Por outro lado, a densidade não influenciou os níveis de hemócitos circulantes nessa espécie. Em *S. frugiperda* e *S. eridania*, observamos respostas específicas à densidade larval. *S. eridania* apresentou mudanças evidentes na coloração, enquanto *S. frugiperda* mostrou variação fenotípica limitada. Em *S. eridania*, a resposta de encapsulamento foi intensificada em densidades mais altas, e ambas as espécies exibiram aumento na contagem de hemócitos na densidade mais elevada, apoiando parcialmente a hipótese da Profilaxia Dependente da Densidade (DDP). Em *S. frugiperda*, fenótipos mais escuros produziram maior quantidade de corpos de oclusão viral após infecção com SfMNPV. Em conjunto, nossos resultados demonstram que alterações fenotípicas induzidas pela densidade podem modular a suscetibilidade do hospedeiro e a replicação viral, e que tais ajustes fenotípicos podem ser mais comuns do que se imaginava, mesmo em espécies sem

comportamento gregário evidente. Assim, expandimos o entendimento sobre a aplicabilidade da DDP em insetos e ressaltamos a importância de considerar características específicas de cada espécie nas interações entre densidade populacional, imunidade e suscetibilidade a patógenos.

Palavras-chave: polifenismo de fase dependente da densidade; epizootias; *Anticarsia gemmatalis*; *Spodoptera spp*; interação inseto-patógeno; resposta imune.

SUMARY

CHAPTER I
GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

Phenotypic plasticity consists of the expression of more than one phenotype by the same genotype in response to environmental variation (Baldwin, 1986; Pfennig et al., 2010; Soeter et al., 2010; Simões et al., 2013). Considered an essential phenomenon for the evolutionary success of insects, it is an important mechanism of ecological adaptation and of great interest for studies in the fields of ecology and evolution, as it allows organisms greater flexibility to survive and adapt to new habitats or variable environments (Via et al., 1995; Lande, 2015; Beaman et al., 2016; Colautti et al., 2017).

The phenotypes resulting from this plasticity can be morphological, physiological, behavioral, biochemical traits, or an interaction among all these processes (Lofeu & Kohlsdorf, 2015; Ayali, 2019). In this sense, the manifestation of density-dependent phase polyphenism in insects is a very clear example of phenotypic plasticity, and refers to changes in phenotype in response to changes in the population density of the same species (Wilson & Cotter, 2009).

During epizootics, the transmission of insect pathogens may occur directly through contact between an infected host and a susceptible host, or indirectly, requiring a vector or an intermediate host (Tanada & Kaya, 1993; Begon et al., 2002). Considering direct transmission, many host-parasite interaction models suggest that the risk of parasite transmission depends on density, such that transmission increases linearly with population density (Anderson & May, 1981; Wilson & Reeson, 1998). In an evolutionary context, if the individual risk of infection increases with population density, it is expected that these organisms will also develop strategies to enhance resistance to the pathogen, as stated by the density-dependent prophylaxis (DDP) hypothesis (Wilson et al., 2002).

According to the DDP hypothesis, in insects that exhibit phase polyphenism, resistance to pathogens will increase in high-density populations, in accordance with the probability of infection (Wilson et al., 2002). Thus, understanding how pathogens act in polyphenic insects (i.e., whether phenotypes influence transmission patterns) can provide important insights into the processes that occur during epizootics (Fuxa & Tanada, 1987; Goulson et al., 1995).

In this General Introduction, we begin with an overview of fundamental concepts for understanding the study, such as phenotypic plasticity, especially the density-

dependent phase polyphenism, the form of plasticity investigated here. We also address the DDP hypothesis and aspects of the insect immune system, with emphasis on mechanisms related to pathogen resistance. Then, we will present examples of these phenomena described in the entomological literature. Finally, we will provide a general overview of our study, highlighting its objectives.

1.1.Density-dependent phase polyphenism

Phenotypic plasticity can be defined as the ability of an organism to express different phenotypes in response to environmental variables (Wolkovich et al., 2013; Lima, 2017). This plasticity can be favorable (adaptive), detrimental, or neutral (Palacio-López et al., 2015). Typical examples of adaptive plasticity include immune responses to parasites and behaviors related to predator avoidance. On the other hand, examples of non-adaptive plasticity include phenotypes that bring no apparent benefits to the individual, generally associated with developmental disturbances caused by resource limitations or environmental stress (Martins et al., 2020).

Phenotypic plasticity has a strong genetic basis, resulting from complex interactions among genes, development, and epigenetics (Fusco & Minelli, 2010; Martins et al., 2020). This phenotypic manifestation represents an important mechanism of ecological adaptation and can occur more restrictively or broadly depending on the characteristics of the individual's genotype (Nijhout, 1999; Lima, 2017).

One widely reported type of phenotypic plasticity in insects is "density-dependent phase polyphenism," which refers to changes in phenotype in response to population density shifts among individuals of the same species (Wilson & Cotter, 2009). These changes may include alterations in color, behavior, morphology, reproduction, development, among others, that contribute to evolutionary success (Pener, 2009; Wang & Kang, 2014). A clear example of the manifestation of different phenotypes, which can be observed according to the density of individuals, is cuticular melanization. In these cases, individuals living in groups (gregarious) tend to have more melanized cuticles than solitary ones (Cotter et al., 2004; Silva et al., 2013).

There are numerous records in the literature of phase polyphenism occurring among insects (Reeson et al., 1998; Pener, 1991; Siva-Jothy, 2000; Barnes & Siva-Jothy,

2000; Wilson et al., 2002; Wilson & Cotter, 2009; Silva et al., 2013; Wen et al., 2025). It has been demonstrated that in Orthoptera, in response to habitat temperature, solitary-phase nymphs of *Locusta migratoria* (Linnaeus, 1758) or *Schistocerca gregaria* (Forsskål, 1775) (Orthoptera: Acrididae) can develop various color phenotypes and changes in morphological and physiological traits as an environmental adaptation mechanism (Hertz & Imms, 1937; Hunter-Jones, 1962; Sugahara et al., 2015).In Coleoptera, there are reports of *Tenebrio molitor* (Linnaeus, 1758) (Coleoptera: Tenebrionidae) showing density-dependent phase polyphenism, with larvae reared at high densities exhibiting color variations ranging from brown to black (Barnes & Siva-Jothy, 2000). In this case, the degree of cuticular melanization was directly associated with pathogen resistance, with darker individuals being more resistant than lighter ones, regardless of rearing density (Barnes & Siva-Jothy, 2000). Additionally, Savvidou and Bell (1994) also observed in *Gnatocerus cornutus* (Fabricius, 1798) (Coleoptera: Tenebrionidae) a significant increase in development time, mortality, and cannibalism incidence as larval density increased. In Lepidoptera, it has been shown that *Spodoptera litura* (Fabricius, 1775) (Lepidoptera: Noctuidae) exhibits increased cuticular melanization and enhanced cuticular immune responses when reared at higher population densities, highlighting its adaptability to population density changes (Wen et al., 2025). Furthermore, it was observed that pathogen resistance in *Spodoptera exempta* (Walker, 1856) (Lepidoptera: Noctuidae) is also phenotypically plastic, and that more melanized cuticles may indicate a more active immune system (Reeson et al., 1998).

Several authors (Reeson et al., 1998; Cotter et al., 2004b; Lee & Wilson, 2006) have shown that *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera: Noctuidae) presents individuals in the gregarious phase with more melanized cuticles during the final larval instars. Similarly, Silva et al. (2013) found that the presence of conspecifics in *Anticarsia gemmatalis* (Hübner, [1818]) (Lepidoptera: Erebidae) induces changes in larval coloration and influences secondary defenses such as encapsulation response, capsule melanization, and hemocyte count. Under more extreme conditions, individuals may exhibit phenotypic changes, although these are not always visible to the naked eye (Wilson & Cotter, 2009).

The records of polyphenism observed in these groups reinforce that insects presenting this phenomenon have greater flexibility to survive and adapt in variable

environments (Colautti et al., 2017), in addition to exhibiting prophylactic plastic responses including increased resistance to pathogens (Silva et al., 2013). This type of plasticity can be especially advantageous in contexts of high population density, where competition for resources is more intense and the risk of parasite and pathogen transmission increases (Anderson & May, 1981). Social or gregarious insects, for example, are particularly exposed to these risks (Rafaluk et al., 2017), which has favored the evolution of defensive strategies such as DDP, according to which increased density induces a preventive immune response (Wilson & Reeson, 1998).

1.2.Density-Dependent Prophylaxis Hypothesis

According to the Density-Dependent Prophylaxis (DDP) Hypothesis, since the infection potential represented by parasites varies with population density, keeping disease resistance mechanisms activated involves high energetic and physiological costs. Thus, prophylactic investment in immune defenses must be flexible, increasing when the risk of infection is high and decreasing when this risk is low. Since these defenses are costly, keeping them continuously activated is not advantageous, especially in low-density contexts, in which the chance of contact with pathogens is reduced. On the other hand, in high densities when the risk of transmission is greater, investment in these defenses becomes advantageous, since it increases the chance of survival in the face of an imminent infection (Wilson & Cotter, 2009).

The DDP hypothesis makes three main predictions: (1) individuals previously exposed to crowded conditions will show lower mortality rates when infected; (2) investment in disease defense mechanisms will increase proportionally with population density; and (3) in natural environments, parasite-induced mortality tends to stabilize (saturate) as host density increases. However, this third prediction is controversial, as it may also result from non-adaptive processes (D'Amico et al., 1996; Dwyer et al., 1997).

According to DDP, insects exhibiting phase polyphenism tend to increase their resistance to pathogens in high-density populations by intensifying cuticular melanization as a defence mechanism. Thus, cuticular melanization is positively associated with phenoloxidase activity (the enzyme responsible for melanin production) in the hemolymph (Reeson et al., 1998).

Some studies provide evidence for the applicability of DDP in insects. For example, Barnes and Siva-Jothy (2000) were the first to report a density-dependent prophylactic response in *Tenebrio molitor*, observing that resistance to the fungus *Metarhizium anisopliae* increased with the degree of cuticular melanism. In Lepidoptera, Reeson et al. (1998) and Wilson et al. (2001) demonstrated that resistance of *Spodoptera exempta* to nucleopolyhedrovirus, and *Spodoptera littoralis* to the fungus *Beauveria bassiana*, also increased according to the intensity of cuticular melanization, which was related to larval density. Similarly, Silva et al. (2013) observed that the presence of conspecifics induces prophylactic phenotypic and immunological responses in *Anticarsia gemmatalis*, while more recent evidence indicates that rearing *Spodoptera litura* at high densities intensifies immune responses in its cuticle and significantly upregulates genes associated with immunity and tyrosine metabolism, suggesting a strategic adaptation to conditions of higher infection risk (Wen et al., 2025).

1.3.Insect immune system

To reduce the risk of infection, in response to selective pressure exerted by pathogens, organisms have developed defense mechanisms (Wilson, 2005). The immune system of insects consists of physical barriers and physiological responses (Wang Tan et al., 2006; Chapman, 2009).

Physical barriers act as the first line of defense, where the cuticle functions as a shield against pathogen entry, while the peritrophic membrane (PM) prevents direct contact of microorganisms and abrasive particles with the cells of the intestinal epithelium (Lehane, 1997; Terra, 2001). When this first barrier is insufficient, the physiological response acts (Vincent & Wegst, 2004). This response is subdivided into two components present in the hemolymph: the cellular response and the humoral response (Schmid-Hempel, 2005; Jaime et al., 2022).

The humoral response includes the production of antimicrobial peptides, reactive oxygen or nitrogen intermediates, and complex enzymatic cascades that regulate coagulation or melanization of the hemolymph (Lavine & Strand, 2002; Strand, 2008; Eleftherianos et al., 2021). In contrast, the cellular response refers to immune reactions mediated by hemocytes, such as phagocytosis, nodulation, and encapsulation (Strand & Pech, 1995; Schmidt et al., 2001). Different types of hemocytes, such as plasmatocytes

and granular cells, perform specific defensive functions by recognizing and eliminating invaders (Lavine & Strand, 2002; Eleftherianos et al., 2021).

The enzyme phenoloxidase (PO), in addition to directly acting in the melanization process (Wilson et al., 2001; Cotter & Wilson, 2002), plays a key role in the insect immune response, especially in the encapsulation reaction (Robb et al., 2003), which occurs in the hemolymph in response to microorganism invasion (Wilson et al., 2002; Moret & Siva-Jothy, 2003). However, maintaining this system imposes a high cost in energetic and nutritional terms (Little et al., 2005).

Besides immunological defense mechanisms against pathogens, insects also have defenses based on non-immune mechanisms, such as fever and other behavioral changes that prevent and combat infections (Elliot et al., 2002; Elliot & Hart, 2010; Parker et al., 2002; Parker et al., 2011). The capacity of an organism to adapt and protect itself against environmental and biological threats through changes in its behavior or ecology is referred to as ecological immunity (Schmid-Hempel, 2005).

In insects, ecological immunity may involve various strategies, including habitat modification, avoidance of areas infested with pathogens, and interaction with other organisms to obtain defensive benefits. The level of investment in defense varies according to the insect, its environment, and the selective pressures it faces (Schmid-Hempel, 2005; Schulenburg et al., 2009).

1.4.Objects of study

The order Lepidoptera is one of the most diverse and abundant among insects, comprising species of great economic importance (Ouaba et al., 2022). Among these species, *Anticarsia gemmatalis* (Hübner, [1818]) (Lepidoptera: Erebidae), *Spodoptera frugiperda* (J.E. Smith, 1797) and *Spodoptera eridania* (Cramer, 1782) (both Lepidoptera: Noctuidae) stand out as significant agricultural pests, especially in the larval stage, in which they cause severe damage to cultivated plants (Pitre & Hogg, 1983; Habib et al., 1983; Sosa-Gómez et al., 1993; Abdullah et al., 2000; Lourenção & Santos, 2016).

This group of insects, whose caterpillars cause the greatest economic losses, shows great variability in size, coloration and shape (Capinera, 2008; Carneiro et al., 2024). In the larval stage, they have three pairs of true legs on the front part of the body

and two to five pairs of prolegs on the posterior part. The adults, in turn, have two pairs of membranous wings covered by scales and a sucking mouthpart. Although they do not cause direct damage to plants, adults are harmful by laying eggs that give rise to new caterpillars, perpetuating the infestation cycle (Carneiro et al., 2024).

The species *A. gemmatalis*, *S. frugiperda* and *S. eridania* are nocturnal lepidopterans and have a holometabolous development, comprising the stages of egg, larva (with up to six instars), pupa and adult (Gallo et al., 2002). The life cycle of each of these species varies according to environmental conditions (Gallo et al., 2002; Sarmento et al., 2002). In *A. gemmatalis*, the egg stage lasts approximately 3 to 5 days. The larval stage lasts 10 to 12 days, and pupation occurs in the soil, lasting about 6 to 10 days (Gazzoni & Yorinori, 1995; Gallo et al., 2002). Adults live on average from 7 to 10 days, and female fecundity ranges from 600 to 1500 eggs, which are laid individually or in small irregular clusters or rows, without scale covering (Costilla, 1988; Gazzoni & Yorinori, 1995; Gallo et al., 2002).

Spodoptera frugiperda has a life cycle of around 30 days under ideal conditions. Females lay egg masses covered with body scales, with an incubation period of 2 to 4 days (Gallo, 2002). The caterpillars go through six instars and, in later stages, may exhibit cannibalistic behavior. The pupal stage occurs in the soil and can last between 7 and 12 days. Adult longevity can reach 10 days, and female fecundity varies from 1,000 to 1,500 eggs per individual (Gallo, 2002; Barros et al., 2010).

Spodoptera eridania has a life cycle similar to other species of the genus (Souza et al., 2014). Females oviposit egg masses on leaves, often covered with scales, with an incubation period of 3 to 4 days (Santos et al., 2010; Souza et al., 2014). The larval stage lasts on average from 12 to 18 days, and the caterpillars may show color variation throughout development (Efrom et al., 2013). Pupation occurs in the soil and lasts from 8 to 10 days. Adult longevity ranges from 7 to 12 days, while female fecundity can vary from 800 to 1300 eggs (Efrom et al., 2013; Souza et al., 2014).

Among the crops affected by these insects, soybean (*Glycine max* (L.) Merr., 1917) is of great importance to the country's economy and is currently one of its most exported products (Vasconcellos et al., 2023). Therefore, much effort has been made to control these lepidopterans, considered important pests of this crop (Vasconcellos et al.,

2023). Infestations of *A. gemmatalis* may compromise soybean production (Fugi et al., 2005), as can *S. frugiperda*, which is responsible for significant damage to this crop (Vasconcellos et al., 2023). Economically, *S. eridania* has also stood out due to its increasing incidence each crop season (Santos et al., 2010; Teodoro et al., 2013; Justus et al., 2022).

In addition to soybean, the incidence of *A. gemmatalis* has already been recorded in cotton (Douglas, 1930), where caterpillars of the genus *Spodoptera* at different developmental stages are also considered a problem (Santos et al., 2010). *Spodoptera frugiperda* may cause damage to young plants through defoliation and floral bud perforation (Santos, 2007; Gallo et al., 2002), and *S. eridania* causes damage from the early stage of floral bud emission, leading to defoliation and damage to the plant's reproductive structures (Santos, 2007).

Spodoptera frugiperda and *S. eridania* are among the main pests responsible for losses in major crops such as corn, soybean, cotton, rice, tomato and wheat (Parra et al., 2021; Ouaba et al., 2022). This broad range of host crops reflects one of the main challenges in managing these pests: the great variety of hosts that these insects find throughout the year, either due to crop rotation or the planting of species with different phenologies nearby, such as soybean, corn and cotton (Santos et al., 2005). The intense exposure of these crops to population pressure from these insects may end up selecting new feeding preferences. For this reason, it is believed that the availability of alternative hosts is enabling the development and persistence of these pests in various crops (Santos et al., 2005; Sá et al., 2009).

