Doctoral Dissertation

Exploring the link between social evolution and immunity: a study using bees

社会進化と免疫の関連性についての考察：ハチを用いた研究から（英文）

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Nguyen Thi Thu Ha.

Contents

Chapter 1.

Immune systems and the evolution of social organisms

Infection by pathogens (defined here broadly to include various pathogens and parasites, including bacteria, fungi, helminths, etc.) pose a significant threat to all living organisms, highlighting the importance of having an efficient immune system capable of recognizing and eliminating pathogens. Consequently, the presence of a robust immune system holds great adaptive value. The immune defense system of individuals is known as personal/individual immunity (Cotter and Kilner, 2010). Personal immunity comprises a range of effects which act at different levels to protect the individual from pathogen threats. Research on individuals has significantly expanded our understanding of the effectiveness of organisms' immune mechanisms in protecting them against parasites and pathogens. For example, these studies have revealed how organisms recognize pathogens through PAMPs (pathogen-associated molecular patterns) and then activate different immune systems to combat various types of pathogens (Medzhitov & Janeway, 2002).

In group living organisms, there is an increased risk posed by pathogens due to high-density populations and frequent interactions among individuals, which create favorable conditions for the transmission of pathogens (Kappeler et al., 2015; Meunier, 2015). Furthermore, high genetic relatedness within social groups creates heightened susceptibility to diseases due to reduced genetic diversity (Kappeler et al., 2015; Meunier, 2015). To combat pathogens, social organisms rely not only on personal immune defenses within each single organism but also on a collective defense system known as social immunity (Cremer et al., 2007; Meunier, 2015; Van Meyel et al., 2018). Developing a social immune system alongside personal immunity is believed to be a response to the higher disease burden faced by group living (Meunier, 2015; Nguyen et al., 2023; Van Meyel et al., 2018). For instance, behaviors like allogrooming play a role in removing parasites present on the bodies of nestmates, and nest sanitation helps eliminate pathogens (Zhukovskaya et al., 2013). In addition, the emergence of social immunity in group-living species can be a factor contributing to the evolution of sociality because social immunity protects against the costs outlined above, enabling more complex and larger societies to arise. They may invest more in immune defenses or have more complex immune systems to cope with the challenges of group living.

This raises the question as to the relationship between the evolution of immunity and the evolution of social behaviour. However, previous rarely address the question of why these immune systems are closely associated with important ecological/evolutionary issues, such as social evolution. Therefore, my research was conducted to explore i) the links between social evolution and

immunity, ii) evolution of host-parasite relationships. Below, I first outline the characteristics of personal immunity and social immunity, then discuss their role in the transition from solitary to social form, and how social context can shape immune responses. Following this, I will explain how eusocial forms can help us to understand the complexity of the evolutionary relationship between host and parasites. I introduce the classic evolutionary dynamic models Arms-Race Dynamics and Fluctuating Selection Dynamics and consider their adaptive importance in host-parasite evolution.

1.1. Immunity and social evolution

1.1.1. Personal and social immune systems

1.1.1.1. Personal immunity

The personal immune system comprises two main components: an immediate 'innate' response and a delayed yet highly specific and long-lasting 'adaptive' response (Milutinović et al., 2016; Schulenburg et al., 2004)

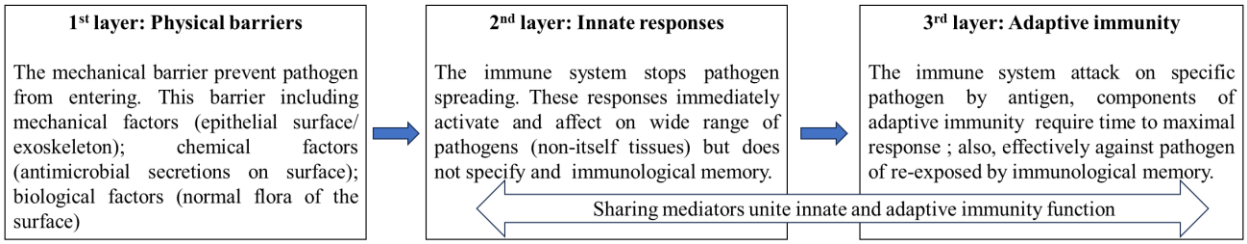

Figure 1. Protection layers of personal immunity. The first layer plays a role in preventing the entry of pathogens into the body. The second layer immediately responses to against pathogens once infected. The third layer helps the body specifically and effectively eliminate pathogens after exposure to a pathogen.

While the innate immune system is present in all living organisms, starting from prokaryotes, adaptive immunity is only found in vertebrates (Danilova, 2006; Dziedziech et al., 2020). Insects, for example, were thought to rely solely on innate immunity due to their lack of an antibody-mediated adaptive immune response which is component of adaptive immunity (Milutinović et al., 2016; Sheehan et al., 2020).

However, empirical studies have indicated that, even though lacking of adaptive immune components, innate immune responses in insects also possess "memory" and "specificity" which

are features of adaptive immune responses in vertebrates (Milutinović et al., 2016; Patel & Chatterjee, 2017; Sheehan et al., 2020). These responses exhibit traits such as lasting effects for a few days, akin to a "memory" response, and specificity resulting from a heightened ability to inhibit pathogens when previously exposed to the same pathogen from an immune priming dose (Cooper & Eleftherianos, 2017).

1.1.1.2. Social immunity

In parallel with personal immunity, social species develop a social immune system which helps to counter higher risks of disease transmission in the social form (Cremer et al., 2007). Social immunity is defined here as cooperative immune defenses which extend beyond the actor to benefit other group members (Nguyen et al., 2023). This immune system plays a role in reducing pathogen transmission, decreasing pathogen load, and countering diseases within the social organization, and functions additively with, or instead of the personal immune system. This immune system brings benefits at both the individual and colony levels. At the individual level, immune behaviors are performed by at least two individuals, where individuals directly benefit from immune behaviors carried out by other individuals within the group (benefit on a personal level). At the colony level, social immunity provides benefits for the survival of the entire colony, thereby indirectly benefiting the individuals within the colony (benefit at the group/colony level).