1.5.Baculovirus

Among the viruses with entomopathogenic potential against Lepidoptera, baculoviruses are the most well-known and studied (Rohrmann, 2019). Due to their host specificity, these viruses represent an important tool in pest management. Besides being effective, they are also a safe alternative for natural enemies, the environment, and human health (Moscardi & Souza, 2002).

Baculoviruses belong to the *Baculoviridae* family and their genome consists of a circular double-stranded DNA, ranging from 80 to 200 kb, surrounded by a rod-shaped

protein capsid, constituting the infectious unit of the virus (nucleocapsid) (Arif, 1986). Characterized by the morphological features of occlusion bodies (OCs), protein structures that protect virions in the environment, they are composed of four main genera: *Alphabaculovirus, Betabaculovirus, Gammabaculovirus* and *Deltabaculovirus* (Harrison et al., 2018; Rohrmann, 2019). The genera Alphabaculovirus and Betabaculovirus are the most widely used in integrated management rules, especially against agriculturally important caterpillars, such as *Spodoptera frugiperda* (Pavan et al., 2024).

The viral particles that infect caterpillars are embedded in a polyhedrin-based protein matrix known as polyhedra or OBs (Rohrmann, 2019). Once ingested by the caterpillar, the polyhedra dissolve in the insect's midgut, releasing viral particles that initiate the infection process (Savio & Pinotti, 2008). Infected caterpillars exhibit behavioral and morphological changes throughout the infection cycle, such as reduced feeding and discoloration of the integument (De Castro et al., 2020). Approximately seven days after infection, the larvae die, remaining attached to the substrate only by their prolegs (Hoffmann-Campo et al., 2000). Upon death, the larval integument ruptures, releasing viral polyhedra into the environment, which serve as inoculum for new infections (Moscardi, 1983; Moscardi & Souza, 2002; De Castro et al., 2020).

The first record of baculovirus infecting *A. gemmatalis* caterpillars in soybean crops dates back to 1972. In the 1980s, the world's largest biological control program using *Anticarsia gemmatalis* multiple nucleopolyhedrovirus (AgMNPV), also known as *Baculovirus anticarsia*, was implemented by the Brazilian Agricultural Research Corporation (EMBRAPA). At its peak, the virus was applied to nearly 2 million hectares of soybean (Moscardi, 1989, 1999; Moscardi & Sosa-Gómez, 1993; Moscardi et al., 2011).

The use of *Baculovirus anticarsia* replaced more than 11 million liters of chemical insecticides and resulted in an estimated savings of around 100 million U.S. dollars (Moscardi & Souza, 2002; Penteado, 2001). Currently, baculovirus-based products, such as *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV), are effective and economically viable alternatives for controlling the fall armyworm (*S. frugiperda*), especially in maize and cotton crops (Sosa-Gómez et al., 2020). Despite the discovery of a new viral isolate with potential to control *S. eridania* (Damascena et al., 2025), no

baculovirus-based commercial product is yet registered in Brazil for this species (MAPA, 2025).

Commercial production of baculovirus products is performed *in vivo*, with insect hosts reared on artificial diets (Grzywacz, Moore, & Rabindra, 2014; Valicente, Tulher, & Barros, 2010b). Consequently, population density may influence large-scale virus production (Silva et al., 2013).

Given the significant economic importance of these lepidopterans and the growing adoption of biological agents for their control, it is essential to deepen our knowledge about the phenotypic plasticity of these insects. In this way, we can understand how variation in population density can influence pathogen resistance, the immune system, and infection dynamics during epizootics, aiming to improve the production of nucleopolyhedrovirus-based biological products and optimize the efficiency of biological control strategies for these insects.

1.6.The Thesis

The thesis is composed of two experimental chapters, both addressing aspects of the interaction between nucleopolyhedroviruses and pest Lepidoptera, with a special focus on the influence of population density on infection dynamics, viral multiplication, and insect immune parameters.

In the first chapter, we investigated how the multiplication of the *Anticarsia gemmatalis multiple nucleopolyhedrovirus* (AgMNPV) can be modulated by phenotypic and population variations in *Anticarsia gemmatalis*, a species that presents density-dependent phase polyphenism. The multiplication of pathogens is a relevant factor during the transmission process in epizootics, as it is directly related to the chances of infection of new hosts (Dwyer, 1991). The greater the number of propagules produced, the greater the chance of dispersal and infection of new hosts (Vasconcelos et al., 2002). Thus, we sought to understand how the virus's ability to replicate in the host can be affected by different phenotypes and population density conditions, and how this can influence subsequent infections. For this purpose, we evaluated viral multiplication parameters, as well as the host's immune system, in order to clarify the mechanisms that favor or limit the production of viral propagules under these different conditions.

In the second chapter, we sought to investigate the occurrence of density-dependent phase polyphenism in *Spodoptera frugiperda* and *S. eridania,* two species of economic importance in the Brazilian agricultural context. This investigation is based on phenotypic changes associated with population density, already described in other species of the same genus (Reeson et al., 1998; Cotter et al., 2004; Lee & Wilson, 2006). For this, morphological analyses were carried out to identify visible changes associated with population density, as well as immunological evaluations to understand possible variations in the immune response under different aggregation conditions. For *Spodoptera frugiperda*, an analysis of susceptibility and multiplication of *Spodoptera frugiperda multiple nucleopolyhedrovirus* (SfMNPV) was also carried out, to understand possible variations in the insect's resistance to the virus and in the replication of the pathogen.

The identification of polyphenism in these species, as well as the understanding of how pathogens interact with species that present this characteristic, can bring relevant contributions both to the evolutionary ecology of Lepidoptera and to the development of more effective strategies in integrated pest management.

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CHAPTER II

POPULATION DENSITY AND LARVAL PHENOTYPE AFFECT BACULOVIRUS MULTIPLICATION IN *ANTICARSIA GEMMATALIS*

Article 1: Population Density and Larval Phenotype Affect Baculovirus Multiplication in *Anticarsia gemmatalis*

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ABSTRACT

The dynamics of epizootics in animal populations can be influenced by characteristics of the host, pathogen, and environmental factors. Among these characteristics, the host population density can be a determining factor in the transmission of pathogens, since high densities increase contact between individuals, increase the probability of infection, and can compromise the immune system. Variations in density can also induce phase polyphenism, in which individuals of the same species develop distinct phenotypes in response to population density. These phenotypes can present different levels of susceptibility to pathogens, affecting the production of new viral propagules and the progression of infections in the population. *Anticarsia gemmatalis* (Lepidoptera: Erebidae) presents density-dependent phase polyphenism, manifesting green, intermediate, and black color phenotypes, in addition to changes in immune defenses. In this study, we investigated how population density and the resulting larval phenotype influence the production of viral propagules during infection by the nucleopolyhedrovirus AgMNPV, a biological control agent for *A. gemmatalis*. For this purpose, larvae were reared in two density treatments, 1 and 4 per container (solitary and gregarious, respectively), and three experiments were conducted. In the first experiment, larvae from both densities were orally inoculated with the virus. The higher density resulted in a higher frequency of black phenotypes, but, unlike previous studies, it did not confer greater resistance to AgMNPV. Under these conditions, greater viral multiplication was observed, suggesting that crowding favors pathogen replication. Green phenotypes survived longer and produced more occlusion bodies, while black ones were more susceptible, despite the likely greater cuticular melanization. In the second experiment, the number of circulating hemocytes was quantified as a measure of the basal immune response. There was no significant difference between treatments, indicating that population density did not affect this immunological parameter. In the third experiment, we evaluated whether the viral material obtained in the first experiment influenced infection in subsequent hosts. The virus from gregarious larvae showed greater virulence and replication. These results demonstrate that density and phenotypic composition influence the dynamics of epizootics, and that the effectiveness of the immune response may depend on pathogen-specific factors. Understanding these interactions is essential to improve integrated pest management strategies and optimize the use of entomopathogenic agents.

Key words: Epizootics; Density-dependent phase polyphenism; Insect-pathogen interaction; Baculovirus, Immune defence

2. INTRODUCTION

Epizootics are outbreaks of infectious diseases that occur in an animal population. The dynamics of these epizootics in insect populations can be influenced by several factors, such as host, pathogen, and environmental characteristics (Vasconcelos et al., 2002; Schmid-Hempel, 2011). The susceptibility of insect hosts to pathogens can vary due to aspects such as population density, behavior, developmental stage, and genetics (Wilson et al., 2002; Knell et al., 1998).

Insect population density is a crucial factor in the transmission dynamics of epizootics, as high densities increase the probability of contact and, consequently, pathogen transmission (Anderson & May, 1981; Wilson et al., 2002). In addition, high densities can lead to stressful conditions that compromise the insects' immune system, increasing their susceptibility to infections (Schmid-Hempel, 2011; Knell et al., 1998). As well as host characteristics, pathogen attributes such as virulence, infectious dose, replication rate, and dispersal capacity play a central role in infection dynamics (Vasconcelos et al., 2002). The higher the level of viral replication, the greater the chances of dispersal and transmission of infectious propagules (Dwyer, 1991). The progression of infection within the host can determine the pathogen's ability to transmit to new hosts (Handel & Rohani, 2015).

Pathogen transmission models suggest that disease progression dynamics are directly related to population density (Anderson & May, 1981; McCallum et al., 2001). As population density increases and resources become scarce, the probability of pathogenic infections also increases (Anderson & May, 1981). In an evolutionary context, the host population is expected to invest in resources to resist these pathogens (Wilson & Cotter, 2009). In this sense, the density-dependent prophylaxis (DDP) hypothesis states that, as the risk of infection changes with population density, the prophylactic investment in resistance mechanisms should also be adjusted (Wilson & Reeson, 1998). Thus, a positive relationship between population density and resistance to pathogens is expected (Wilson & Cotter, 2009).

Density-dependent phase polyphenism in insects is an adaptive phenomenon, where individuals of the same species can exhibit different morphological, physiological, and behavioral forms in response to variations in population density (Pener, 2009; Wang

& Kang, 2014). The manifestation of these phenotypes may include changes in coloration, behavior, morphology, and immune defense, among others, and this phenomenon is widely reported in Lepidoptera (Reeson, 1998; Cotter et al., 2004b; Lee et al., 2006; Silva et al., 2013; Wen et al., 2025).

Anticarsia gemmatalis (Hübner, [1818]) (Lepidoptera: Erebidae) is a species characterized by presenting density-dependent phase polyphenism (Silva et al., 2013). The presence of conspecifics can result in the manifestation of different color phenotypes (green, intermediate, and black) and changes in the insect's immune defenses (encapsulation response, capsule melanization, and hemocyte counts; Silva et al., 2013).

Diseases caused by an entomopathogenic virus belonging to the Baculoviridae family (AgMNPV - *Anticarsia gemmatalis multiple nucleopolyhedrovirus*) occur naturally in its populations, resulting in epizootics that lead to a reduction in the population density of this pest (de Castro et al., 1999; Valicente, 2019). In this sense, entomopathogenic viruses play a fundamental role in the biological control of several agricultural pests, being especially relevant in the management of defoliating caterpillars in soybean crops (Moscardi, 1999).

As in other models, it is believed that baculovirus transmission is density-dependent, with infection rates proportional to the number of hosts (Anderson & May, 1981). Thus, studies investigating characteristics capable of influencing infection dynamics such as disease progression and the production of dispersal structures (multiplication/production of viral particles) are essential for understanding the processes involved in epizootics (Fuxa & Tanada, 1991; Goulson et al., 1995). Furthermore, this understanding can assist in improving strategies for microbial control programs (Silva et al., 2013).

In this context, we investigated the interaction between AgMNPV, a nucleopolyhedrovirus widely used in one of the largest biological control programs in the world (Moscardi, 1989, 1999), and *Anticarsia gemmatalis*, a species that presents density-dependent phase polyphenism (Silva et al., 2013) and is considered one of the main pests of soybean crops (Bortolotto et al., 2015). Our main focus is to evaluate whether environmental conditions (solitary or gregarious) and the phenotype expressed by the

larvae influence the dynamics of infection, through the amount of viral propagules produced during the course of the disease.

Our aim was to understand better the interactions between population density, phenotype, and immune response in larvae of a polyphenic lepidopteran (*Anticarsia gemmatalis*) infected with baculovirus. In the first experiment, we evaluated the resistance of *A. gemmatalis* to viral infection and quantified the multiplication of the pathogen by estimating the number of occlusion bodies (OBs) produced per infected larva at the caterpillar rearing densities, as well as the phenotypes they expressed. In the second experiment, we characterized the immune response by measuring the density of circulating hemocytes in the hemolymph, since these cellular components are directly involved in immune defenses, both in cellular responses and in the regulation of humoral mechanisms (Feng et al., 2021). Finally, in the third experiment, we investigated the infectivity of the viral material produced in the previous treatments, testing the hypothesis that the origin (host density and phenotype) and the amount of occlusion bodies (OBs) generated during the initial infection affect the ability of the virus to initiate and sustain new infections in subsequent hosts, evaluating whether these viral populations present distinct characteristics and, possibly, greater infectivity in relation to the original virus.

Our hypothesis is that crowded conditions promote greater resistance to the virus and, as a consequence, lead to a reduction in the production of viral particles. We hope to understand whether there is a loss of virulence or infectious capacity associated with the environment of origin of the virus, which may have important implications for the dynamics of infection in epizootics.

3. MATERIAL AND METHODS

2.1.Insect colony

The *Anticarsia gemmatalis* colony was established in the Laboratory of Insect-Microbe Interactions at the Universidade Federal de Viçosa in 2024. The insects were obtained from Pragas.com® - Piracicaba, SP. Adults were kept in a 50 × 50 cm wooden cage lined with sheets of A4 bond paper used as an oviposition substrate (Figure 1). These sheets were removed every two days for egg collection. Adults were fed *ad libitum* with a nutritional solution consisting of honey (20.0 g), beer (350 ml), sucrose (50 g), ascorbic acid (1.05 g), nipagin (1.05 g) and water (650 ml) (adapted from Hoffman-Campo et al.,

1985). The diet was provided in a cotton pad placed at the bottom of the cage, on a Petri dish.

The collected sheets with eggs were kept in 1-liter transparent plastic pots until neonate larvae hatched. They were then transferred with the help of a fine-bristled brush to 50 ml plastic cups containing an artificial diet based on textured soy protein, white beans, wheat germ, brewer's yeast, nipagin, ascorbic acid, sorbic acid, formaldehyde, agar-agar, casein, tetracycline and vitamin solution (adapted from Hoffman-Campo et al., 1985) (Figure 1).

Figure 1- Rearing of *Anticarsia gemmatalis.* The insect colony was kept in a climate-controlled chamber (25 ± 1 °C; relative humidity 60% ± 10% and photoperiod of 14 hours).

2.2.*Anticarsia gemmatalis Multiple Nucleopolyhedrovirus* **(AgMNPV)**

Anticarsia gemmatalis Multiple Nucleopolyhedrovirus (AgMNPV) was obtained from CNPSo-EMBRAPA. To produce a stock suspension (2×10^9 polyhedra ml^{-1}), it was multiplied in *A. gemmatalis* caterpillars. For this purpose, the caterpillars were individualized in 100 ml plastic pots and kept in a climate-controlled chamber (25 ± 1 °C; relative humidity 60% ± 10% and photoperiod of 14 hours), until the tenth day post-hatching (approximately fourth instar). To infect the insects, an artificial diet was prepared, similar to that used in the rearing (above), but without the addition of formaldehyde so that the virus would not lose viability (Gupta et al., 2007). A piece of this diet (approximately $1cm^3$) was fed to each caterpillar. On the surface of each of these blocks, 20 µl of viral suspension was pipetted at 1×10^8 polyhedra ml^{-1} (i.e. 5×10^6 polyhedra per block of diet). After 24 hours, by which time the insects had completely

consumed the diet blocks, the caterpillars were returned to their normal dietary treatment (see above).

Caterpillar mortality was monitored daily. Those with baculovirus symptoms (at *ca.* seven days) were collected and macerated using a porcelain mortar and pestle with distilled water. This were then filtered through gauze and the broth resulting from this process was subjected to centrifugation for 3 minutes at 3,000 rpm. Subsequently, the precipitate was discarded and the supernatant was subjected to centrifugation at 8,000 rpm for 20 minutes (Batista-Filho, 1997). The precipitate obtained was suspended in distilled water and applied to the Neubauer chamber for confirmation and counting of viral polyhedra. The material was then stored until use in experiments.

2.3.*Anticarsia gemmatalis* density treatments

It has been shown that a single conspecific is sufficient to trigger density-dependent phenotypic changes in *A. gemmatalis* (Silva et al., 2013). Thus, instead of using a series of densities, we chose two densities (based on Silva et al., 2013 and Costantin et al, 2022) to simulate the solitary and gregarious environment, and stimulate the expression of the phenotypes (green, intermediate and black). This also allows us to ensure the hygiene of the containers until the end of the experiment. To set up these treatments, *A. gemmatalis* eggs were removed from the rearing and kept at 25 ± 1 °C, 60% ± 10% relative humidity and a 14-h photoperiod. After hatching (maximum of 24 h), the caterpillars were placed in 100 ml plastic pots, at two densities: 1 or 4 per pot. They were kept on artificial diet under the same rearing conditions described above until used in the tests. This procedure was adopted for the experiments described below. In density 4 treatments, only one insect, randomly selected, was used for the experiments, in order to avoid pseudoreplication (Silva et al., 2013).

2.4.Determination of phenotypes

One component of density-dependent variation in the *A. gemmatalis* phenotype is the different color phenotypes: green, intermediate or black (Silva et al., 2013) (Figure 2). Phenotypes were determined visually on the ninth day after the start of the experiments, when we were about to start handling the caterpillars, as excessive handling can trigger changes in the phenotype (Costantin et al., 2022).The dorsal regions of the caterpillars were photographed with a digital camera in a portable mini photo studio (USB, 60 × 60 × 60 cm, 140 LEDs), with a neutral white background and standardized lighting, in order to ensure consistency in capturing colors between images. We took into account the color

of the head capsule and body, where for the green phenotype, we considered the caterpillar with an olive-green body with prominent black spots and a head capsule with colors ranging from green to yellow; for the intermediate phenotype, we considered those with black spots arranged on the dorsum and subdorsal, with a yellow-orange coloration of the head capsule; and for the black phenotype, we considered those with a dark body and a yellow-orange head capsule (adapted from Fescemyer & Hammond, 1986; Silva et al., 2013) (see figure 2). This procedure was adopted in the three experiments.

Figure 2- Caterpillars of *Anticarsia gemmatalis* expressing the three color phenotypes. A) Green; B) Intermediate and C) Black. Author's personal archive.

2.5.Experiment One: Susceptibility to AgMNPV and quantification of viral production

The number of virus particles (OBs) produced during the infection process can help in understanding the processes that occur during epizootics (Fuxa &Tanada, 1991; Goulson et al., 1995). Understanding this can also be informative for the production of OBs in commercial bioinsecticide production (Paiva, 2013).

For this purpose, the insects were kept at two densities (1 and 4), with or without baculovirus inoculation, i.e. a 2 × 2 factorial design. Forty-five 10-day-old caterpillars (n = 180) were used per treatment. Twenty-four hours before inoculation with AgMNPV, the caterpillars were kept individually and left without food. Subsequently, the stock viral suspension (see item 2.2) was diluted in Tween 80® (0.01% v/v) to a concentration of 1 × 10^8 polyhedra ml^{-1} for use in the experiment. An aliquot of 20 µl of this viral suspension was pipetted onto the surface of a soybean leaf disc (1 cm in diameter), resulting in a dose

of 2×10^6 polyhedra per disc for the virus treatments. Caterpillars that did not consume the entire leaf disc during this period were excluded from the experiment to ensure that all individuals ingested a uniform number of viral particles. After this period, a soybean leaf disc of the same size was inoculated with 20 µl of Tween 80® (0.01% v/v) and supplied to the control treatments.

One day after inoculation, the caterpillars were given artificial diet again (see 2.1). Mortality was assessed daily until death or pupation. Dead caterpillars with typical baculovirus symptoms or that did not respond to physical stimulus with a brush (approximately from the third day) were collected for counting polyhedra.

Cadavers were collected, weighed on an analytical balance (Shimadzu AY220) and macerated with the aid of a porcelain mortar and pestle containing distilled water. The viral suspensions obtained from these larvae were filtered through gauze and 1 ml of these suspensions were added to a test tube containing 9 ml of Tween® 80 (0.01% v/v) for dilution. The OBs were then counted in a Neubauer® chamber with the aid of an optical microscope at 400 × magnification. Two counts were performed for each repetition and the mean was taken to calculate OB/larva. These values were adjusted by the caterpillars' weights to give OB/g. The remaining viral suspensions were stored and frozen at -20 °C for use in experiment three.

2.6.Experiment Two: Hemocyte counting bioassay

Changes in insect immunological parameters can be considered a phenotypic adjustment of insects, for example, the increase in hemocyte density (Wilson et al., 2002; Cotter et al., 2004). In this assay, insects were kept at two densities (1 and 4) as above, using 30 10-day-old caterpillars (n = 60) per density. A 2.5 µl sample of hemolymph was collected from each caterpillar using a fine needle, piercing a small hole near the first pair of legs. Subsequently, the sample was added to an Eppendorf tube with 20 µl of anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM NaEDTA and 41 mM citric acid, pH 4.5) (adapted from Ibrahim & Kim, 2006). A 10 µl aliquot of the suspension was added to a Neubauer® chamber to count the total number of hemocytes under an optical microscope at 400 × magnification. The final value was based on the average of the counts of the two aliquots (adapted from Ibrahim & Kim, 2006).

2.7.Experiment three: Evaluation of Virulence and Multiplication Capacity of OBs From Experiment 1

The objective of this assay was to investigate whether the viral material obtained in experiment one differs in terms of its impact on subsequent infection dynamics, focusing on two main parameters: virulence, measured by the time required to cause larval death, and viral multiplication, estimated by the number of OBs produced per infected individual. To compare the extremes of viral production, we selected four insects that had died in each density treatment (densities 1 and 4). Of these, we selected two insects with the highest and two with the lowest OB production per larva. The selected replicates were: Density 1: Lower production: VS15 and VS27 (≈ 6×10^6 OBs/larva); Higher production: VS6 and VS8 (≈ 2×10^8 OBs/larva), Density 4: Lower production: VG29 and VG35 (≈ 2×10^6 OBs/larva); Higher production: VG3 and VG20 (≈ 3×10^8 OBs/larva).

These samples were used to evaluate the virulence of the viral material produced, through a new standardized bioassay. For that, the insects used were obtained from the stock and kept individually in 100 ml plastic cups at a density of four per pot (as above), until the experiment was performed. Twelve replicates were used per treatment, including the virus-free control. On the tenth day, the phenotypes were recorded and the larvae were kept without diet for 24 hours.

After this period, viral suspensions standardized at a concentration of 1×10^6 polyhedra/ml were prepared from each treatment. Soybean leaf discs were inoculated with 20 µl of this suspension, as above. It is important to note that the inoculum was equivalent to 2×10^4 polyhedra per disc, two orders of magnitude lower than in the original assay. This was so as to allow inclusion of the treatments from the insects that had produced the lower numbers of OBs. Larvae were allowed to feed for 24 hours on this material and on the following day again given artificial diet (see item 2.1). Mortality was evaluated daily until death or pupation, and the procedures for collection and counting of OBs followed the methodology described in section 2.5.

2.8.Statistical analyses

Statistical analyses were conducted using R software (4.4.1). The effects of larval density and larval phenotype on viral multiplication (OB/larva and OB/g) and hemocyte number were verified using Generalized Linear Models (GLM) followed by analysis of variance (ANOVA). The phenotypic frequency observed in the experiments was compared between treatments using the χ^2 test. The data were initially subjected to the

Shapiro-Wilk normality test, and since none of them met the normality parameters, a Quasi-Poisson distribution was assumed for subsequent analyses. Differences between treatments were compared using an F-test ($p > 0.05$). When necessary, t-tests were performed for multiple comparisons between pairs of treatments ($p < 0.05$). Survival data were used to obtain survival curves using Kaplan-Meier estimators and Median Lethal Times (LT50). Curves were compared using χ^2 Log-Rank tests and pairwise comparisons between treatments were performed with Bonferroni corrections ($p < 0.05$).

4. RESULTS

3.1 Phenotypic frequencies

Phenotypic frequency was assessed in all three experiments. In Experiment 1, we observed no significant differences between phenotypic proportions ($\chi^2_{(2)} = 2.38$; $p = 0.3041$) (Figure 3). *A. gemmatalis* caterpillars expressed all three color phenotypes (green, intermediate, and black) at both densities (1 – solitary and 4 – gregarious). The intermediate phenotype was the most frequent at both densities (54.4% in both). The frequency of green caterpillars varied from 31.1% at density 1 to 37.8% at density 4, while the black phenotype was more frequent in solitary caterpillars (14.4%) than in gregarious caterpillars (7.8%).

In Experiment 2, we observed significant differences in phenotypic frequencies between the two densities ($\chi^2_{(2)} = 16.82$; $p < 0.001$) (Figure 3). Density 1 showed a predominance of the green phenotype (80.0%), followed by the intermediate (16.7%) and black (3.3%). At density 4, the distribution was more balanced, with a higher frequency of black caterpillars (36.7%), followed by 33.3% of intermediate phenotype caterpillars and 30.0% of green phenotype caterpillars. In the third experiment, carried out only at density 4, there were also significant differences in the distribution of phenotypic frequencies ($\chi^2_{(3)} = 74.89$; $p < 0.001$). The intermediate phenotype was the most frequent (41.6%), followed by green (37.8%) and black (20.6%) (Figure 3).

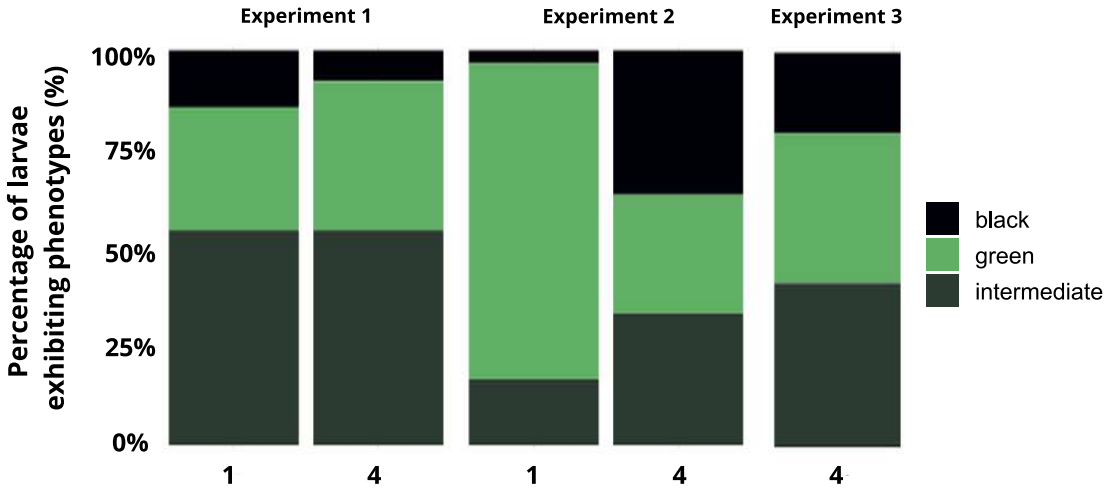

Figure 3- Distribution of the percentage of *Anticarsia gemmatalis* caterpillars that expressed the three color phenotypes (green, intermediate and black), according to population density treatments. The caterpillars were reared in 100 ml plastic pots containing: one caterpillar per pot (solitary condition) or four caterpillars per pot (gregarious condition), as described in the methodology. The phenotypic frequency data refer to the individuals used in the following experiments: Experiment 1: Susceptibility to AgMNPV and quantification of viral production; Experiment 2: Hemocyte density; Experiment 3: Virulence and multiplication of OBs obtained in Experiment 1. In this last experiment, only caterpillars from density treatment 4 (gregarious condition) were used.

3.2 Experiment One: Susceptibility to AgMNPV and quantification of viral production

In the bioassay of susceptibility to AgMNPV and quantification of viral production, we evaluated the survival of *A. gemmatalis* for ten days (240 hours) in four treatments. No mortality was observed in the control groups (Control-Solitary and Control-Gregarious) throughout the evaluation period, contrasting with significant mortality in the inoculated treatments ($\chi^2_{(3)} = 256.83$; $p < 0.001$). Controls were excluded from subsequent analyses. Among inoculated insects, density did not significantly affect survival time; mean survival was 134.4 ± 7.2 hours for the Baculovirus-Solitary group and 158.4 ± 6.7 hours for the Baculovirus-Gregarious group ($\chi^2_{(1)} = 3.74$; $p = 0.053$) (Figure 4A).

Analysis of survival times according to caterpillar phenotype (considering only inoculated individuals) revealed a significant effect of phenotype ($\chi^2_{(2)} = 9.45$; $p = 0.008$). Caterpillars with the black phenotype exhibited the shortest mean survival time (120 ± 13.68 hours), which differed significantly from the green phenotype (160.8 ± 9.12 hours) ($\chi^2_{(1)} = 7.41$; p = 0.0065), but not from the intermediate phenotype (139.2 ± 0.25 hours). Caterpillars with the green phenotype tended to survive longer than those with the intermediate phenotype ($\chi^2_{(1)} = 3.59$; $p = 0.058$), although this difference was marginally non-significant (Figure 4B).

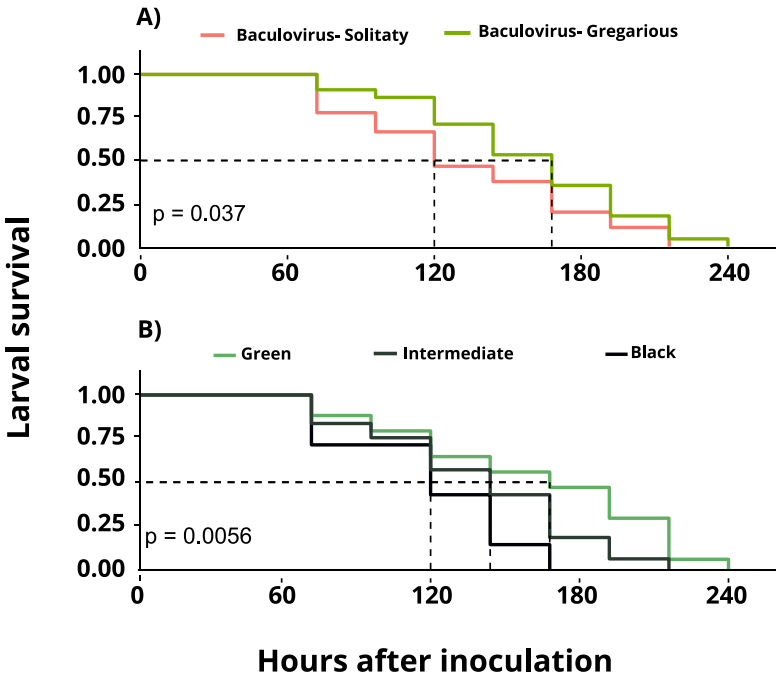

Figure 4- Survival curves of *Anticarsia gemmatalis* caterpillars inoculated with AgMNPV in: A) In two densities B) In different phenotypes. Caterpillars were reared at two densities per 100 ml plastic pot and the effect of density (1 and 4, solitary and gregarious treatments respectively) and phenotype (green, intermediate and black) on daily mortality were evaluated. For this purpose, we had four treatments, two baculovirus treatments were inoculated with a viral suspension of 1×10^8 polyhedra/mL and the control treatments with only distilled water plus Tween 80® (0.01% v/v). Survival analyses are presented in the text.

There was no density x phenotype interaction in the amount of viral propagules produced per caterpillar ($F_{(1,84)} = 0.1508$; $p = 0.860248$). We therefore considered density and phenotype separately as independent variables. Viral multiplication was almost twice as high in insects reared at density 4 (Baculovirus-Gregarious) than at density 1 (Baculovirus-Solitary) ($F_{(1,88)} = 6.4880$; $p = 0.01268$) (Figure 5A).

When considering the phenotypes, pairwise comparisons showed that viral multiplication did not differ between the black and intermediate phenotypes ($F_{(1,88)} = 0.1386$; $p = 0.7106$), however, viral multiplication was twice as high in the green phenotype than in the other two ($F_{(2,86)} = 5.9520$; $p = 0.003824$) (Figure 5B).

Given the results above, we decided to investigate if the phenotype-dependent pattern was expressed more in one of the density treatments versus the other, so we examined the data from the two density treatments separately. The previous result (above) did not hold in this instance, giving marginally non-significant test statistics and broadly similar patterns across the phenotypes for the two densities. Specifically, we observed that for both solitary caterpillars (Baculovirus-Solitary) and gregarious caterpillars (Baculovirus-Gregarious) there was no difference in the yield of viral propagules produced between the three phenotypes (Respectively ($F_{(2,42)}$= 2.8836; p= 0.06707 and $F_{(2,42)}$= 2.8515; p=0.06899) (Figure 5C,D).

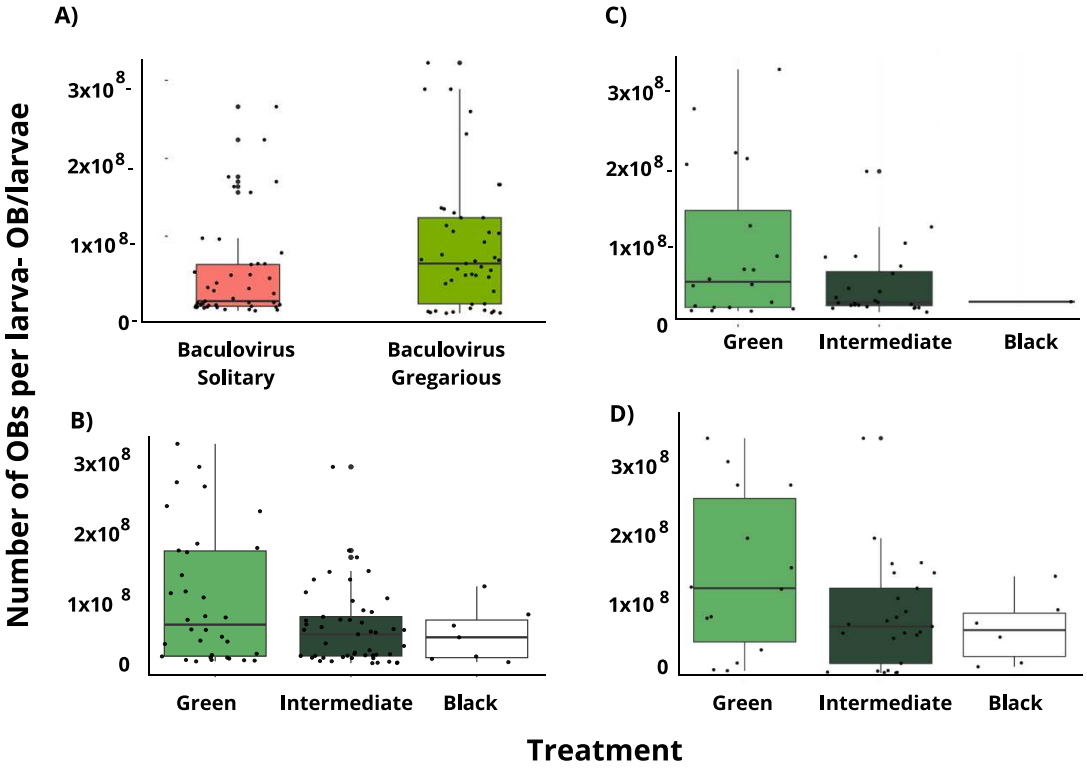

Figure 5- Number of viral propagules (OBs/larva) produced by *Anticarsia gemmatalis* caterpillars inoculated with AgMNPV. A) Comparison between two population densities: solitary condition (1 caterpillar/pot) and gregarious condition (4 caterpillars/pot); B) Comparison between the three color phenotypes: green, intermediate and black (combined densities); C) Number of OBs per caterpillar among the phenotypes in solitary condition (density 1); D) Number of OBs per caterpillar among the phenotypes in gregarious condition (density 4). The OB count was performed from aliquots of the viral suspension extracted from caterpillars killed by infection. The samples were placed in an improved Neubauer chamber and analyzed under an optical microscope. The caterpillars were reared in 100 mL plastic pots containing one (density 1 – Baculovirus-Solitary) or four (density 4 – Baculovirus-Gregarious) caterpillars per container. The effect of population density and color phenotype on the number of occlusion bodies produced per caterpillar was evaluated.