Benefits on an individual level

Allogrooming behavior

Various animals engage in grooming behaviors to remove pathogens, parasites, and microbial contaminants from the bodies of other group members. Allogrooming involves mutual grooming between group members, which aids in the removal of pathogens from their nestmates' bodies (Zhukovskaya et al., 2013).

Trophallaxis behavior

In some social insects, individuals can prime their immune systems by exposure to non-pathogenic microorganisms or components of pathogens (Powell et al., 2014). This immune priming enhances their resistance to subsequent infections. In addition, social insects can transfer immune molecules, such as antimicrobial peptides or antibodies, through trophallaxis (food sharing) or direct contact, which helps to protect nestmates from pathogens.

Benefits at the group/ colony level

Antimicrobial secretions

Some social insects produce antimicrobial substances, such as antimicrobial peptides, chemical compounds, or secretions from specialized glands (Gómez-Moracho et al., 2017). These substances help to inhibit the growth of pathogens and protect the colony from infections.

Collect antibiotic component

Many insect species collect materials containing antibacterial properties, such as resin from plants, and combine them with other components for nest construction to inhibit bacterial growth in nesting habitats (Meunier, 2015).

Hygienic behavior

The colony exhibits behaviors to maintain a clean and disease-free environment (Spivak & Gilliam, 1998). This can involve the removal and disposal of dead individuals, waste materials, or infected individuals from the nest or hive.

Self-exclusion

Individuals infected with diseases exhibit self-exclusion behavior or are eliminated by other members. This behavior helps eliminate pathogens and prevent their spread within the group (Van Meyel et al., 2018).

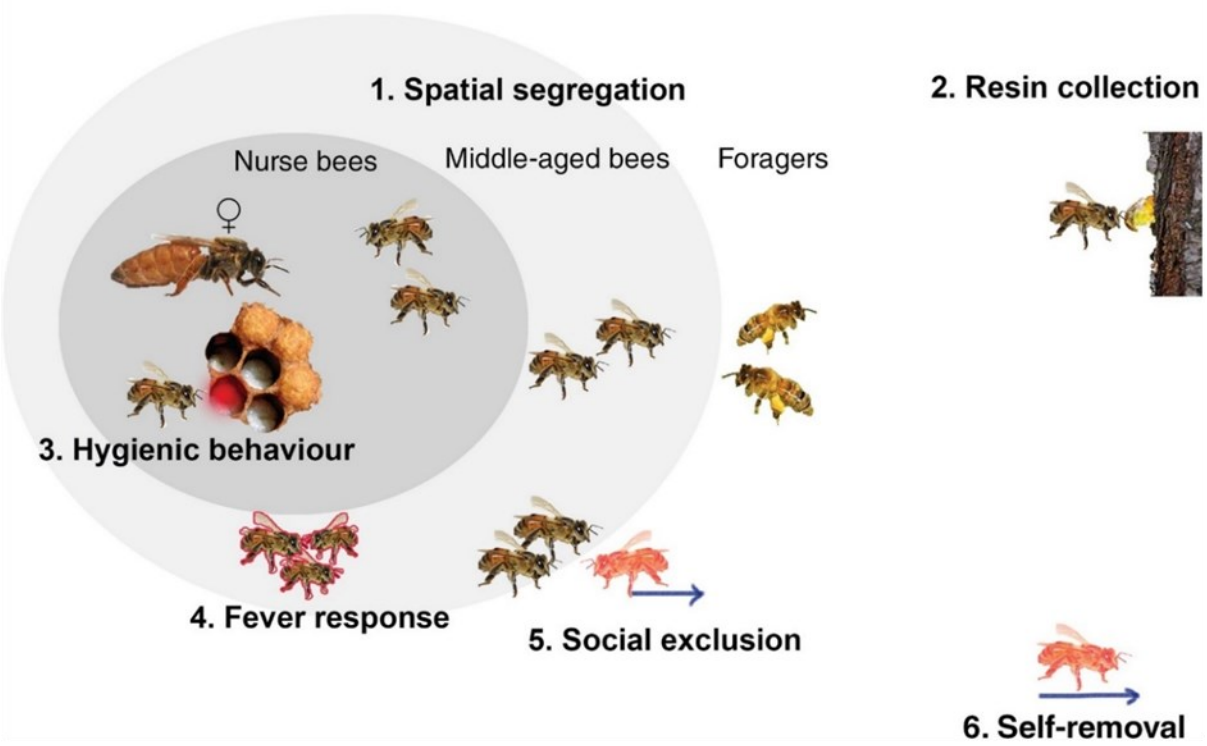

Figure 2. Social immune system of the honey bee (Gómez-Moracho et al., 2017)**.** Social immune behaviors are conducted by workers in the nest including hygienic behavior to maintain a clean and disease-free environment; fever response which is the constant flapping of wings to raise the temperature in the nest to fight against temperature sensitive fungi; social exclusion and self-removal to eliminate pathogens and prevent their spread within the group. Bees collect resin which contains antimicrobial components to build their nest to help inhibiting the growth of pathogens and protect the colony from infections.

1.1.2. Links between the evolution of immunity and sociality

According to life-history theory, immune responses have evolved while considering costs and benefits (Ardia et al., 2012; Cotter et al., 2010). Personal immunity and social immunity are both responses to pathogens, so individuals with stronger personal immunity may allocate more resources to their own immune defenses, but this could lead to reduced investment in social immunity (Meunier, 2015; Siva-Jothy et al., 2005). Research on the sub-social burying beetle *Nicrophorus vespilloides* has shown that a decrease in the antibacterial activity of anal exudates (social immunity) is caused by an increased investment in wound repair (personal immunity) (Cotter et al., 2010). The anal exudates protect resources on the carcass from microbial competitors,

then provide benefits not only for larval fitness, but also for incoming breeder on these carcasses. The focus on "personal immunity" and the reduced expression of social immunity implies that organisms do not invest in both immune systems at the same time, and individual immunity may be traded off with social immunity in their energy investment strategies.

Based on this trade-off hypothesis, Van Meyel et al. (2018) proposed that evolution is of social immunity can occur via two different frameworks; i) the Eusocial framework and ii) the Group-living framework.