When examining the data according to OB by caterpillar weight rather than by host, there was again no interaction between density and phenotype ($F_{(2,84)}$ = 2.3831; p = 0.09847) so we examined the two possible independent variables (density and phenotype)

separately. Any effect of density was weakened to non-significance ($F_{(1,88)}$= 3.7794; p=0.05523) (Figure 6A).

In terms of caterpillar phenotypes, the data by weight followed the same pattern, qualitatively, as when observing viral multiplication per larva. Thus, there was a significant difference in viral multiplication ($F_{(2,86)}$ = 4.0938; p = 0.02011) (Figure 6B). The black and intermediate phenotypes were similar in terms of viral yield ($F_{(1,88)}$ = 0.117; p = 0.7331), although the green phenotype demonstrated a production of polyhedra per gram of caterpillar greater than the other two phenotypes ($F_{(1,88)}$ = 6.4695; p = 0.01274).

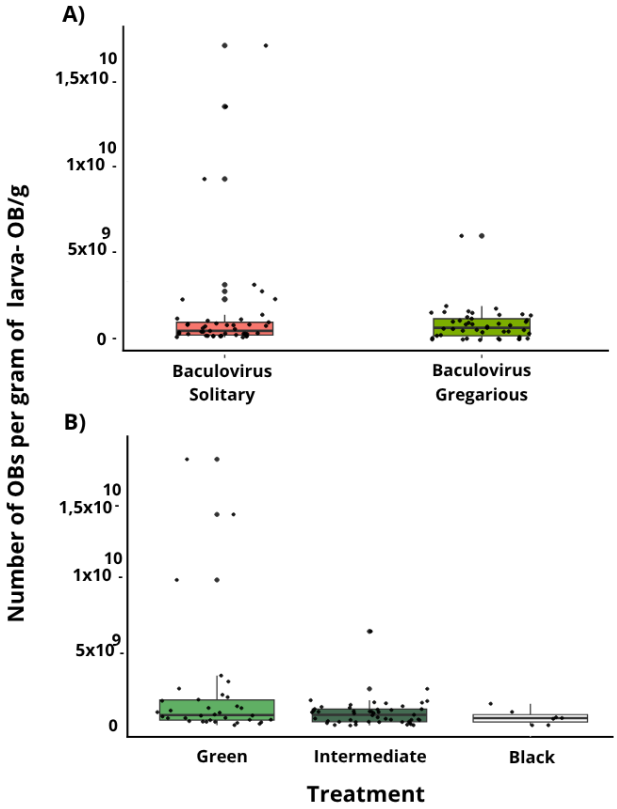

Figure 6- Number of viral propagules produced per gram (OB/g) in caterpillars of *A. gemmatalis*; A) In two densities B) In different phenotypes. To calculate this parameter, the cadavers were weighed and an aliquot of the viral suspension obtained from virus-killed caterpillars was placed in an improved Neubauer chamber for counting OBs per gram of caterpillar. The caterpillars were reared at densities 1 and 4 (Baculovirus-Solitary and Baculovirus-\gregarious respectively) per 100 ml plastic container, and the effect of the two rearing densities and the three color phenotypes (green, intermediate and black) on the number of viral polyhedra obtained from each caterpillar was evaluated.

3.4 Experiment: Hemocyte density

For hemocyte densities, there was no interaction between density and phenotype ($F_{(2,54)}$ = 0.9959; p = 0.3761). Furthermore, there were no significant effects of rearing density ($Z_{(1,58)}$ = 1.049; p = 0.294) or color phenotypes ($F_{(2,56)}$ = 0.4062; p= 0.6682) on hemocyte densities (Figure 7A,B).

We evaluated the number of hemocytes of each larval phenotype, at the two rearing densities. Thus, we observed that both solitary caterpillars (Hemocyte Solitary- $F_{(27)}$=0.3315; p=0.7208 and gregarious caterpillars (Hemocyte Gregarious- $F_{(27)}$=1.5382; p=0.233 (Figure 7C). The number of hemocytes did not vary according to the larval phenotype (Figure 7D).

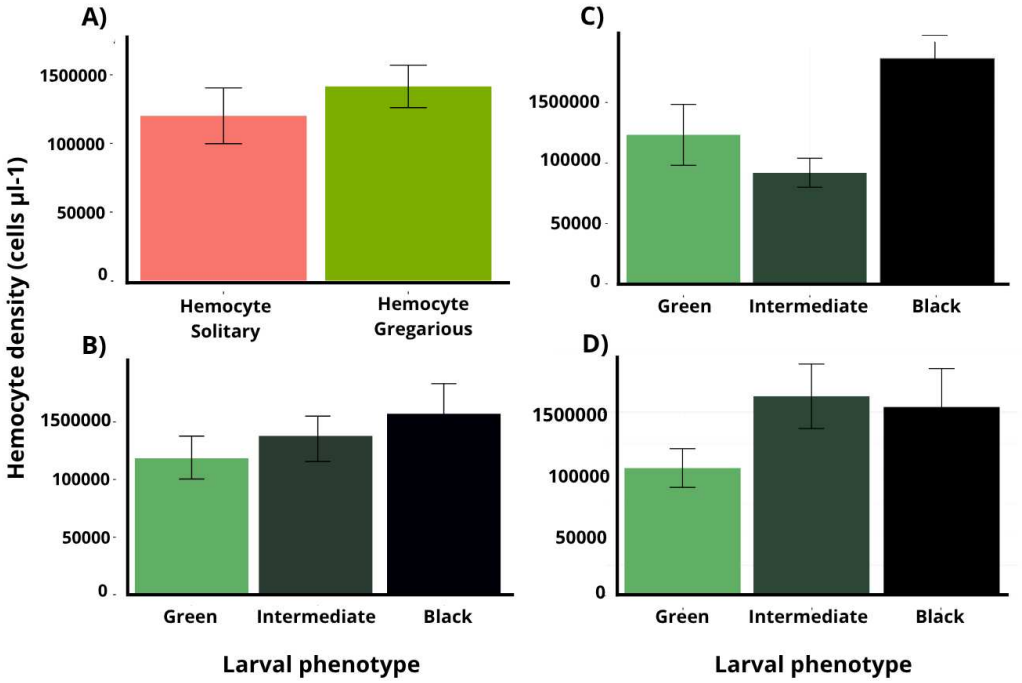

Figure 7- Density of hemocytes (μL^{-1} cells) present in the hemolymph of *Anticarsia gemmatalis* caterpillars reared under different conditions: A) Comparison between the two population densities (1 – solitary; 4 – gregarious); B) Comparison between the three coloration phenotypes (green, intermediate and black), regardless of density; C) Comparison between phenotypes in solitary condition (density 1); D) Comparison between phenotypes in gregarious condition (density 4). To count the hemocytes, an aliquot of hemolymph was extracted from each caterpillar (n = 30 per density), placed in a Neubauer chamber and analyzed under an optical microscope. The caterpillars were reared in 100 mL plastic jars containing one (density 1) or four (density 4) caterpillars per jar. The effect of population density and color phenotype (green, intermediate and black) on the density of hemocytes in the hemolymph was evaluated.

3.5 Experiment three: Virulence and Multiplication of OBs Obtained in Experiment 1

We investigated whether the viral material obtained in Experiment 1 differs in its impact on the dynamics of subsequent infection, considering two main components: virulence, represented by the time required to cause larval death, and viral multiplication, expressed by the number of occlusion bodies (OBs) produced per infected individual. Note the significant variation among viral isolates, evidenced by the median survival times, approximately 96 hours for isolates VG20 and VS27, and 192 hours for VS8 (Figure 8A).

Regarding viral production, there was a statistical difference between treatments in the number of OBs per caterpillar ($F_{(7.88)} = 2.20$; $p = 0.041$) (Figure 8B). Among the isolates tested, VG3 ($t_{(88)} = 2.98$; $p = 0.0038$), VG35 ($t_{(88)} = 2.52$; $p = 0.0136$) and VS8

($t_{(88)} = 2.48$; $p = 0.0152$) have higher amounts of OBs per caterpillar compared to the other treatments.

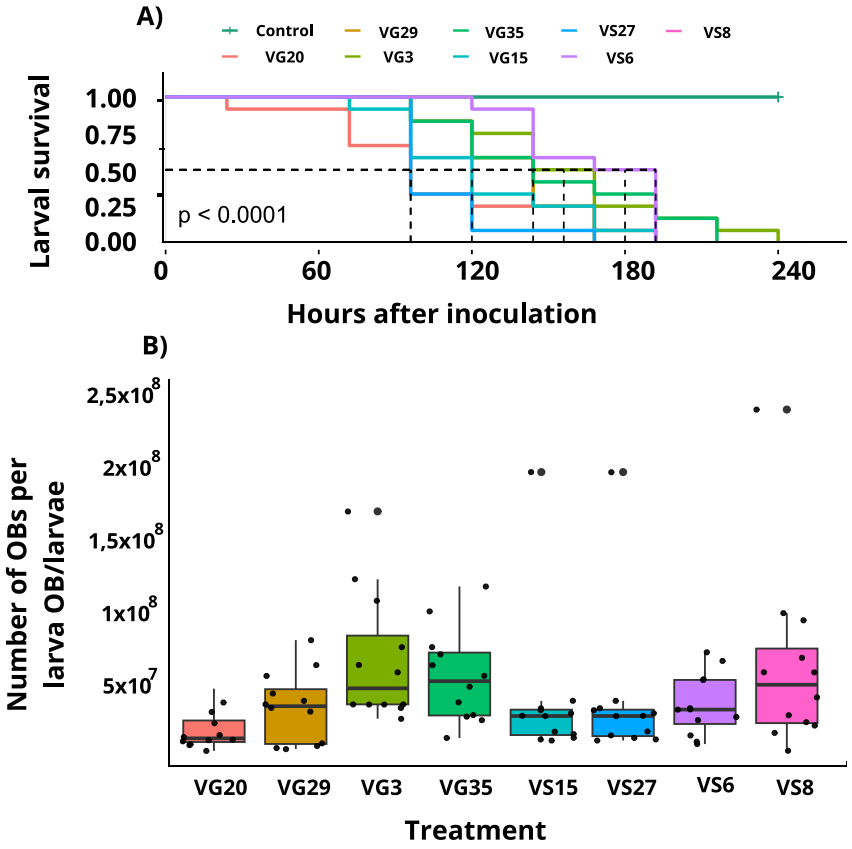

Figure 8. A) Survival curves of *Anticarsia gemmatalis* caterpillars inoculated with different AgMNPV isolates from caterpillars kept under different population densities (solitary or gregarious). B) Number of viral propagules produced per caterpillar (OB/larva) after inoculation with the same isolates.
Four isolates were selected from each density condition: two from caterpillars that, in the previous generation, showed lower production of occlusion bodies (OBs) and two with higher production. For solitary caterpillars, the selected isolates were: VS15 and VS27 (low production, ≈ 6×10^6 OBs/larva) and VS6 and VS8 (high production, ≈ 2×10^8 OBs/larva). For gregarious caterpillars: VG29 and VG35 (low production, ≈ 2×10^6 OBs/larva) and VG3 and VG20 (high production, ≈ 3×10^8 OBs/larva). Inoculation was performed with a viral suspension containing 1×10^8 OBs/mL (equivalent to 2×10^6 OBs per caterpillar). Control treatments received only distilled water with Tween 80® (0.01% v/v). For OBs counting, an aliquot of the viral suspension obtained from virus-killed caterpillars was placed in an improved Neubauer chamber and counted in a specific optical microscope.

DISCUSSION

We investigated here how density of conspecifics, and the resultant phenotype expressed by the larvae of *A. gemmatalis*, might influence susceptibility to AgMNPV and also affect the number of viral propagules produced during the infection process.

This way, we observed that larvae expressed all three color phenotypes (green, intermediate, and black) at both densities; however, the effect of density on phenotypic frequency varied among experiments (Figure 3). The increase in the frequency of black

larvae under higher density conditions observed in Experiment 2 is consistent with previous studies, which reported an increase in the proportion of dark larvae under crowding conditions (Anazonwu & Johnson, 1986; Fescemyer & Hammondi, 1986; Silva et al., 2013; Silva et al., 2016; Costantin et al., 2022).

Environments with higher population densities present a greater risk of pathogen transmission (Myers & Cory, 2015). In this context, individuals at high densities may reallocate physiological resources to strengthen their immune defenses, which is known as density-dependent prophylaxis (DDP) (Reeson et al., 1998). Based on previous studies (Silva et al., 2013, 2016; Costantin, 2022), we expected to observe a similar pattern here. However, we observed that density did not have a significant effect on larval resistance (Figure 4A).

It has been shown that *A. gemmatalis* larvae reared under crowding conditions are more resistant to AgMNPV than those reared in isolation (Silva et al., 2013, 2016; Costantin, 2022). In contrast, Anderson & May (1981); Hochberg (1991); Kim & Muturi (2013) suggest that crowded environments may induce physiological stress, compromising the immune response and making individuals more susceptible to infections. Regardless of density, larval phenotype was a significant factor in determining survival time after inoculation with AgMNPV (Figure 4B).

In Lepidoptera, cuticular melanization is generally associated with higher resistance to pathogens, especially bacteria and fungi (Reeson et al., 1998; Cotter et al., 2004). Studies with *Spodoptera exempta* and *Helicoverpa armigera* have shown that individuals with darker phenotypes (higher cuticular melanization) exhibit greater resistance to baculovirus infections. This resistance is correlated with higher activity of the enzyme phenoloxidase (PO), activated by the proPO system, which is fundamental to the insect innate immune response (Reeson et al., 1998; Cotter et al., 2004; Wang et al., 2020; Wen et al., 2025). However, our results indicate that larvae with lower cuticular melanization (green phenotype) survived longer after being inoculated with AgMNPV, unexpectedly (see Figure 4B).

Cuticular melanization, besides acting as a physical barrier, reflects a more active regulation of the proPO system, resulting in higher production of cytotoxic compounds and pathogen encapsulation (Reeson et al., 1998; Cotter et al., 2004; Wang et al., 2020;

Zdybicka-Barabas et al., 2025). In *Spodoptera littoralis*, dark larvae exhibited higher PO activity and melanotic encapsulation response, representing a higher immune investment compared to light larvae (Reeson et al., 1998; Cotter et al., 2004; Wen et al., 2025). The proPO system is triggered by cascades of serine proteases, leading to the conversion of proPO into active PO, which catalyzes the formation of melanin and compounds toxic to pathogens (Yuan et al., 2017; Wang et al., 2020; Zdybicka-Barabas et al., 2025). Baculovirus, in turn, has evolved mechanisms to suppress this response, such as inducing serpins (serpin-5 and serpin-9), which inhibit key proteases and reduce melanization, facilitating infection (Ardisson-Araujo et al., 2015; Rohrmann et al., 2013; Ji et al., 2022).

The insect immune system is based on two main innate responses: cellular immunity, which includes processes such as phagocytosis, encapsulation, and nodulation; and humoral immunity, which involves the production of antimicrobial peptides, activation of the melanization cascade, and other signaling pathways (Strand & Pech, 1995; Schmidt et al., 2001; Lavine & Strand, 2002; Strand, 2008; Eleftherianos et al., 2021). Therefore, viral manipulation of melanization can compromise an essential part of humoral defense, making insects more susceptible to infection (Yuan et al., 2017).

Three life history traits of nucleopolyhedroviruses are studied to understand their adaptation during epizootics: pathogenicity, mortality rate and number of particles produced after the insect's death (production) (Farrar & Ridgway, 2000). In our study, we investigated viral production through virus multiplication parameters. Since baculoviruses are principally transmitted horizontally through the release of new occlusion bodies into the environment after host death, the greater the production of polyhedra, the greater the chance of encountering and infecting new hosts, especially under high population density conditions (Cory & Myers, 2003). In our study, we observed that viral multiplication was higher in larvae reared at high density (Figure 5A), and that larvae of the green phenotype, although more resistant to AgMNPV infection, showed a higher rate of viral multiplication (OB/larva) than the other phenotypes (Figure 5B).

Some authors have observed that the efficiency of nucleopolyhedrovirus transmission increases with the increase in larval density of *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae) in cabbage (Vasconcelos, 1996) and granulovirus in *Plodia interpunctella* (Hübner, 1813) (Lepidoptera: Pyralidae) (Knell et

al., 1998). According to Anderson & May (1981), as population density increases and resources become limited, the likelihood of infections by pathogenic agents also increases. In the case of *Anticarsia gemmatalis*, larvae may exhibit the three color phenotypes even in the field, infesting soybean plants (Sosa-Gómez et al., 2014). Among them, the green phenotype showed the longest survival time after infection, but also produced a higher number of viral particles. This suggests that, although they survive longer, these larvae may significantly contribute to virus spread, releasing a greater amount of occlusion bodies into the environment. Thus, the high viral production observed in the green phenotype indicates that these individuals may play a central role in the virus transmission dynamics to subsequent generations (Vasconcelos et al., 2002).

In addition to host characteristics, pathogen traits such as virulence, infectious dose, replication rate and dispersal capacity play a central role in infection dynamics (Vasconcelos et al., 2002). In *Trichoplusia ni* (Hübner, [1803]) (Lepidoptera: Noctuidae), the relationship between OB production, larval weight and time to death is positive, with larvae that die later producing more virus per unit of larval weight. This implies that viral replication and tissue infection accompany host growth (Hernández-Crespo et al., 2001). In our study, we did not observe differences in viral polyhedra production per gram of larva (OB/g) between the two densities and among the three phenotypes, although we expected that the green phenotype, which showed later death, would present greater OB production per gram of larva (Figure 6). Insects that take longer to die tend to be larger and, therefore, produce more viral propagules (Hodgson et al., 2001). In our study, larvae of the green phenotype took longer to die (160.8 ± 9.12 hours) and showed a greater amount of OBs produced. Thus, viral yield would be predictable based on the mortality rate, with lower production in the black phenotype, which died faster (Hernández-Crespo et al., 2001; Burden et al., 2000).

Dushoff & Dwyer (2001) argue that a faster mortality rate indicates that the virus is dispersed earlier by the host, creating potential for early secondary disease transmission. However, this view is contested by Hodgson et al. (2001), who claim that early death of insects by NPVs may result in the production of less infectious OBs (Hernández-Crespo et al., 2001; Hodgson et al., 2001). If a pathogen becomes less virulent, the chances of transmission of these propagules are reduced (Dwyer, 1991).

Hemocytes play a central role in the insect immune defense against baculovirus infections, acting both in cellular responses and in the regulation of humoral mechanisms (Feng et al., 2021). The Density-Dependent Prophylaxis (DDP) Hypothesis proposes that as the risk of infection increases with population density, organisms should invest more in prophylactic defense mechanisms, thus establishing a positive relationship between density and resistance to pathogens (Wilson & Cotter, 2009). Although our study did not specifically assess hemocyte density in infected larvae, we did not observe an increase in hemocyte numbers in individuals reared at higher density, a result that contradicts one of the central premises of DDP (Figure 7). However, these findings are in line with the literature, in which previous studies with *Anticarsia gemmatalis* also did not identify significant variations in hemocyte density as a function of population density (Silva et al., 2016; Costantin et al., 2022).

By analyzing the material obtained in the first experiment, we found that the virus originating from larvae kept in solitary conditions, which showed longer survival time in the previous generation, also resulted in longer life in larvae infected in the subsequent generation (Figure 8A). On the other hand, three of the four isolates from gregarious individuals, which had previously shown a higher rate of viral multiplication, maintained this pattern, promoting a significant increase in the number of OBs per larva compared to the other treatments (Figure 8B). Our findings indicate that the characteristics of the source host, such as survival time and intensity of viral replication, directly influenced the virus's performance in the subsequent infection.

Garretson et al., 2018 state that successive passages of baculovirus in cell culture may lead to reduced protein expression and genomic changes, such as deletions and mutations, affecting virus stability (Garretson et al., 2018). These genetic changes from generation to generation have direct implications for its effectiveness as a biological control agent and the evolution of host resistance (López-Ferber et al., 2025).

The dissemination of baculovirus during epizootics results from a complex interaction between biological, environmental and spatial factors, requiring multidisciplinary approaches for its understanding and effective management (Mihaljevic et al., 2018; Mihaljevic et al., 2020). Several studies suggest that baculoviruses manipulate and exploit their hosts to maximize their own multiplication, altering insect behavior and physiology until they die and facilitate viral dispersion (Wang & Hú, 2019).

In this sense, our results suggest that, in a scenario of AgMNPV epizootic in populations of *A. gemmatalis*, larvae with the green phenotype may play a crucial role in the dynamics of viral dissemination. By showing greater survival after infection, these individuals continue to develop, which may result in a greater load of occlusion bodies (OBs) at the time of death. This accumulation of viral particles in the environment increases the risk of infection of new hosts, favoring the maintenance and intensification of the disease over time (Vasconcelos et al., 2002). Thus, even though initially more tolerant to infection, these larvae may act as important amplifiers of the pathogen in the population.

Considering that in our study and in others (Fescemyer & Hammondi, 1986; Silva et al., 2013; Silva et al., 2016; Costantin et al., 2022), population density influenced the frequency of phenotypes, with a predominance of the black phenotype at higher densities, in soybean crops, the phenotypic structure of the population may vary and, consequently, impact the dynamics of disease transmission. In high-density populations, the greater proportion of individuals with the black phenotype, associated with lower survival after infection, may limit viral replication time and thus restrict pathogen dissemination (Hernández-Crespo et al., 2001; Hodgson et al., 2001). On the other hand, in populations composed mostly of green larvae, which survive longer and produce a greater number of OBs, the environmental viral load tends to be higher, favoring the intensification and persistence of the epizootic.

The fact that black phenotype larvae showed greater susceptibility to baculovirus, even with characteristics associated with a more active immune response, such as cuticular melanization, indicates that the effectiveness of the immune response may strongly depend on the nature of the pathogen. Baculoviruses, such as AgMNPV, have specific immune evasion mechanisms that inhibit fundamental processes such as melanization, rendering strategies that would be effective against other pathogen groups, such as bacteria and fungi, ineffective (Schmid-Hempel, 2011; Yuan et al., 2017; Ji et al., 2022).

Many species of noctuids exhibit density-induced phase polyphenism as an adaptive strategy (Reeson et al., 1998; Wilson & Cotter, 2004), which reinforces the need for more studies focused on the dynamics of viral diseases in these systems. Future investigations into the molecular and immunological mechanisms associated with differential susceptibility between phenotypes may improve the use of baculoviruses as a

biological control tool, optimizing the selection of viral isolates with greater replication potential and field efficacy.

5. CONCLUSION

We observed that population density is associated with a higher frequency of black caterpillars at higher densities of *Anticarsia gemmatalis*. However, contrary to the Density-Dependent Prophylaxis (DDP) Hypothesis, a higher density did not result in greater resistance to the AgMNPV baculovirus. On the contrary, we observed greater viral multiplication at higher densities, suggesting that crowding conditions may favor viral replication due to increased physiological stress predisposing individuals to more severe infections. Furthermore, we observed that the viruses produced during the initial infection are fully capable of initiating and sustaining infections in subsequent hosts.Furthermore, the green and black phenotypes showed different responses to AgMNPV, with green caterpillars surviving longer but producing a greater number of occlusion bodies (OBs) before dying. Our results suggest that the population structure in terms of phenotypes may influence the dynamics of epizootics, where populations dominated by green caterpillars could facilitate the intensification and perpetuation of the viral outbreak. The observation that black caterpillars, despite potentially having a stronger immune response due to melanization, are more susceptible to AgMNPV, indicates that the effectiveness of the immune response may be influenced by pathogen-specific factors.

Understanding the phenotypic composition and immune responses of natural populations is essential to improve integrated pest management strategies and maximize the potential of entomopathogenic agents. Therefore, further studies on the physiological, molecular, and immunological mechanisms associated with differential susceptibility between phenotypes may enhance the use of baculoviruses as a biological control tool, especially in the selection of viral isolates with greater replication potential and field efficacy.

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CHAPTER III

INFLUENCE OF POPULATION DENSITY AND LARVAL COLORATION ON IMMUNOLOGICAL PARAMETERS OF *SPODOPTERA FRUGIPERDA* AND *SPODOPTERA ERIDANIA*

Article 2: Influence of Population Density and Larval Coloration on Immunological Parameters of *Spodoptera frugiperda* **and** *Spodoptera eridania*

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ABSTRACT

Density-dependent phase polyphenism is the ability of some insects to alter their phenotype in response to changes in population density, triggering transitions between solitary and gregarious phases. These transitions involve marked changes in behavior, physiology, development, reproduction, and coloration, and can result in morphological and physiological modifications that directly influence immune response and susceptibility to infections. In this study, we investigated whether two economically important agricultural pests, *Spodoptera frugiperda* and *Spodoptera eridania*, exhibit any degree of phenotypic plasticity in response to population density. To investigate the effects of population density on phenotypic and immunological traits, larvae were reared at four densities (1, 2, 4, or 8 individuals per container). In the first experiment, we evaluated cuticle melanization through larval coloration. *Spodoptera eridania* showed significant differences in coloration in both experiments in which it was evaluated (encapsulation and hemocyte assays), with increased melanization observed at intermediate densities. In *S. frugiperda*, larval coloration did not show expressive variations as a function of density. In the second experiment, we analyzed the encapsulation response by measuring the area and the intensity of melanization of the capsule formed around a nylon filament simulating a parasite. The capsule area was not influenced by density in either species. However, the intensity of capsule melanization significantly increased in *S. eridania* at higher densities. In the third experiment, we assessed the density of circulating hemocytes, which increased significantly in both species at the highest density (8 individuals per container), supporting the hypothesis of Density-Dependent Prophylaxis (DDP). Additionally, in *S. frugiperda*, susceptibility to the baculovirus SfMNPV was evaluated. Population density did not significantly influence mortality or survival time after infection. However, individuals with darker coloration produced a higher number of occlusion bodies, suggesting that more melanized phenotypes were more susceptible to viral replication. These findings provide evidence that density-dependent phenotypic plasticity and immune modulation can occur even in species that do not exhibit typical gregarious behavior, expanding our understanding of the applicability of DDP in insects and highlighting the importance of considering species-specific traits in the interactions among population density, immunity, and pathogen susceptibility.

Key words: Phenotypic plasticity; Population density; *Spodoptera spp*; Immune response; Melanization; Hemocyte density

1. INTRODUCTION

A notable example of phenotypic plasticity is density-dependent phase polyphenism in insects, in which changes in population density trigger transitions between solitary and gregarious phases, with marked differences in behavior, physiology, development, reproduction, and coloration (Pener, 2009; Wang & Kang, 2014; Ayali et al., 2019). This phenomenon is best known in locusts, such as those of the genus *Schistocerca* (Orthoptera: Acrididae). In these insects, exposure to high population densities can trigger behavioral, coloration, and morphological changes, leading to the formation of migratory swarms, while at low densities individuals remain solitary and inconspicuous (Song, 2011; Gotham & Song, 2013; Pocco et al., 2019; Ayali et al., 2019). This phenomenon is also known in Lepidoptera, most markedly within the genus *Spodoptera* (Reeson et al., 1998; Cotter et al., 2004; Wen et al., 2025).

In some lepidopterans, high population densities can trigger these phenotypic changes without any evidence to date of behavioural changes or swarming (Silva et al. 2013). Indeed, density-dependent phase polyphenism has been documented in various insect groups (Yuan et al., 2020) and there is increasing evidence that this type of plasticity may occur in species that do not exhibit such evident visible changes (Li et al., 2020; Bono et al., 2022; Yuan et al., 2020). It is an open question, then, to what degree this phenomenon occurs in species for which visible changes in phenotype are undocumented. Here we address this in some lepidopterans of particular importance as agricultural pests.

In species that do show visible changes in phenotype with density, such as *Mythimna separata* (Walker, 1865) (Lepidoptera: Noctuidae), *Spodoptera exempta* (Walker, 1856) (Lepidoptera: Noctuidae) and *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera: Noctuidae), it has been shown that larvae reared at high densities present greater cuticular melanization (Zhang et al., 2020; Reeson et al., 1998; Cotter et al., 2004) and activation of genes linked to immunity, especially in the Toll and IMD pathways (Wen et al., 2025). In addition, greater activity of the enzyme phenoloxidase (PO) is observed, associated with melanization and the immune response, resulting in greater

resistance to pathogens, especially entomopathogenic viruses and fungi. In some studies, insects experimentally kept at high densities, such as *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae) and *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae), also demonstrated increased PO activity, total hemocyte counts and lysozyme activity compared to those reared at lower densities (Kong et al., 2020; Sheehan & Kavanagh, 2017). Similarly, *Anticarsia gemmatalis* (Hübner, [1818]) (Lepidoptera: Erebidae) in the presence of conspecifics, presents changes in caterpillar coloration and alterations in secondary immunological defenses, such as the encapsulation response (Silva et al., 2013).

Insects are frequently exposed to the risk of infection by pathogens (Wilson & Cotter, 2009). As a result, cohabitation with conspecifics becomes one of the main sources of disease transmission, as frequent contact can facilitate the spread of pathogens, especially those transmitted by direct contact (Holt & Pickering, 1985). Pathogen transmission in insects is positively correlated with population density, implying that the risk of infection increases as density also increases (Anderson & May, 1981; Jiang et al., 2024; Opare et al., 2023). Crowding conditions act as signals of increased vulnerability to infection, leading individuals to redirect physiological resources that would normally be allocated to growth or reproduction in order to strengthen their immune defenses (Kong et al., 2016; Kong et al., 2020). This phenomenon is called Density-Dependent Prophylaxis (DDP), a fundamental adaptive response to the greater likelihood of infection in densely populated environments (Wilson & Cotter, 2009).Several studies in Lepidoptera document that individuals raised in crowded environments exhibit more robust immune responses, suggesting that this physiological adjustment is induced by population density (Reeson et al., 1998; Cotter et al., 2004; Silva et al., 2013; Sheehan & Kavanagh, 2017; Kong et al., 2020; Zhang et al., 2020). Among the documented species are *Spodoptera exempta* and *Spodoptera littoralis* (Reeson et al., 1998; Cotter et al., 2004).

The *Spodoptera* complex includes several species of caterpillars that are agricultural pests of great economic importance (Parra et al., 2021). *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae), known as fall armyworm, and *Spodoptera eridania* (Stoll, [1782]) (Lepidoptera: Noctuidae), the southern armyworm, have a wide range of host plants, including high-value crops such as corn, soybean, and

cotton (Santos et al., 2010; Ouaba et al., 2022; Zhang et al., 2023). The high reproductive capacity of these species, combined with resistance to different environmental conditions, favors their distribution and makes their management difficult (Parra et al., 2021; Goergen et al., 2016; Hilliou et al., 2021).

The management of these pests has been challenging due to the resistance developed to insecticides (Hilliou et al., 2021; Amezian et al., 2021). Control strategies include the use of biological agents such as egg parasitoids (*Telenomus remus* (Nixon, 1937) (Hymenoptera: Platygastridae) and *Trichogramma* spp. (Hymenoptera: Trichogrammatidae)) (Fortes et al., 2023), Cry proteins from *Bacillus thuringiensis* (Santos et al., 2009), and baculoviruses, such as *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV), effective in controlling fall armyworm (*S. frugiperda*) (Sosa-Gómez et al., 2020).

Given the importance of these species as agricultural pests and the use of pathogens in their management, their response to variable densities, particularly potential changes in disease resistance, is of great significance. Thus, this study aimed to investigate whether *Spodoptera frugiperda* and *Spodoptera eridania* exhibit any degree of density-dependent phase polyphenism, either through phenotypic color changes or immune responses, in order to obtain more information on the mechanisms underlying density-dependent plasticity. Understanding the resistance mechanisms of these pests is essential for predicting outbreaks and improving integrated pest management programs, contributing to the reduction of insecticide use and the promotion of sustainable alternatives (Hilliou et al., 2021; Wan et al., 2021). Thus, we sought to better understand the immune system of these species at different densities, through total hemocyte counts, encapsulation responses, and in the case of *S. frugiperda*, resistance to SfMNPV. Knowing that density can result in more robust immune responses in some *Spodoptera* species (Reeson et al., 1998; Cotter et al., 2004), we expect these species to show responses proportional to density, in line with the Density-Dependent Prophylaxis hypothesis.

2. MATERIALS AND METHODS

2.1. Insects and microorganisms

Spodoptera frugiperda and *Spodoptera eridania* were obtained from the company Pragas.com® - Piracicaba, SP, and colonies were established in the Insect-Microorganism Interactions Laboratory at the Federal University of Viçosa in 2024, in a climate-controlled room (Temperature 25 ± 1 °C; relative humidity 60% ± 10% and photoperiod of 14:10 light:dark) (Figure 1). Adults were kept in PVC cages, lined inside with A4 paper sheets, used as a substrate for oviposition. These sheets were removed every two days to collect the egg masses. The adults were fed *ad libitum* with a nutritional solution composed of honey (20.0 g), beer (350 ml), sucrose (50 g), ascorbic acid (1.05 g), nipagin (1.05 g), and water (650 ml) (adapted from Hoffman-Campo et al., 1985). This was offered on cotton placed at the bottom of the cage, in a Petri dish.

The collected A4 paper sheets with the eggs were kept in 1-liter transparent plastic containers until hatching of the neonate larvae. These were transferred with the aid of a fine-bristle brush to plastic trays with 16 wells (30 × 20 cm), containing artificial diet based on textured soy protein, white beans, wheat germ, brewer's yeast, nipagin, ascorbic acid, sorbic acid, formaldehyde, agar-agar, casein, tetracycline, and vitamin solution (adapted from Hoffman-Campo et al., 1985).

The *Spodoptera frugiperda multiple nucleopolyhedrovirus* (SfMNPV) isolate used in the experiment corresponds to the commercial formulation Destroyer, obtained from the market.

Figure 1- Rearing of A) *Spodoptera frugiperda;* B) *Spodoptera eridania*. The insect colony was kept in a climate-controlled chamber (25 ± 1 °C; relative humidity 60% ± 10% and photoperiod of 14 hours).