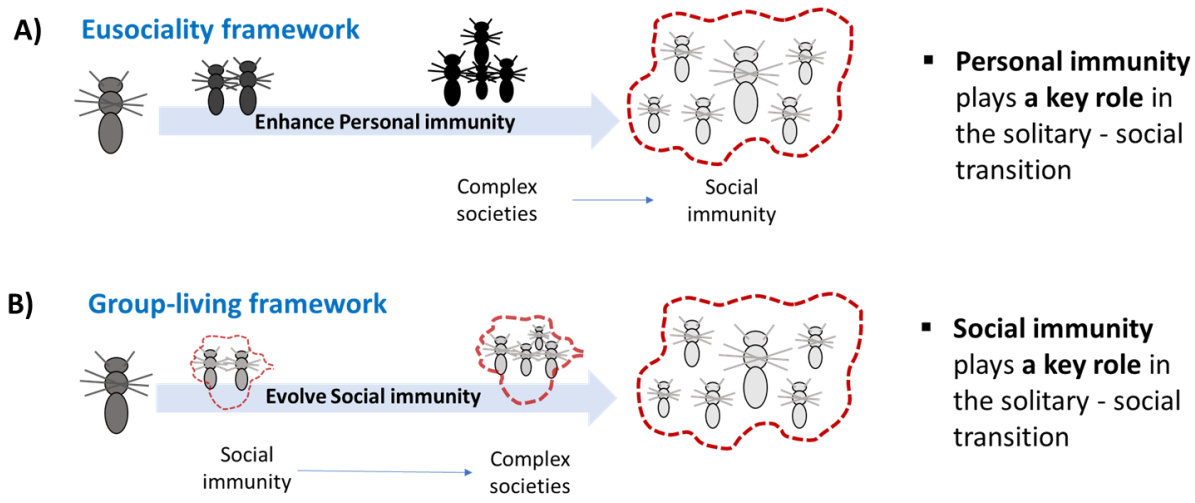

Figure 3. Eusocial framework and Group living framework. The gradient color of each bee indicates how strong of personal immunity, darker means stronger and paler means weaker. The read dashed outline indicates social immunity with bolder dashed means stronger social immunity and fainter dashed means weaker social immunity. A) Eusocial framework. B) Group-living framework.

Eusocial framework (Figure 3A)

Eusocial species exhibit an obligate reproductive division of labor, with only a few individuals (queens and males) responsible for reproduction, while the rest of the colony members are sterile workers (Cremer & Sixt, 2009). Therefore, their inclusive fitness is determined by colony survival, not individual survival. According to the Eusocial framework, such complex societies evolved prior to the development of social immunity. Social immunity, in turn, is a consequence of eusociality, and allows such large societies to be maintained (Van Meyel et al., 2018).

The emergence of social immunity in eusocial forms can contribute to colony survival by shifting higher risks of disease transmission on the worker caste. Rather than all individuals facing equal disease risks within the society, the worker caste assumes the primary responsibility for engaging in activities with elevated exposure to pathogens, such as foraging. When these individuals become infected, they may be removed from the colony, which helps reduce disease pressure and improves the chances of survival for the entire colony. Under the eusociality framework, social immunity is thus considered a product of social evolution, emerging after complex societies form. As social immunity is a secondary product derived from eusociality.

However, as non-eusocial group-living species lack an obligatory division of labor like eusocial forms, we might expect that they should rely on personal immunity while transitioning from solitary to social lifestyles. As group living increases the risk of pathogen infection (see above), individuals in non-eusocial groups should increase investment in personal immunity relative to solitary individuals.

The enhanced individual immunity in the face of high disease risk associated with group living is known as Density-Dependent Prophylaxis (DDP) (Wilson & Cotter, 2009). Density-Dependent Prophylaxis is a form of phenotypic plasticity in which individuals up-regulate their parasite resistance mechanisms under conditions of higher density to counteract the increased risk of pathogen infection (Wilson & Cotter, 2009). Immune investment of individuals can therefore be expected to vary depending on density. Density serves as a signal indicating a higher risk of disease transmission, prompting individuals within the group to adapt in response to this threat. The hypothesis is supported in a wide range of insect families (Wilson & Cotter, 2009). For example, in desert locusts, *Schistocerca gregaria*, stronger fungus resistance is found in individuals in the *gregaria* phase (form swarms of migration) than that in *solitaria* phase. Across species, experiments conducted across various taxa in the Hymenoptera, which encompass a broad spectrum of sociality ranging from solitary to semi-social and eusocial, have highlighted significant differences in bacterial resistance between solitary and social species, particularly, personal immunity increasing progressively with complexity and density of social organization (Stow et al., 2007).

Group-living framework (Figure 3B)

As an alternative explanation to the Eusocial framework, the Group-living framework proposes that social behaviour and social immunity co-evolved. This idea is based on the fact that social immunity is not only found in eusocial species but also exists in non-eusocial species that exhibit group living, such as the secretion of antifungal chemicals by group-living beetles *Dendroctonus rufipennis* to inhibit fungal growth within their nests, or the hygienic behavior of removing frass in the subsocial cockroach *Cryptocercus punctulatus* (Meunier, 2015). This evidence raises a question about the role of social immunity in a non-eusocial organisms (Meunier, 2015; Van Meyel et al., 2018).

Could the emergence of social immunity in non-eusocial species play a more significant role in the social evolution, rather than just serving as a replacement or mimicry of personal immunity in eusocial forms as conceptualized by Cremer et al., 2009 and Van Meyel et al. 2018?

The Group-living framework assumes that social immunity is established in the early stages of group-living as an alternative investment strategy traded-off with personal immunity. In this scenario, social immunity co-evolves with social behaviour. (Meunier, 2015; Van Meyel et al., 2018). The research on eusocial species reveals a decreased investment in personal immunity when compared to solitary species. For instance, highly eusocial *Apis mellifera* has approximately one-third of the immune gene families found in solitary *Drosophila* and *Anopheles* (Evans et al., 2006). This reduced investment on personal immunity is also supported by physiological studies of 12 Lepidoptera species, which found higher levels of hemocytes and phenoloxidase activity in solitary species compared to gregarious species (Wilson et al., 2003). These findings also support the idea of a trade-off between personal immunity and social immunity, resulting in a diminished investment in personal immunity under different social circumstances (eusocial versus solitary) or varying population densities (gregarious versus solitary).

The presence of social immune traits in both non-eusocial and eusocial organisms suggests that social immunity is an ancestral phenomenon and does not have eusociality as a prerequisite as initially thought. In addition, the reduced investment in personal immunity suggests the importance of social immunity in the evolution of sociality. Therefore, we might expect that organisms would rely on social immunity during the transition from solitary to social states, and as a consequence, the investment in personal immunity would decrease due to the trade-off with social immunity.

I investigate the likely importance of these two frameworks in the evolutionary transition between social and solitary behaviour using two facultatively social bee species in Chapter 2 and 3. As a secondary aim of my PhD studies, I also investigated how social immune systems might respond to changes in immune challenge (Chapter 3). Besides, I use eusocial species to understand the evolution of host-parasite relationships (Chapter 4). Below, I explain how the relationship between host and parasite can be elucidated by consideration of two canonical evolutionary dynamic models: Arms-race dynamics and Fluctuating selection dynamics. These are two classic models used when considering the host-parasite relationships from a population perspective.

1.2. Evolution of host-parasite relationships

The interactions between hosts and parasites give rise to diverse immune responses through the dynamics of Arms-races and Fluctuating selection. Independent of the cost of immune defense, these approaches can explain the dynamics that contribute to the diversity in immune responses from a population perspective. These models assume that coevolution between hosts and parasites can result in genetic structure and diversity within host and parasite populations (Siva-Jothy et al., 2005).

Arms-race dynamics (ARD): In this model, there is no frequency-dependent selection, and both the host and parasite continually accumulate adaptive mutations to gain an advantage over each other (Gandon et al., 2008). The term "arms-race" reflects the idea that both parties are engaged in constant competition to improve their defenses and infectivity. As a result, this leads to an ongoing escalation of adaptations in both the host's immune system and the parasite's ability to evade it (Gandon et al., 2008). The directional selection for increased resistance and infectivity may lead to a reduction in diversity within the host-parasite populations (Quigley et al., 2012).

Fluctuating selection dynamics (FSD): In contrast, the FSD model involves negative frequency-dependent selection (Gandon et al., 2008). In this model, the frequencies of host and parasite genotypes fluctuate over time and space, and the success of a particular genotype depends on its relative abundance in the population. If a genotype becomes too common, it becomes more vulnerable to the actions of the opposing genotype, leading to a decrease in its fitness (Gandon et al., 2008). As a result, rare host genotypes are favored through negative frequency-dependent selection, which helps maintain genetic variation within a host population (Siva-Jothy et al., 2005). According to this hypothesis, parasites should become adapted to their local hosts, and they may

not be as efficient at infecting different host types (Siva-Jothy et al., 2005). This dynamic process of fluctuating selection ensures that genetic diversity remains preserved among host populations.

Overall, the host-parasite co-evolutionary processes have far-reaching implications for the genetic structure and diversity of host and parasite populations. These encompasses both competitive dynamics and specialized interactions, contributing to the intricate interplay between hosts and parasites in various ecological contexts.

1.3. Study model and aims of this study

1.3.1. Study models

The understanding of the relationship between social evolution and immune function has been limited by the choice of appropriate study model systems. These limitations may arise from confounding factors that impact personal immunity, such as basic life history, and the magnitude of intrinsic immune responses of individuals when comparing across species. Typically, research among species in Hymenoptera has encountered an exception when the majority of species show an increasing trend of antimicrobial resistance with the complexity of their societies whereas an eusocial species exhibited weaker antimicrobial activities compared to semi-social species, and this could be influenced by life history differences such as habitat, leading to varied antimicrobial behaviors in adaptation to their respective environments (Stow et al., 2007). In addition, eusocial models are considered inappropriate for the early stage of the transition from solitary to social due to the potential reproductive division of labor, which can lead to an imbalance in the trade-off between individual and social immunity. Particularly, the difficulty arises from the sterility of the majority of group members in eusocial colonies, making it challenging to distinguish the fitness of donors and recipients (Meunier, 2015). To address this challenge, studying non-eusocial species is an ideal approach, as their group members have measurable individual fitness and specially may undergo both solitary and group-living phases in their life cycle (Meunier, 2015). Therefore, I use the facultative bees *Ceratina okinawana* and *Lasioglossum baleicum*, non-eusocial species, as study models to explore the relationship between social context and personal immune efficacy. In these species, both social and solitary forms exist in the same population, allowing us to compare social context without interspecific cofounding effects. Due to their ability to switch between solitary and social form, these bees can provide insights into the early stages of social evolution.

Social behaviour is thought to have evolved multiple times from solitary ancestors in the Halictidae (Brady et al., 2006), while in *Ceratina*, a simple social form existed in the ancestral lineage, was demonstrated a reversal to a solitary form several times (Rehan et al., 2012). In both groups, various species maintain a social polymorphic state that is likely long-term and may give them benefits in variable environments (Wcislo & Danforth, 1997). The polymorphism of this bee species presents a versatile framework for revealing the adaptability of immune responses across varying social structures, without being restricted by the social constraints (such as mandatory division of labor or obligatory reproductive roles).

On the other hand, studies on the host-parasite relationships are mainly interested in host adaptation. This study provides the opposite view, in terms of parasites, by using the *Apis* - *Varroa* system. This model is a good one to examine this relationship because the host species are closely related and have similar life-histories but have different evolutionary relationships with the parasite. In *Apis cerana* we have a long history of *Varroa* infection and an adapted immune response. In *Apis mellifera* we have a recent shift and high susceptibility (Beaurepaire et al., 2015). This suggests that the social immune response has adapted to this threat in one species but not the other. Furthermore, we might expect different evolutionary adaptation levels within species, where exposure has been for a different amount of time. Further investigation of the immune evolution in *Apis cerana* requires clarification of the relationships between the host species and parasites, as evidence on this has been contradictory. In this study I therefore take the first step in investigating host-parasite relationships among *Apis* and *Varroa* mites in Vietnam, where *Varroa* infects both host species in sympatry.