2.2. Density treatments

For the experiments, neonate caterpillars (maximum of 24 hours) were placed in 100 ml plastic pots, at four densities: 1, 2, 4 or 8 per pot (Silva et al., 2013). The insects were maintained on an artificial diet (adapted from Hoffman-Campo et al., 1985) under the same rearing conditions described above until they were used in the tests. During the experiments, only one randomly chosen insect was used from each jar, in order to avoid pseudoreplication (Silva et al., 2013). Since *Spodoptera frugiperda* is a species that exhibits cannibalistic behavior (Lu et al., 2024), the cannibalism rate was measured at densities 2, 4 and 8.

2.3. Larval color phenotypes

Density-dependent phenotypic variation in insects is often manifested by differences in body coloration (Shen et al., 2024). To evaluate the effect of rearing density on caterpillar coloration, a photographic record was taken on the ninth day after the start of the experiment. The dorsal regions of the caterpillars were photographed with a digital camera in a portable mini photo studio (USB, 60 × 60 × 60 cm, 140 LEDs), with a neutral white background and standardized lighting, in order to ensure consistency in color capture between images. The degree of cuticle melanization was obtained by converting the images to a grayscale scale from 0 to 255, where 0 represents absolute black (the darkest possible) and 255 represents absolute white (the lightest possible), using the average gray intensity value per image as a measure of melanization using the ImageJ software (NIH, Bethesda, USA) (adapted from Dubovskii et al., 2010). This procedure was adopted in all three experiments performed.

2.4. Encapsulation responses

One of an insect's immune defenses when subjected to invasion by a parasite is the triggering of the encapsulation response (Dubovskiy et al., 2016). To assess whether the density of conspecifics influences this response, the larvae were subjected to simulated parasitism. For this, a sterile fragment of nylon filament (5 mm long × 0.25 mm in diameter) was inserted into the dorsal region of the first thoracic segment of 15 larvae per density of *Spodoptera frugiperda* (n = 60) and 30 larvae per density of *Spodoptera eridania* (n = 120) at 10 days of age (adapted from Silva et al., 2013).

After 24 hours, the larvae were dissected and the removed filaments were mounted on slides for analysis. The samples were photographed using a Motic Images Plus 3.0 camera attached to a Nikon optical microscope at 400× magnification. Two variables were analyzed using IMAGEJ 1.42q software (adapted from Berggren, 2009): (i) the area of the capsule formed around the nylon filament, measured by the area occupied by the encapsulating cell layer; and (ii) the degree of melanization of the capsule, obtained by converting the images to a grayscale scale (see above),using the mean grayscale intensity value per image as a measure of melanization (adapted from Dubovskii et al., 2010).

2.5. Hemocyte density

Changes in insect immunological parameters, such as increased hemocyte density, are examples of phenotypic responses that allow insects to respond to environmental challenges, such as increased population density or exposure to pathogens (Wilson et al., 2002; Cotter et al., 2004). Hemolymph extraction was conducted on a group of 30 10-day-old caterpillars for each density of the two species (n = 120 for each species).

A 2.5 µl sample of hemolymph was collected from each caterpillar using a fine needle, piercing a small hole near the first pair of legs. Subsequently, the sample was added to an Eppendorf tube containing 20 µl of anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM NaEDTA and 41 mM citric acid, pH 4.5) (adapted from Ibrahim & Kim, 2006). A 10 µl aliquot of this suspension was added to a Neubauer® chamber to count the total number of hemocytes under an optical microscope at 400× magnification. The final value was based on the average of the two aliquot counts (adapted from Ibrahim; Kim, 2006).

2.6. Susceptibility of *Spodoptera frugiperda* to SfMNPV and viral multiplication

Spodoptera frugiperda is highly susceptible to SfMNPV (*Spodoptera frugiperda multiple nucleopolyhedrovirus*), making the virus a promising tool in the biological management of this agricultural pest (Bentivenha et al., 2024). No virus was available for *S. eridania*. Twenty-four hours before inoculation with SfMNPV, 30 nine-day-old larvae from each density treatment of *Spodoptera frugiperda* (n = 120) were kept individually and fasted. After this period, a piece of artificial diet (approximately 0.5 cm^3 (equivalent to 0.5 g) without added formaldehyde was provided to the larvae; formaldehyde was left

out in order to avoid inactivation of the virus (Paiva, 2013). For viral treatment, the commercial product was diluted in Tween 80® (0.01% v/v) at a concentration of 1×10^8 polyhedra/ml, and a 20 µl aliquot of this viral suspension was applied to the surface of the diet (i.e. 2×10^6 polyhedra per piece). In the control treatments, each piece received 20 µl of distilled water containing Tween 80® (0.01% v/v). The insects were exposed to the treated diet and fed for 24 h.

Subsequently, the larvae were fed artificial diet (adapted from Hoffman-Campo et al., 1985), and mortality was assessed daily until death or pupation. Larvae that died with typical baculovirus symptoms such as lethargy and discoloration of the larval tegument (De Castro et al., 2020), or that did not respond to physical stimulation with a brush (approximately from the seventh day onward) were collected to count viral occlusion bodies. The cadavers were collected and macerated using a porcelain mortar containing Tween® 80 (0.01% v/v) to release the polyhedral occclusion bodies from inside the larvae (Paiva, 2013). The viral suspensions obtained from these larvae were filtered through gauze, and 1 ml of these suspensions was added to a test tube containing 9 ml of Tween® 80 (0.01% v/v) for dilution. The OBs were then counted in a Neubauer® chamber using a Nikon optical microscope at 400× magnification. Two counts were performed for each replicate, and the average was taken to calculate OBs per larva (OB/larvae).

2.7. Statistical analysis

Statistical analyses were conducted using the R software (R Core Team, 2024), adopting a significance level of 5%. Initially, the data were subjected to analysis of variance (ANOVA) with F-test. Subsequently, the normality of residuals was assessed using the Shapiro–Wilk test, and homoscedasticity was evaluated. When the assumptions of normality and homogeneity of variances were violated, alternative models with appropriate distributions were employed.

To evaluate the effects of population density on larval coloration of the two species, the data were analyzed using generalized linear models (GLMs) with Gamma distribution and logarithmic link function, since the residuals did not meet the assumptions of normality. Differences between treatments were assessed using the chi-

square test (χ^2), followed by multiple comparisons between density levels using the Tukey test ($p < 0.05$).

The effects of larval coloration and population density on immunological parameters, and on the cannibalism rate in *S. frugiperda*, were evaluated using GLMs, using the appropriate distribution family for each variable. The two explanatory variables (larval coloration and population density) were analyzed independently in separate models. For the analysis of the area and intensity of melanization of the nylon, a model with Gamma distribution and logarithmic link function was adopted, while for hemocyte counts, a model with normal (Gaussian) distribution was used. Differences between treatments were assessed using F tests (for data with normal distribution) or chi-square tests (χ^2) (for data with alternative distributions).

For the analysis of the susceptibility of *S. frugiperda* to the virus, a survival analysis was conducted using the Cox proportional hazards model, in order to test the effect of treatments on larval survival time. The analysis of the number of occlusion bodies (OBs) per larva, used as a measure of viral multiplication, was performed using GLMs, adopting the quasi-Poisson family to correct for overdispersion.

3. RESULTS

3.1. Larval color phenotypes

Larval coloration was evaluated as an indicator of cuticle melanization across the three experiments conducted: (i) capsule melanization and encapsulation response, (ii) hemocyte density, and (iii) susceptibility of *Spodoptera frugiperda* to SfMNPV and viral multiplication (only for *S. frugiperda*).

Spodoptera eridania

Spodoptera eridania exhibited variations in cuticle melanization in response to population density. In the first experiment (Figure 2A), larval coloration differed significantly among treatments. Compared to density 1 (isolated larvae), individuals reared at densities 2 and 4 showed lighter coloration (coef. = –0.208; p = 0.0069 and coef. = –0.163; p = 0.0324, respectively), indicating reduced cuticle melanization under moderate crowding. At density 8, the estimated coloration value was numerically higher

than that of density 1, although this difference was not statistically significant (coef. = 0.107; p = 0.1586).

In the second experiment (Figure 2B), population density significantly affected larval coloration ($F_{(3,116)} = 9.83$; $p < 0.001$). Larvae reared in isolation (density 1) had significantly higher grayscale values compared to those reared at higher densities, indicating a lighter cuticle and reduced melanization under solitary conditions.

Spodoptera frugiperda

Spodoptera frugiperda showed an increase in larval coloration (i.e., lighter cuticle and reduced melanization) in response to increasing density in only one of the three experiments. In experiment 1 (Figure 2C), larval density had no significant effect on larval coloration ($F_{(3,56)} = 1.515$; $p = 0.2206$).

In experiment 2 (Figure 2D), population density significantly influenced larval coloration ($\chi^2_{(3)} = 7.14$; $p = 0.0411$), indicating a global effect of crowding on coloration. However, individual comparisons with density 1 (isolated larvae) did not reveal statistically significant differences: density 2 (coef. = 0.068; $p = 0.0707$), densities 4 and 8 ($p = 0.98$ and $p = 0.33$, respectively).

In experiment 3 (Figure 2E), population density had a statistically significant effect on larval coloration ($\chi^2_{(3)} = 16.1155$; $p < 0.001$). Compared to density 1 (isolated larvae), those reared at densities 4 and 8 exhibited lighter cuticle coloration (coef. = –0.104; $p < 0.001$ and coef. = –0.144; $p < 0.001$, respectively), indicating reduced melanization under these crowding conditions. In contrast, density 2 did not differ from density 1 ($p = 0.625$), suggesting that intermediate levels of crowding did not alter larval coloration patterns.

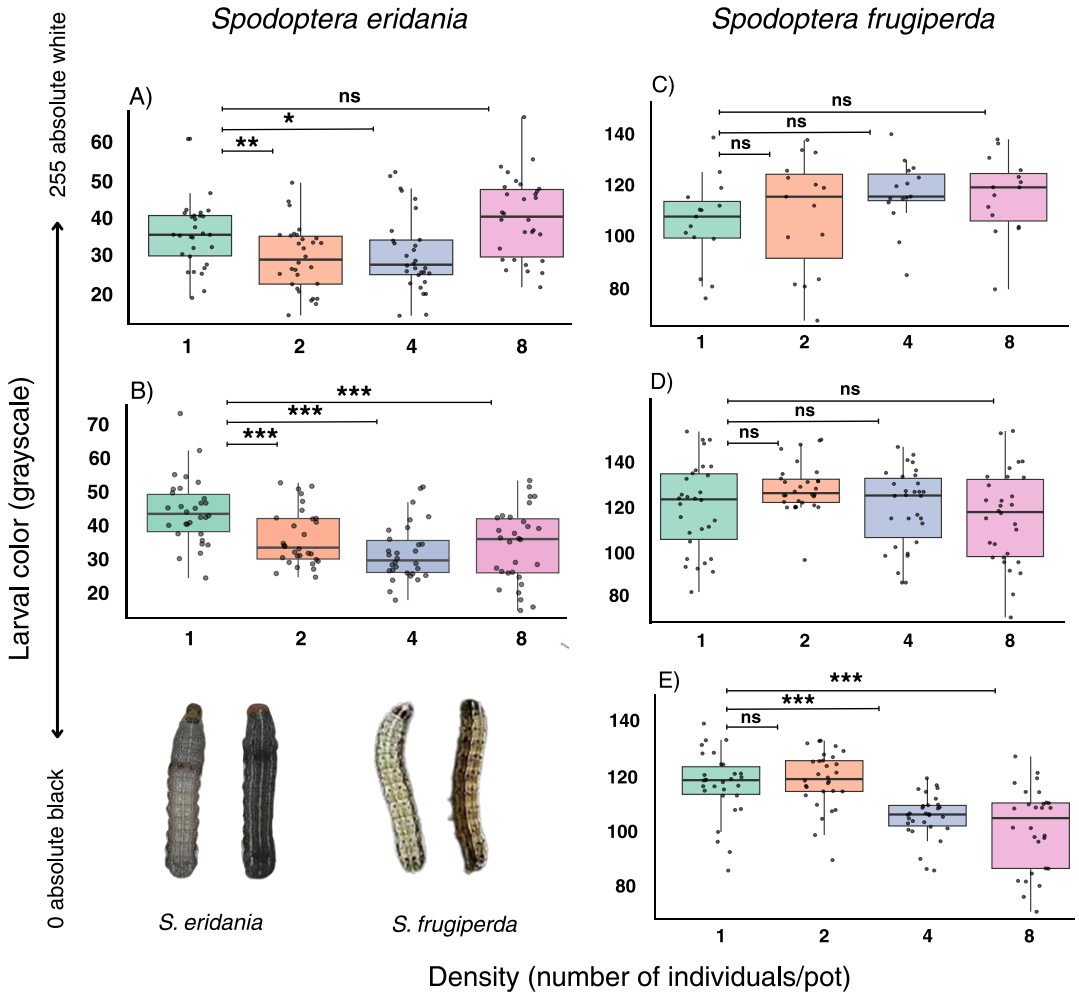

Figure 2- Colour phenotype, assessed as grayscale, in response to rearing densities, of larvae of the lepidopterans *Spodoptera eridania* and *Spodoptera frugiperda*, Shown are phenotypes from the five assays conducted here. (A, C) Experiment 1 - Encapsulation response (A: *S. eridania*, C: *S. frugiperda*); (B, D) Experiment 2 - Hemocyte density (B: *S. eridania*, D: *S. frugiperda*) ; (E) Experiment 3 - Susceptibility of *S. frugiperda* to SfMNPV and viral multiplication . Larvae were reared at four densities: 1, 2, 4, or 8 individuals per pot. To assess the effect of rearing density on larval coloration, photographs were taken on day 9. The images were converted to grayscale values ranging from 0 to 255, where 0 represents absolute black (darkest possible) and 255 represents absolute white (lightest possible). As an indicator of melanization level, the mean grayscale intensity per image was used (adapted from Dubovskii et al., 2010). Representative images illustrate visible differences in larval coloration across density treatments. Statistical significance: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns = not significant ($p > 0.05$).

3.2. Experiment One and Experiment Two: Encapsulation Responses and Hemocyte Density

Spodoptera eridania

In the first bioassay, we evaluated two immune parameters: (i) the area of the capsule formed around the nylon filament, measured by the extent of encapsulating cell coverage; and (ii) the degree of capsule melanization, determined by converting images to a grayscale scale (0 to 255), where 0 represents absolute black and 255 absolute white.

The mean grayscale intensity per image was used as an indicator of melanization level (adapted from Dubovskii et al., 2010).

Analysis of capsule area revealed that larval density had no significant effect on melanization in *Spodoptera eridania* (Figure 3A). Compared to density 1 (isolated larvae), the p-values were as follows: density 2 ($p = 0.799$), density 4 ($p = 0.862$), and density 8 ($p = 0.0677$). In contrast, capsule melanization intensity was significantly influenced by larval density and coloration. Larvae reared at densities 4 ($p = 0.0069$) and 8 ($p < 0.001$) exhibited greater melanization intensity compared to isolated larvae, while density 2 showed no significant difference ($p = 0.729$) (Figure 3B). Additionally, larval coloration had a marginally significant effect ($p = 0.0579$), suggesting that individuals with darker cuticles tend to mount a stronger melanotic immune response. Model term analysis confirmed that both population density ($\chi^2(3) = 4.33$, $p = 0.0046$) and larval coloration ($\chi^2(1) = 1.31$, $p = 0.0474$) significantly contributed to variation in melanization intensity.

In experiment two, larval density significantly affected the number of circulating hemocytes. Larvae at density 8 showed a significant increase in hemocyte count compared to isolated larvae ($p = 0.0018$), while those at densities 2 ($p = 0.146$) and 4 ($p = 0.254$) did not differ significantly. Larval coloration had no significant effect on hemocyte number ($p = 0.729$) (Figure 3C).

Spodoptera frugiperda

For *Spodoptera frugiperda*, the analyses considered population density, larval coloration, and cannibalism as factors. In the first bioassay, none of these significantly affected the area of capsule melanization. Compared to isolated larvae, the results were: density 2 ($p = 0.213$), density 4 ($p = 0.655$), and density 8 ($p = 0.278$) (Figure 3D). Similarly, neither larval coloration ($\chi^2_{(1)} = 0.311$, $p = 0.101$) nor cannibalism ($\chi^2_{(1)} = 0.311$, $p = 0.101$) had a significant impact on capsule area.

Capsule melanization intensity was also unaffected by any of the three factors. Compared to density 1, values were: density 2 ($p = 0.661$), density 4 ($p = 0.213$), and density 8 ($p = 0.412$) (Figure 3E). Larval coloration ($p = 0.162$) and cannibalism ($p = 0.399$) did not show statistically relevant associations with this parameter.

In contrast, in experiment two population density significantly influenced hemocyte numbers ($\chi^2_{(3)} = 33.618$, $p < 0.001$). Larvae reared at densities 8 ($p < 0.001$) and 4 ($p = 0.0103$) exhibited significantly higher hemocyte counts than isolated individuals, while density 2 showed only a non-significant trend ($p = 0.099$) (Figure 3F). Neither larval coloration ($\chi^2_{(1)} = 0.317$, $p = 0.468$) nor cannibalism ($\chi^2_{(1)} = 0.468$, $p = 0.379$) significantly affected hemocyte levels.