1.3.2. Aims of the study

This dissertation addresses two principal questions to explore the links between social evolution and immunity and evolution of host-parasite relationships:

1. How do immune systems cope with the transition from solitary to social behavior? Under aim, I investigate two sub-questions:
    - How does social context influence immune efficacy?
    - Does social context influence the mechanistic basis of the immune response to pathogen challenge?
2. How do host-parasite relationships evolve in a eusocial context?

The dissertation consists of five chapters that address the principal study questions. In Chapter 1, I present the concepts of personal immunity and social immunity, and their roles in the social context. This chapter establishes the framework for all the discussions in the thesis and clarifies the research questions being explored. Chapter 2 is conducted using the *Ceratina okinawa* model, where the comparison between personal immunity in a social context and in a solitary context is made to answer the question of how the social context influences immune efficacy. Chapter 3 is conducted on the *Lassioglosum baleicum* model. This experiment assesses gene expression in response to pathogens under changes in social context, transitioning from social to solitary conditions. Understanding the regulation of gene expression between different social contexts will help answer whether immune systems respond differently to changes in the social environment. Chapter 4 involves an experiment on the *Apis*-*Varroa* model to answer the question of how the host-parasite relationship evolves in a eusocial context. The genetic structure of *Varroa* mites will be examined to understand their interactions with the host. Chapter 5 is a general discussion and describes some potential research questions that should be explored in the future.

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Chapter 2.

The influence of social context on personal immunity

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2.1. Introduction

Living in societies precipitates advantages through specialization and cooperation but comes at the cost of increased resource competition and a heightened threat from pathogens. Pathogens (defined here broadly to include viruses, bacteria, protozoans, helminths and fungi) pose a greater threat to groups because transmission is more likely at higher densities and among genetically related individuals (Alexander, 1974; Brahma et al., 2022; Hamilton, 1987; Kappeler et al., 2015; Meunier, 2015; Schmid-Hempel, 1998). To combat these heightened risks, animals living in groups have been reported to increase investment in personal immunity (ie: density dependent prophylaxis or DDP; Reeson et al., 1998; Wilson and Reeson, 1998). Ant queens co-operatively founding nests, for example, have higher investment in personal immunity than solitary foundresses (Brütsch et al., 2017), and cross-species studies of wasps, bees and thrips, have shown that the efficacy of cuticular antimicrobials increases with group size (Hoggard et al., 2011; Stow et al., 2007; Turnbull et al., 2010; but see Penick et al., 2018;). Group-living species can also employ a diverse array of cooperative immune defences which extend beyond the actor to benefit other group members. These defences are collectively known as 'social immunity' (Cremer et al., 2007), and include mutual grooming, exclusion of infected individuals, and use of antimicrobial secretions (Bordes et al., 2007; Bos et al., 2012; Cremer et al., 2007; Meunier, 2015; Reber et al., 2011; Rosengaus et al., 2004; Stockmaier et al., 2021). Social immunity can ameliorate the need for personal immunity, and investment in social immunity may trade-off against investment in personal immunity because of the cost of mounting immune responses (Cotter and Kilner, 2010; Meunier, 2015).

Social immunity was initially considered a characteristic of highly eusocial societies with obligatory group-living. However, this perspective has since been questioned on the grounds that it is based on several misconceptions, including mounting evidence for social-immune defences in non-eusocial organisms (Van Meyel et al., 2018). This raises the question of whether social immunity can be considered a secondary trait that arose following the evolution of complex

obligatory social systems (the 'eusociality framework' (Cremer et al., 2007; Cremer et al., 2018; Schmid-Hempel, 2017)), or an ancestral trait which may have facilitated the transition from solitary to group living, and which can thus be considered an important factor in the evolution and elaboration of social behaviour ('group living framework' (Cotter and Kilner, 2010; Meunier, 2015; Van Meyel et al., 2018)).

Further elucidation of the relationship between social evolution and immune function has been constrained by the limitations of appropriate model systems, including potential confounding effects arising from cross-species comparisons (Evans et al., 2006; Hoggard et al., 2011; Stow et al., 2007; Turnbull et al., 2010), transient life-history stages (Brütsch et al., 2017), the use of sub-social species, which we might expect to have limited social immune behaviours (Wilson et al., 2003), and use of highly eusocial species, which are not suitable for studies of early stages in social evolution (Cotter and Kilner, 2010; Meunier, 2015). In this study, we provide a new perspective on this question by exploring the relationship between social context and immune efficacy in a facultatively social organism: the small carpenter bee *Ceratina okinawana*. In this species, both social and solitary forms exist in the same population, allowing us to compare social context without inter-specific cofounding effects. We use a novel immune assay to explore how personal immunity varies between individuals from solitary and social colonies, allowing us to shed new light on the importance of social immunity in the transition between solitary and social behaviour.

2.2. Methods

2.2.1. Model system

Entire colonies of *Ceratina okinawana* were collected in October 2021 in stems of *Miscanthus sinensis* from Tokashiki island, Japan (26.19611°N, 127.3606°E). Nests were opened in the laboratory and adults maintained in identical laboratory conditions in artificial nests until immune assays (supplemental material, figure S1). Colonies were classified as solitary (one adult female) or social (>1 adult female; supplemental material, data file 1). We measured size characteristics (weight, wing length and head width) of bees and quantified mandibular wear and wing wear as proxies of age (supplemental material, figure S2). In the absence of information on how the presence different brood stages or sexes of adult bee might influence immune investment, we use total 'group size' (number of all brood and adults in the nest) as a measure of the density of individuals in the nest.

2.2.2. Immune assays

For immune testing, we used a single adult female bee from each nest (n = 24 social and n = 32 solitary). For social nests, we selected a random individual or the larger individual when there was a clear size difference, under the assumption this was more likely to be the original founding queen and thus more directly comparable to solitary nesting bees (see also electronic supplementary material). We quantified personal immune efficacy by assessing the capacity for bee haemolymph to suppress bacterial growth, using a novel immune assay (supplemental material, supplementary methods). Briefly, whole bee haemolymph was extracted into a collection buffer by centrifuging bees after removing antennae. To this mix, we added *Escherichia coli* modified to grow in antibiotic medium, allowing us to avoid contamination from microorganisms naturally present in bees. This mixture was incubated for 15 hrs in antibiotic medium, and growth of *E. coli* quantified each hour over the subsequent six hours as the change in the absorbance in this mixture using a spectrophotometer at 600 nm. As a control, we used a mix of buffer and bacteria (n = 10).

2.2.3. Statistical analysis

Social and solitary colony bee size and wear characters, brood numbers, and nest lengths were compared using Wilcoxon signed rank tests, while brood composition of nests was compared using a Fischer's exact test. Immune efficacy was quantified as the total change in absorbance over the experimental duration. This was compared between treatments (social and solitary) and the control using Kruskall-Wallace test and Dunn post-hoc test using the dunn.test package of R (Dinno, 2017). We explored relationships between the log transformed values of the change in absorbance and demographic and individual factors using a generalized linear model (GLM), which included social status, individual size, mandibular wear, and number of group members (adults and brood) as factors and the interaction between group size and social status. We report test statistics from type-II analysis of variance on the output from the GLM, generated with the *Anova* function of the *car* package for R (Fox and Weisberg, 2019). All analyses were performed in R version 4.0 (R Core Team, 2022).

2.3. Results

2.3.1. Contrasts between social and solitary nests

Individuals from social and solitary nests did not differ in size or wear characteristics (figure 1a) and there was no difference in the number of broods between social types (figure 1b). The nests of social colonies were longer (figure 1c; Wilcoxon's $W = 614$, $p = 0.001$), and social and solitary colonies differed in brood composition (Fischer's exact test, $p = 0.033$), though both types of colonies had representatives of all brood stages (figure 1d). Measures of adult size (wing length, head width and weight) were correlated with each other (Pearson's $\rho > 0.91$, $p < 0.001$) as were measures of wear ($\rho = 0.55$, $p < 0.001$), though there was no relationship between size and wear (electronic supplementary material, figure S3). We thus selected head-width and mandibular-wear as representative measures of size and age for use in GLM analysis.

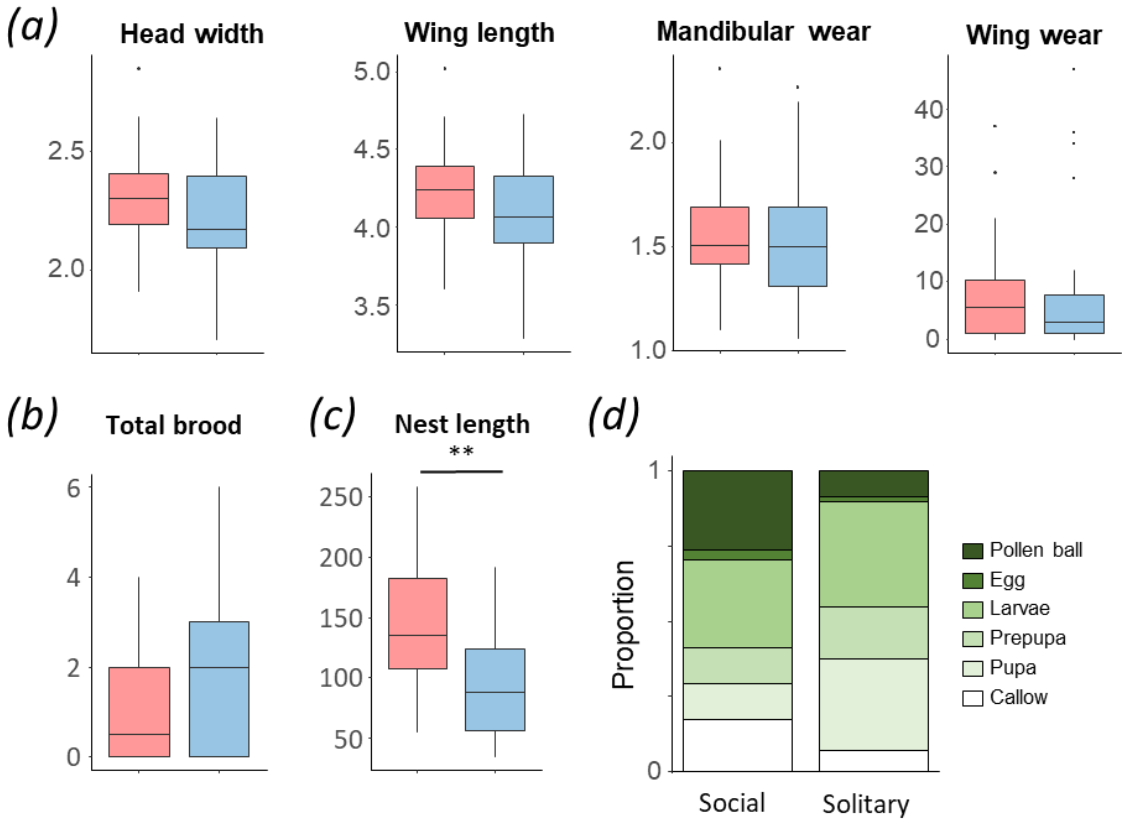

Figure 1. Comparative characteristics for (a) adult females, (b) brood number, (c) nest length and (d) brood composition, from social (red) and solitary (blue) nests. Boxes indicate interquartile range, solid horizontal lines medians, whiskers minimum and maximum values, and points indicate outliers. Head width, wing length and nest length are in millimetres, wing wear and total brood are counts, mandibular wear is a ratio of width to height, and brood composition is the proportion of total brood.

2.3.2. Immune efficacy in social and solitary individuals

Bacterial growth was suppressed by bee haemolymph, and this effect was greater in individuals from social nests than those from solitary nests (figure 2a). Control samples showed relatively low variance, indicating the assay approach yielded consistent results where expected, and that immune efficacy varied considerably among individuals from both solitary and social nests. However, differences between treatments were consistent with a density effect, as GLM analyses indicated that immune efficacy increased with group size (Figure 2b; $\chi^2 = 6.071$, $p = 0.013$) while there was no significant effect of other factors and no interaction between group size and social status (supplemental material, table S5).