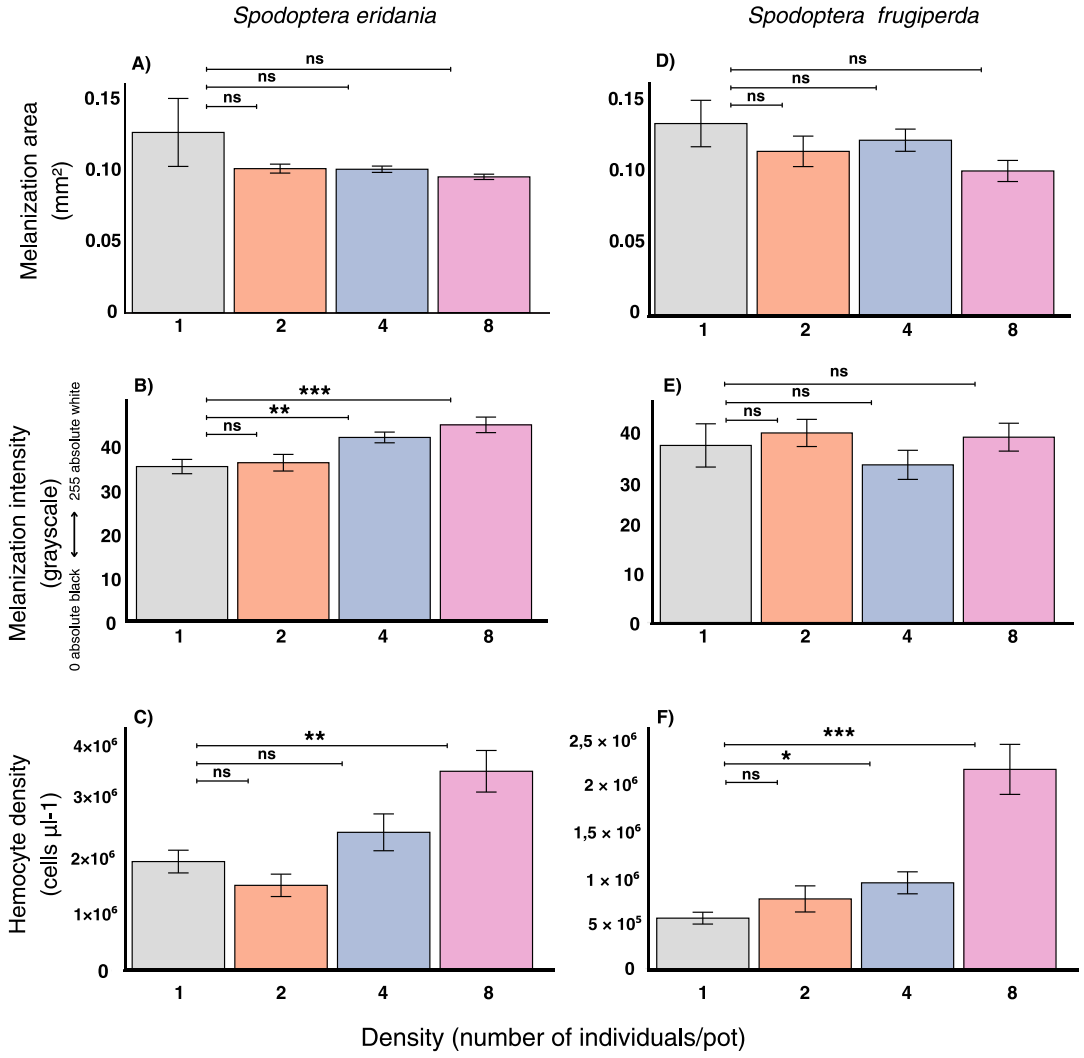

Figure 3- Immune responses according to rearing density in larvae of the lepidopterans *Spodoptera eridania* and *Spodoptera frugiperda*. (A, D) Area of capsule melanization (mm²) (A: *S. eridania*, D: *S. frugiperda*); (B,E) Degree of capsule melanization (gray scale, 0 = black, 255 = white) (B: *S. eridania*, E: *S. frugiperda*); (C,F) Density of hemocytes in the hemolymph (C: *S. eridania*, F: *S. frugiperda*). Larvae were reared at four densities: 1, 2, 4 or 8 individuals per pot. For encapsulation response assays, 30 larvae per density of *S. eridania* (n=120) and 15 larvae of *S. frugiperda* (n=60) had a sterile nylon filament (5 mm × 0.25 mm) inserted into the dorsal region of the first thoracic segment at 10 days of age. After 24 h, the filaments were removed and mounted on slides to measure: (A, D) melanized area (mm²) and (B, E) melanization intensity (gray scale from 0 = black to 255 = white). For hemocyte counting assays (C, F) an aliquot of hemolymph was extracted from each caterpillar of both species (30 larvae/density), placed in a Neubauer chamber and counted under an optical microscope. Statistical significance: *** $p < 0.001$; ** $p < 0.01$;* $p < 0.05$; ns = not significant.

3.3. Experiment three: Susceptibility of *Spodoptera frugiperda* to SfMNPV and viral multiplication

The third experiment (Susceptibility of *Spodoptera frugiperda* to SfMNPV and viral multiplication) was performed only with *S. frugiperda* caterpillars. For this, we used a Cox proportional hazards model, considering density one as the reference category. None of the analyzed variables showed a statistically significant effect on the risk of death (density two (p = 1.000), density four (p = 0.874), density eight (p = 0.923), and cannibalism (p = 0.978), indicating that the different population densities and cannibalism did not significantly alter the larval mortality rate over time. According to the analysis, the mean survival times were similar between treatments: 203.28 hours for density eight, 200.88 hours for density four, and 201.6 hours for densities one and two, demonstrating that the progression of the baculovirus infection and the associated mortality followed similar patterns among the experimental groups (Figure 4A).

We evaluated the effects of population density, cannibalism rate, and larval coloration on the production of occlusion bodies (OBs) in *Spodoptera frugiperda*, excluding the control group from the analyses. We observed that population density did not have a statistically significant effect on OB production ($\chi^2_{(3)}$ = 5.24; p = 0.2299). However, using density one as the reference category, we observed that densities two and four did not differ significantly from density one (densities 2 and 4 p = 1.000 in both cases, density 8 p = 0.6277) (Figure 4B). Finally, larval coloration showed a significant effect on OB production ($\chi^2_{(1)}$ = 7.11; p = 0.0156), suggesting that the degree of melanization may directly influence viral multiplication. This analysis indicated that larval coloration had a negative effect on occlusion body production (coefficient = −0.0106 ± 0.0055; z = −1.915; p = 0.0579), suggesting that darker-colored larvae, and therefore more melanized, tend to present higher viral multiplication.

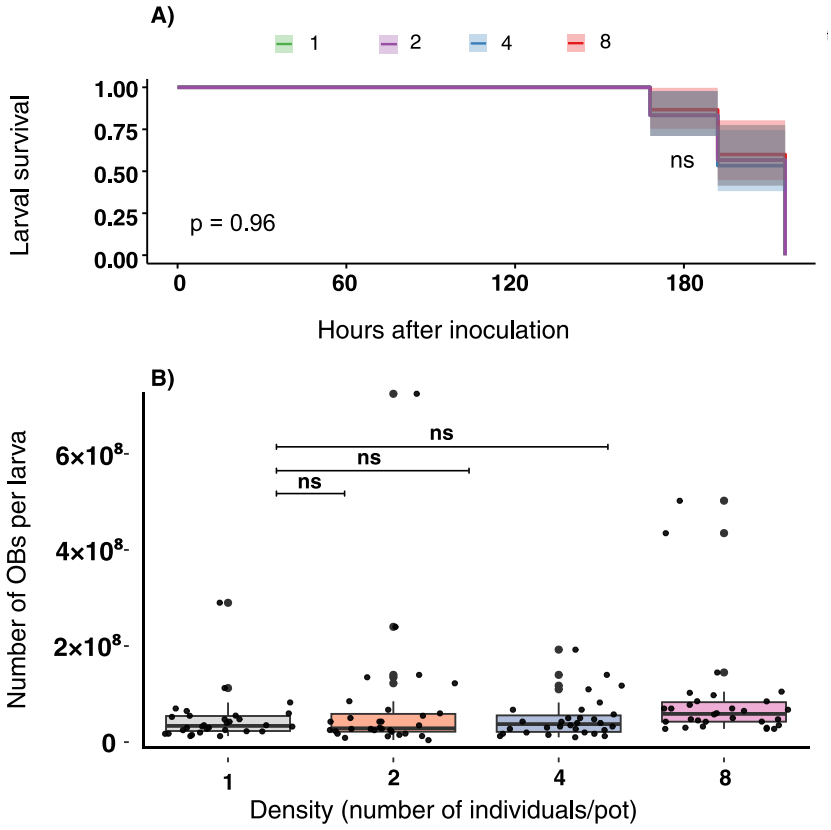

Figure 5- Effects of rearing density on susceptibility and viral production in *Spodoptera frugiperda* larvae inoculated orally with the baculovirus SfMNPV (2×10^6 occlusion bodies or OBs per larva). (A) Survival curves of *S. frugiperda* larvae reared at four different densities (1, 2, 4, or 8 larvae per pot) and inoculated with SfMNPV and (B) Number of viral propagules (OBs) produced per larva after infection with SfMNPV at the same four rearing densities. Statistical significance: *** $p < 0.001$; ** $p < 0.01$;* $p < 0.05$; ns = not significant ($p > 0.05$).

4. DISCUSSION

We aimed to investigate whether *Spodoptera frugiperda* and *Spodoptera eridania* exhibit any degree of density-dependent phase polyphenism, either through phenotypic changes in coloration or immune responses. Our results indicated that, in *S. eridania*, larval coloration was significantly influenced by density. Furthermore, in both species, we observed that immune responses were modulated according to population density. As these species are not known to form aggregations in nature, especially *Spodoptera frugiperda*, which exhibits cannibalistic behavior, our findings indicate that density-mediated phenotypic adjustments may occur more frequently than previously believed.

Density-dependent phase polyphenism refers to the ability of some insects to alter their phenotype in response to changes in population density (Pener, 2009; Wang & Kang, 2014). These changes can result in behavioral, physiological, developmental,

reproductive, and coloration traits (Reeson, 1998). In our study, we observed that larval coloration of *S. eridania* varied with population density, with larvae at higher and intermediate (more crowded) densities exhibiting darker coloration (i.e., greater melanization) (Figure 2A,B).

As in our study, several lepidopteran species, such as *Spodoptera exempta*, *Spodoptera littoralis, Mythimna separata, Anticarsia gemmatalis* and *Spodoptera litura*, showed increased cuticular melanization when reared at high population densities, characterizing a classic phase polyphenism (Zhang et al., 2020; Reeson et al., 1998; Cotter et al., 2004b; Lee & Wilson, 2006; Silva et al., 2013; Wen et al., 2025).

Cuticular melanization in insects is a multifunctional physiological process, essential not only for pigmentation, hardening, and waterproofing of the cuticle, but also for the innate immune response (Hiruma & Riddiford, 2009; Sugumaran & Barek, 2016). This process involves metabolic pathways that share precursors such as tyrosine and requires the activation of key enzymes such as phenoloxidase (PO), which are essential for both melanin production and defense against pathogens (Wilson et al., 2001; Hiruma & Riddiford, 2009; Zdybicka-Barabas et al., 2025). The overlap between the mechanisms involved in cuticular melanization and immunity suggests that, under certain conditions, a differentiated allocation of metabolic resources among crucial physiological functions, such as growth, reproduction, and defense, may occur, modulating the phenotypic expression of multiple traits (Wilson & Cotter, 2009).

High population density environments are characterized by increased competition for resources and a higher risk of pathogen transmission (Boots, 2000; Myers & Cory, 2015). Under these conditions, individuals may reallocate physiological resources normally directed toward growth or reproduction to strengthen their immune defenses. This phenomenon is known as density-dependent prophylaxis (DDP) (Reeson, 1998 et al 1998).

Modulation of cuticular melanization may represent an alternative adaptive strategy, allowing the reallocation of metabolic resources to more urgent physiological functions, such as immune defense or increased growth rate (Cotter et al., 2008; Lee et al., 2008). In this context, the reduction in cuticular melanization observed in *S. eridania* at the highest density, compared to intermediate densities (2 and 4), may reflect an

adaptive adjustment to the stress imposed by high population densities, prioritizing more effective or metabolically less costly immunological mechanisms.

In support of this hypothesis, we observed that at density 8, *S. eridania* showed increased melanization of the capsule formed around the nylon filament inserted into the dorsal region of the larvae, simulating (Figure 3A). These data suggest a possible reallocation of metabolic resources from cuticular melanization to more specific cellular responses, such as encapsulation. In contrast, *S. frugiperda* did not show significant variations in encapsulation responses between the densities evaluated (Figure 3D,E). Although cannibalism is a common behavior in this species, this factor did not influence any of the immunological parameters analyzed (Figure 3D,E,F).

Similar results were reported by Silva et al. (2013), who observed a higher degree of encapsulation in larvae of *Anticarsia gemmatalis* maintained at high densities, using the same experimental model with nylon filament to simulate the presence of a microparasite. These findings reinforce the hypothesis that the presence of conspecifics can enhance the encapsulation response, possibly as an adaptation to the increased risk of infection in crowded environments, in which the stress caused by crowding acts as an inducer for the activation of cellular immunity (Anderson & May, 1981; Hochberg, 1991).

The encapsulation response, often associated with capsule melanization, is one of the main lines of cellular immune defense in insects (Cerenius et al., 2008; Dubovskiy et al., 2016). This process begins within the first minutes after penetration into the hemolymph, and capsules can continue to form over 2 to 24 hours (Dubovskii et al., 2010; Carton et al., 2008). The number of circulating hemocytes in the hemolymph is also a crucial component of cellular immunity (Eleftherianos et al., 2021).

We observed a significant increase in this parameter in *Spodoptera eridania* (density 8) and *Spodoptera frugiperda* (densities 4 and 8) (Figure 3C,F). Similar patterns were reported by Dubovskiy et al. (2013) in *Galleria mellonella* and by Wen et al. (2025) in *Spodoptera litura*. These results reinforce the role of crowding in the activation of cellular immunity, even when not linked to coloration effects or cannibalistic behavior.

In addition to their role in cellular responses, hemocytes also participate in humoral immunity, being responsible for the production of antimicrobial peptides (AMPs), lysozymes, and lectins (Hultmark, 2003; Sumathipala & Jiang, 2010). Specific subtypes, such as oenocytoids, produce the enzyme pro-phenoloxidase (pro-PO), the precursor of active phenoloxidase and essential for the activation of the melanization cascade (Carlos & Michel, 2010; Wago, 1980).Thus, it is possible that the increase in hemocyte density in *S. eridania* (density 8) contributed directly to the intensification of the melanization response, characterizing a reinforced immune response to the stress caused by greater crowding, in accordance with the density-dependent prophylaxis (DDP) hypothesis.

Some studies have investigated the effects of population density on pathogen resistance in lepidopteran larvae, revealing that rearing at high densities can increase resistance to entomopathogenic viruses in species such as *Mythimna separata* (Kunimi & Yamada, 1990), *Spodoptera exempta* (Reeson et al., 1998), and *Anticarsia gemmatalis* (Silva et al., 2013). In *S. exempta*, for example, larvae reared at high densities not only showed greater resistance to the baculovirus SeMNPV (*Spodoptera exempta nucleopolyhedrovirus*), but also exhibited increased cuticular melanization, a possible indicator of a more intense immune activation (Reeson et al., 1998). Based on these findings in related species or with similar response patterns, it was expected that *Spodoptera frugiperda* larvae reared at higher densities would also show greater resistance to SfMNPV.

Although we did not observe a direct effect of population density or cannibalism on resistance to viral infection in *Spodoptera frugiperda* (Figure 5A), larval coloration significantly influenced the production of occlusion bodies (OBs), with darker larvae producing a significantly higher quantity of viral particles (Figure 5B). This finding is particularly interesting in light of previous discussions, since although melanization is often associated with a more robust immune response, as observed in *S. exempta* (Reeson et al., 1998) and *A. gemmatalis* (Silva et al., 2013), in *S. frugiperda* greater pigmentation did not translate into higher resistance to the virus.

It is known that baculoviruses possess effective mechanisms for suppressing melanization in hosts, such as inhibition of phenoloxidase activation and interference in signaling pathways associated with innate immunity (Ardisson-Araujo et al., 2015;

Rohrmann et al., 2013; Yuan et al., 2017; Ji et al., 2022). In this context, it is possible that the greater melanization observed in *S. frugiperda* does not reflect an effective immune response to viral infection.

Taken together, our results reinforce that immune and phenotypic responses modulated by population density may occur even in species that do not exhibit typical gregarious behavior, such as *S. frugiperda* and *S. eridania*, challenging the traditional view that density-dependent prophylaxis (DDP) is restricted to gregarious insects. The expression of these responses, however, seems to be species-specific, resulting from complex interactions between host physiology and the nature of the immune challenge.

Overall, our findings are consistent with DDP, according to which an increase in population density acts as a signal of imminent infection risk, inducing a strategic reallocation of resources to strengthen immune defenses. We observed evidence of density-dependent phase polyphenism, especially in *S. eridania*, with intensification of encapsulation response and increased hemocyte density under higher crowding conditions. In *S. frugiperda*, although an increase in hemocyte density was also recorded at high densities, no significant effects of density were observed on larval coloration or viral infection resistance.

Considering that baculoviruses possess effective mechanisms to actively suppress host defenses, it is plausible that the greater pigmentation observed in *S. frugiperda* represents a nonfunctional phenotype in the face of viral infection.

5. CONCLUSION

Our study demonstrated that both *Spodoptera eridania* and *Spodoptera frugiperda* exhibit immune responses modulated by population density, albeit in a species-specific manner. In *S. eridania*, a clear pattern of phenotypic plasticity was observed, characterized by significant changes in larval coloration, increased capsule melanization, and elevated hemocyte density under crowded conditions. In contrast, *S. frugiperda* showed no marked variation in larval coloration according to density, although an increase in hemocyte density was noted at high densities.

Notably, darker Spodoptera frugiperda larvae produced a greater number of SfMNPV occlusion bodies (OBs), indicating that the melanized phenotype, contrary to

expectations, may represent a condition of higher susceptibility to infection rather than increased resistance.