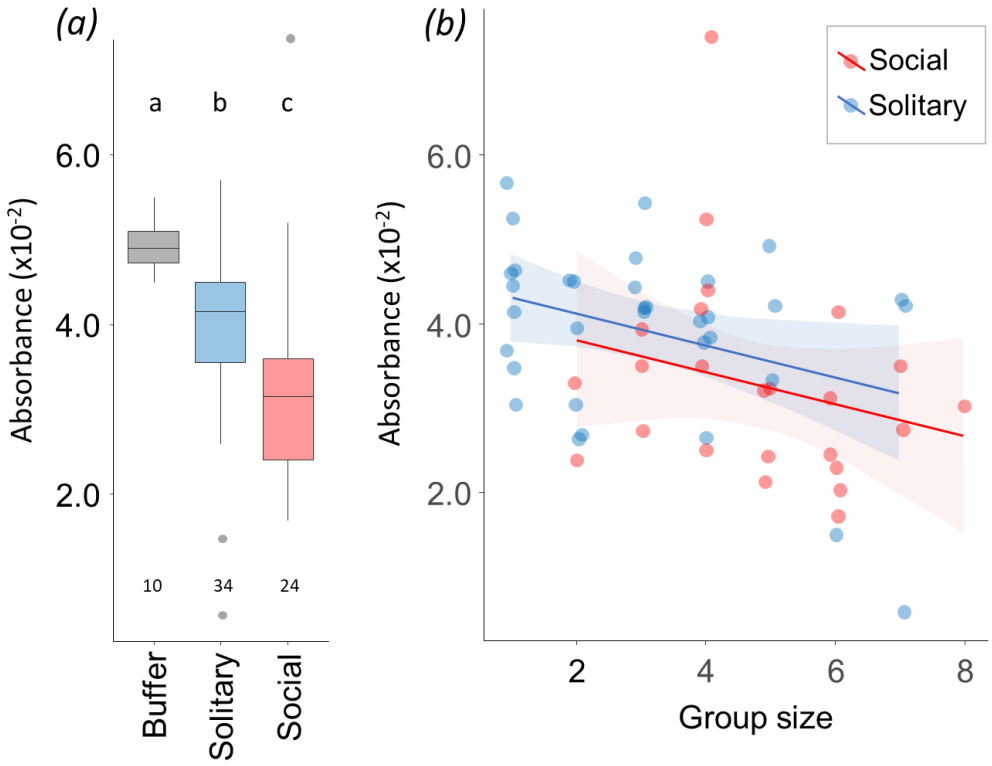

Figure 2. (*a*) Boxplot of total change in absorbance for two treatment and the control group. Sample sizes are included above the x-axis. Letters above bars indicate significant differences among groups based on Kruskal-Wallace and Dunn post-hoc tests. (*b*) Jitter plot of relationship between overall change in absorbance and group size (number of adults and brood in the nest). Blue dots and line indicate solitary colonies and red dots and line indicate social colonies. Shaded areas denote 95% confidence intervals of the regression lines. Change in absorbance was

quantified as the change in optical density at 600 nm of the medium containing haemolymph and bacteria, with higher values indicating greater bacterial growth and thus lower immune efficacy.

2.4. Discussion

We use a novel assay technique to demonstrate that bees from social and solitary nests in a facultatively social bee exhibit patterns consistent with differences in immune efficacy, with social nesting bees having higher antibacterial activity than solitary nesters. However, bees from larger colonies (more adults and brood) also had higher immune efficacy than those in smaller colonies, suggesting that differences in immune efficacy among social and solitary nests can be explained by a density-dependent prophylactic effect (Wilson and Reeson, 1998) rather than an influence of social status per se. We interpret this pattern as indicative of an increase in personal immunity in response to the greater risk of infection posed by larger groups. Previous studies have shown that lower immune efficacy in solitary individuals can result from the stress of being alone (Kohlmeier et al., 2016) (though this can also arise from stress associated with group living (Kappeler et al., 2015)). Additionally, while our experimental protocol effectively precluded of social immune effects after the bacterial challenge, this did not exclude the influence of social immune effects occurring prior to experimentation, such as immune-priming through trophallaxis (Casillas-Pérez et al., 2022; Masri and Cremer, 2014). However, the above alternatives are unlikely given that i) the costs of mounting an immune response mean that investment in personal immunity can generally be expected to limit investment in social immunity (Cotter et al., 2010; Cotter et al., 2013), and ii) the relationship between immune efficacy and group size showed no evidence of a marked shift across the social/solitary boundary and was largely linear, as expected from density-related effects.

Density dependent prophylactic effects have been documented in a wide range of non-social insects, but are also found in social species (reviewed in Wilson and Cotter, 2009). Similarly, social immunity, once considered the hallmark of eusocial organisms, has been found in sub-social species (Van Meyel et al., 2018), blurring the expected association between social behaviour and social immunity. Comparative studies across different kinds of social systems have shown increases in individual immune efficacy with increasingly complex social groups of related species (Hoggard et al., 2011; Stow et al., 2007; Turnbull et al., 2010; but see Wilson et al., 2003), but cannot disentangle the effects of group-size from those of social-context, and are complicated by

life-history differences among study species. The present study is the first study to our knowledge to investigate changes in immune efficacy in a socially polymorphic species, allowing us to investigate the role of social immunity in the early stages of social evolution without the potential confounding effects of interspecific comparisons. Our results indicate a reliance on personal immune systems over the transition from solitary to social behaviour in this species. These findings are consistent with the idea that social immunity evolved secondarily, following the evolution of group-living (the 'Eusociality framework') rather than being an ancestral feature which facilitated the evolution of sociality ('Group-living framework') (Van Meyel et al., 2018).