These results evidence the occurrence of density-dependent phase polyphenism, especially regarding immune responses and external phenotypic traits, reinforcing the density-dependent prophylaxis (DDP) hypothesis even in species with cannibalistic or non-gregarious behavior. Thus, we expand the understanding of DDP applicability in insects and highlight the importance of considering species-specificity in the interactions between population density, immunity, and pathogen susceptibility.

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FINAL CONSIDERATIONS

The results obtained in this thesis demonstrate that population density directly influences phenotypic characteristics and immune responses in agriculturally important lepidopterans, also affecting the dynamics of baculovirus infection. The results obtained in this thesis demonstrate that population density directly influences the phenotypic characteristics and immune responses of agriculturally important lepidopterans, also affecting the dynamics of baculovirus infection. Different phenotypes of *Anticarsia gemmatalis* were shown to have distinct susceptibilities to infection, directly influencing viral multiplication. These results reinforce the importance of considering the phenotypic composition of natural populations when evaluating the dynamics of epizootics and the potential dissemination of biological control agents.

In the species *Spodoptera frugiperda* and *Spodoptera eridania*, modulation of the immune response was observed as a function of larval density, although in a species-specific manner. *S. eridania* showed clear phenotypic plasticity, with changes in larval coloration, increased melanization of the encapsulation capsule, and elevated hemocyte density under high-density conditions. In *S. frugiperda*, although no expressive changes in coloration were observed, there was an increase in hemocyte density under higher population densities. Notably, darker phenotypes of *Spodoptera frugiperda* produced more OBs following SfMNPV infection, suggesting greater susceptibility associated with this coloration.

Overall, these results provide evidence that density-dependent phase polyphenism can occur even in species without typical gregarious behavior, and that such phenotypic changes are associated with variations in larval coloration as well as in immune response. These findings expand the applicability of the Density-Dependent Prophylaxis (DDP) hypothesis in insects and highlight the importance of considering species-specific traits when investigating the relationship between population density and immune resistance.

From a practical perspective, understanding how population densities affect the dynamics of insect-pathogen interactions is essential to improving integrated pest management strategies. Knowledge generated about the interactions between larval phenotypes, immune response, and viral performance can help select more efficient viral isolates and contribute to the development of bioinsecticides with greater potential for

dissemination and persistence in target populations. Future studies exploring the expression of key immune genes and pathways, especially those associated with the Toll pathway, IMD, and tyrosine metabolism, as well as phenoloxidase (PO) activity in the hemolymph and cuticle, may elucidate the physiological and molecular mechanisms underlying the differences between phenotypes.

APPENDIX A

Supplementary material Chapter II

Descriptive statistics of *Anticarsia gemmatalis*

Phenotype	N	OB/larva	SD	SE	CI
Black	7	4x 10^7	4.1x10^7	2x10^7	4x10^7
Green	34	1x10^8	9.7x10^7	2x10^7	3x10^7
Intermediate	49	5x 10^7	5.7x10^7	8x10^6	2x10^7

Table 1A- Mean values of viral propagules produced per caterpillar (OB/larva), standard deviation (SD), standard error (SE), and confidence interval (CI) in the three color phenotypes of *Anticarsia gemmatalis* caterpillars inoculated with AgMNPV, at two rearing densities (1 and 4 caterpillars per pot, referred to as Baculovirus Solitary and Baculovirus Gregarious, respectively). OB/larva = occlusion bodies per larva (used as an indicator of viral multiplication); SD = standard deviation (measure of dispersion of values around the mean); SE = standard error (estimate of the precision of the mean, calculated as SD/√n); CI = confidence interval (95% confidence margin around the mean, calculated as SE × 2.042 for n = 45).

	Phenotype	N	OB/larvae	SD	SE	CI
Baculovirus Solitary	Black	1	1.1x10^7	*NA*	*NA*	*NA*
	Green	20	7.2x10^7	8x10^7	1.8x10^7	3.8x10^7
	Intermediate	24	3.3x10^7	4x10^7	7.4x10 ^{6}	1.5x10^7
	Phenotype	**N**	**OB/larvae**	**SD**	**SE**	**CI**
Baculovirus Gregarious	Black	6	4.9x10^7	4x10^7	1.7x10^7	4.4x10^7
	Green	14	1.2x10^8	1x10^8	2.6x10^7	5.7x10^7
	Intermediate	25	7.1x10^7	7x10^7	1.3x10^7	2.8x10^7

Table 2A- Average values of viral propagules produced per caterpillar (OB/larva), standard deviation (SD), standard error (SE), and confidence interval (CI) in the three color phenotypes of *Anticarsia gemmatalis* caterpillars inoculated with AgMNPV, at two rearing densities (referred to as Baculovirus Solitary and Baculovirus Gregarious). OB/larva = occlusion bodies per larva (used as an indicator of viral multiplication); SD = standard deviation (measure of dispersion of values around the mean); SE = standard error (estimate of the precision of the mean, calculated as SD/√n); CI = confidence interval (95% confidence margin around the mean, calculated as SE × 2.042 for n = 45).

Phenotype	N	OB/g	SD	SE	CI
Green	34	2,1x 10^9	3,8x10^9	6,6x10^8	1,3x10^9
Intermediate	49	8,6x10^8	9,4x10^8	1,3x10^8	2,7x10^8
Black	7	6,4x10^8	4,9x10^8	1,9x10^8	4,5x10^8

Table A3- Average values of viral propagules produced per gram of caterpillar (OB/g), standard deviation (SD), standard error (SE), and confidence interval (CI) in the three color phenotypes of *Anticarsia gemmatalis* caterpillars inoculated with AgMNPV, at two rearing densities (referred to as Baculovirus Solitary and Baculovirus Gregarious). OB/g = occlusion bodies per gram of caterpillar (indicator of viral yield normalized by biomass); SD = standard deviation (measure of variability of values around the mean); SE = standard error (estimate of the precision of the mean, calculated as SD/√n); CI = confidence interval (95% confidence margin around the mean, calculated as SE × 2.042 for n = 45).

Descriptive statistics of *Anticarsia gemmatalis*

Treatment	N	Hemocytes	SD	SE	CI
Hemocyte Solitary	30	1.20×10^6	1.11×10^6	2.03×10^5	4.15×10^5
Hemocyte Gregarious	30	1.41×10^6	8.45×10^5	1.54×10^5	3.15×10^5
Black	12	1.57×10^6	9.93×10^5	2.86×10^5	6.31×10^5
Green	33	1.17×10^6	1.07×10^6	1.86×10^5	3.80×10^5
Intermediate	15	1.38×10^6	7.64×10^5	1.97×10^5	4.23×10^5

Table A4- Average values of hemocyte counts, standard deviation (SD), standard error (SE), and confidence interval (CI) in *Anticarsia gemmatalis* caterpillars reared at two different densities and expressing three color phenotypes. SD = standard deviation (measure of variability around the mean); SE = standard error (precision of the mean estimate, calculated as SD/√n); CI = confidence interval (95% confidence margin around the mean, calculated as SE × 2.042 for n = 30).

Treatment	*dF*	*Deviance*	*F*	*P>F*
Hemocyte Solitary	27	699415	0.3315	0.7208 ns
Hemocyte Gregarious	27	1432907	1.5382	0.233 ns

Table A5- Effects of larval phenotype (green, intermediate, or black) on hemocyte counts of *Anticarsia gemmatalis* caterpillars reared at two densities (1 and 4 caterpillars per container, referred to as Baculovirus Solitary and Baculovirus Gregarious, respectively). SD = standard deviation (measure of variability around the mean); SE = standard error (precision of the mean estimate, calculated as SD/√n); CI = confidence interval (95% confidence margin around the mean, calculated as SE × 2.042 for n = 30).

Treatment	N	OB/Larvae	SD	SE	CI
Control	12	NA	NA	NA	NA
VG20	12	$1x10^7$	$1x10^7$	$4x10^6$	$9x10^6$
VG29	12	$3x10^7$	$2x10^7$	$7x10^6$	$2x10^7$
VG3	12	$6x10^7$	$5x10^7$	$1x10^7$	$3x10^7$
VG35	12	$5x10^7$	$3x10^7$	$9x10^6$	$2x10^7$
VS15	12	$3x10^7$	$5x10^7$	$1x10^7$	$3x10^7$
V27	12	$3x10^7$	$5x10^7$	$1x10^7$	$3x10^7$
VS6	12	$3x10^7$	$2x10^7$	$6x10^6$	$1x10^7$
VS8	12	$5x10^7$	$4x10^7$	$1x10^7$	$2x10^7$

Table 6A- Average values of viral propagules produced per caterpillar (OB/larvae) standard deviation (SD), standard error (SE) and confidence interval (CI) in nine treatments of the *Anticarsia gemmatalis* caterpillars inoculated with AgMNPV. OB/larva = occlusion bodies per larva (indicator of viral multiplication); SD = standard deviation (dispersion of values around the mean); SE = standard error (precision of the mean, calculated as SD/√n); CI = confidence interval (95% confidence margin, calculated as SE × 2.042 for n = 12).

APPENDIX B

Supplementary material Chapter II

Descriptive statistics of *Spodoptera eridania*

Spodoptera eridania					
Experiment one: melanization response and capsule encapsulation					
Treatment	**N**	**Larvae color (Mean)**	**SD**	**SE**	**CI (±95%)**
Density 1	30	35.44	9.48	1.73	3.54
Density 2	30	28.80	8.50	1.55	3.17
Density 4	30	30.10	10.04	1.83	3.75
Density 8	30	39.45	10.58	1.93	3.95
Experiment two: Hemocyte density					
Treatment	**N**	**Larvae color (Mean)**	**SD**	**SE**	**CI (±95%)**
Density 1	30	42.84	9.82	1.79	3.66
Density 2	30	34.84	8.31	1.51	3.10
Density 4	30	30.50	8.47	1.54	3.16
Density 8	30	32.48	10.98	2.00	4.10

Table B1. Descriptive statistics of *Spodoptera eridania* larval coloration at different rearing densities in two experiments: Experiment One- Melanization response and capsule encapsulation, and Experiment Two-Hemocyte density. N- number of caterpillars evaluated; Larvae color (mean)- mean grayscale intensity; Standard deviation (sd)- dispersion of data around the mean; Standard error (se)- precision of the mean estimate (sd/√n); Confidence interval (ci) – 95% confidence margin around the mean (calculated as se × 2.042 for n = 30). Lower mean values indicate higher melanization (i.e., darker larvae).

Descriptive statistics of *Spodoptera eridania*

Spodoptera eridania					
Experiment one: melanization response and capsule encapsulation					
Melanization area mm²					
Treatment	**N**	**Nylon area (Mean µm²)**	**SD**	**SE**	**CI (±95%)**
Density 1	30	122.65	126.60	23.11	47.27
Density 2	30	98.00	16.76	3.06	6.25
Density 4	30	97.58	11.70	2.13	4.37
Density 8	30	92.40	9.85	1.79	3.68
Experiment one: melanization response and capsule encapsulation					
Melanization intensity (grayscale)					
Treatment	**N**	**Capsule Color (Mean)**	**SD**	**SE**	**CI (±95%)**
Density 1	30	22.53	5.72	1.04	2.13
Density 2	30	23.09	6.65	1.21	2.48
Density 4	30	26.78	4.28	0.78	1.60
Density 8	30	28.62	6.21	1.13	2.32
Experiment two: Hemocyte density					
Treatment	**N**	**Hemocyte Density (Mean cells µl -1)**	**SD**	**SE**	**CI (±95%)**
Density 1	30	1.87×10^6	1.07×10^6	1.95×10^5	3.99×10^5
Density 2	30	1.46×10^6	1.06×10^6	1.93×10^5	3.94×10^5
Density 4	30	2.37×10^6	1.74×10^6	3.17×10^5	6.48×10^5
Density 8	30	3.42×10^6	1.96×10^6	3.58×10^5	7.32×10^5

Table B2- Descriptive statistics of immunological parameters in *Spodoptera eridania* caterpillars reared at different population densities in two experiments. For each density treatment (1, 2, 4 and 8 caterpillars per pot), the table shows: N- number of caterpillars evaluated, Mean-mean value of the measured variable, SD-standard deviation (dispersion around the mean), SE-standard error of the mean (SD/√n), CI (±95%)-confidence interval, calculated as SE × 2.042 for n = 30. All values are presented in their original units; hemocyte density is presented in scientific notation.

Descriptive statistics of *Spodoptera frugiperda*

Spodoptera frugiperda					
Experiment one: melanization response and capsule encapsulation					
Treatment	**N**	**Larvae color (Mean)**	**SD**	**SE**	**CI (±95%)**
Density 1	15	106.86	17.93	4.63	9.93
Density 2	15	110.40	23.70	6.12	13.12
Density 4	15	118.83	13.98	3.61	7.74
Density 8	15	117.92	16.23	4.19	8.99
Experiment two: Hemocyte density					
Treatment	**N**	**Larvae color (Mean)**	**SD**	**SE**	**CI (±95%)**
Density 1	30	120.33	19.46	3.55	7.27
Density 2	30	128.81	10.78	1.97	4.02
Density 4	30	120.21	16.98	3.10	6.34
Density 8	30	116.05	20.30	3.71	7.58
Experiment three: Susceptibility of *Spodoptera frugiperda* to SfMNPV and viral multiplication					
Treatment	**N**	**Larvae color (Mean)**	**SD**	**SE**	**CI (±95%)**
Control	30	119.71	7.68	1.40	2.86
Density 1	30	119.00	11.94	2.18	4.46
Density 2	30	120.58	10.28	1.87	3.83
Density 4	30	107.28	8.07	1.47	3.01
Density 8	30	135.50	8.80	1.60	3.29

Table B3. Descriptive statistics of *Spodoptera frugiperda* larval coloration at different rearing densities in two experiments: Experiment One- Melanization response and capsule encapsulation, Experiment Two- Hemocyte density and Experiment three- Susceptibility of *Spodoptera frugiperda* to SfMNPV and viral multiplication N- number of caterpillars evaluated, Larvae color (mean)- mean grayscale intensity, Standard deviation (sd)- dispersion of data around the mean, Standard error (se)- precision of the mean estimate (sd/√n), Confidence interval (ci) – 95% confidence margin around the mean (calculated as se × 2.042 for n = 30).

Descriptive statistics of *Spodoptera frugiperda*

Spodoptera frugiperda					
Experiment one: melanization response and capsule encapsulation					
Melanization area mm²					
Treatment	**N**	**Nylon area (Mean µm²)**	**SD**	**SE**	**CI (±95%)**
Density 1	15	130.96	61.47	15.87	34.04
Density 2	15	111.94	40.30	10.40	22.31
Density 4	15	119.55	29.37	7.585	16.26
Density 8	15	98.54	27.87	7.19	15.43
Experiment one: melanization response and capsule encapsulation					
Melanization intensity (grayscale)					
Treatment	**N**	**Capsule Color (Mean)**	**SD**	**SE**	**CI (±95%)**
Density 1	15	37.99	17.97	4.64	9.95
Density 2	15	40.67	11.28	2.9	6.25
Density 4	15	33.82	12.096	3.12	6.69
Density 8	15	39.76	11.58	2.99	6.41
Experiment two: Hemocyte density					
Treatment	**N**	**Hemocyte Density (Mean cells µl -1)**	**SD**	**SE**	**CI (±95%)**
Density 1	30	5.58×10^5	3.58×10^5	6.53×10^4	133.62
Density 2	30	7.70×10^5	7.90×10^5	1.44×10^5	295.07
Density 4	30	9.48×10^5	6.65×10^5	1.21×10^5	248.39
Density 8	30	2.20×10^6	1.51×10^6	2.76×10^5	565.11
Experiment three: Susceptibility of *Spodoptera frugiperda* to SfMNPV and viral multiplication					
Treatment	**N**	**OB per larvae (Mean)**	**SD**	**SE**	**CI (±95%)**
Control	30	NA	NA	NA	NA
Density 1	30	4.78×10^7	5.10×10^7	9.32×10^6	1.91×10^7
Density 2	30	7.21×10^7	1.33×10^8	2.43×10^7	4.98×10^7
Density 4	30	4.94×10^7	4.19×10^7	7.66×10^6	1.57×10^7
Density 8	30	8.74×10^7	1.08×10^8	1.97×10^7	4.02×10^7

Table B4. Descriptive statistics of immune responses and viral susceptibility in *Spodoptera frugiperda* caterpillars reared at different population densities in three experiments. For each density treatment (1, 2, 4 and 8 caterpillars per pot), the table presents: N-number of caterpillars evaluated, Mean- mean value of the measured variable (e.g. area, gray scale, hemocytes, OBs), SD- standard deviation (dispersion of values), SE- standard error of the mean (SD / √n), CI (±95%)- 95% confidence interval around the mean, calculated as SE × t, with t = 2.145 for n = 15 and t = 2.042 for n = 30. Values are presented in original units or in scientific notation, as appropriate. Lower mean values indicate higher melanization (i.e., darker larvae).

Descriptive statistics of *Spodoptera frugiperda*

Treatment	N	Events	rmean	SE	Median	CI (± 95%)
Control	30	0	10.00	0.00	NA	NA
Density 1	30	30	8.40	0.13	9	8
Density 2	30	30	8.40	0.13	9	8
Density 4	30	30	8.36	0.13	9	8
Density 8	30	30	8.46	0.13	9	8

Table B5-. Descriptive statistics of time to death (days after inoculation) of *Spodoptera frugiperda* caterpillars reared at different densities and inoculated with SfMNPV. N- total number of individuals observed, Events- number of death events recorded (e.g. due to infection), rmean- restricted mean survival time (mean time to death within the observation window), SE- restricted standard error of the mean, Median- mean survival time (in days), CI (±95%)- confidence interval for the median (not available for the control group due to the absence of events). The control group showed no mortality during the observation period; therefore, its average time is fixed at the maximum follow-up time (10 days), with undefined median and confidence interval.