While we are able to demonstrate an overall effect of group size on personal immune efficacy across social and solitary colonies in *C. okinawana*, some limitations of our study await further investigation. Firstly, our immune assay approach does not permit disentangling effects of haemolymph quality and quantity, and the relative importance of these factors deserves further investigation. Secondly, the haemolymph extraction protocol precludes an assessment of the reproductive status of adults, which may be important given the possible trade-off between investment in immunity and reproduction (Schwenke et al., 2016). Therefore, although we targeted larger (and thus more likely to be reproductive (Okazaki, 1987)) individuals in social nests, the potential influence of this trade-off remains unclear. Thirdly, we show that brood number and composition do not differ markedly between social and solitary colonies but cannot rule out the possibility that brood of different developmental stages influence immune investment differently. Experimental manipulation of brood composition and/or comparative study of immune expression during colony ontogenetic development are thus needed. Finally, our findings are correlative and would benefit from experimental validation, such as through manipulations of group size.

Density dependent prophylaxis is a form a phenotypic plasticity (Wilson and Cotter, 2009), and as such may befit socially polymorphic species. The degree of flexibility offered by this mechanism is evidenced by studies of bumblebee workers, which can rapidly shift immune function in response to change in social context (Ruiz-González et al., 2009). As transitions from solitary to social life are likely to progress through a facultative stage (Shell and Rehan, 2018), a single system offering flexibility may be more adaptive at this stage than switching between different forms of immunity. Further studies of socially polymorphic species will help establish the generality of these patterns.

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2.5. Supplementary Methods

2.5.1. Model system

Ceratina okinawana is a facultatively social bee native to the southern Ryukyu Islands of Japan. This species inhabits hollowed out stems of Chinese silver grass (*Miscanthus sinensis*), forming linear nests with separated brood cells. Social nests occur during the brood rearing season, and in Naha in Okinawa, this species can have three broods a year (Okazaki, 1987). Nests can contain >2 adult females, and multiple egg-laying females may be found in the same nest (Okazaki, 1987; Sakagami and Maeta, 1995). Sociality is facultative in this species and both social and solitary nests can be found in the same population.

Bees in this study were collected in early of October 2021, Tokashiki island, Japan (26^{o} 11' 46" N, 127^{o} 21' 38" E). Nests were collected late at night or before dawn when it can be expected that all adults are in the nest.

Nests were returned to Tokyo Metropolitan University, where they were opened, and adults transferred to artificial nests. Demographic information on nest contents can be found in supplementary data file 1.

Bees were maintained in artificial nests consisting of 25x2x1cm hardwood stick with a 6mm groove cut down most of its length which was covered with transparent plastic. Bees were fed sugared water and kept at room 25^{o}C until haemolymph extraction (figure S1).

Figure S1. *Ceratina okinawana* in artificial laboratory nests.

2.5.2. Morphological measurements

Bee size

Adult size was quantified using three measures, i) weight, ii) wing length, and iii) head width (figure S2). The weight of bees following haemolymph extraction was measured using on a Vibra XFR scale. Head width was calculated as the broadest part of the head in frontal view going through the base of both antennae (Figure S1). Wing length was calculated as the average of WL1 and WL2 (Figure S1) for each wing. All lengths were measured using Image-J software on photos obtained from a Mokose UC70 USB camera attached to a Micronet YS05T dissecting microscope. Measures were calculated with reference to a 0.01 mm scale stage micrometer. Each individual measure was repeated twice (except for head width which was taken three times) and an average calculated across measurements for each individual. Wing and wear measures were then averaged across left and right sides. These measures were highly repeatable, with a proportionate error (proportion of average absolute difference to mean) of <1% for larger measures (wing length and head size) and 3-5% for small-scale measures (mandible measurements; Table S1).

Table S1: Proportionate differences from means for repeated morphometric measures.

Measure	Mean±SD
Left wing length	0.35±0.27
Right wing length	0.38±0.27
Mandible height	5.72±5.51
Mandible width	2.55±2.20
Head width	0.27±0.16

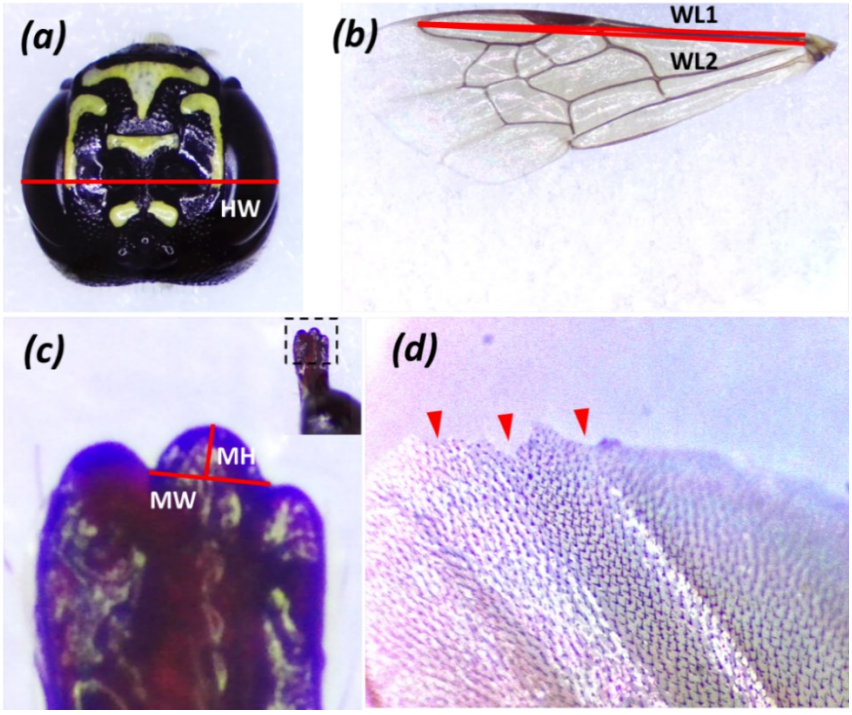

Figure S2. Size and wear measurements for bees. Red lines indicate different measurements taken for (a) head width, (b) wing length and (c) mandible width (MW) and height (MH). (d) shows a fore-wing margin with nicks indicated by arrows.

Bee age class

As it is difficult to quantify the age of adult bees, we used two wear measures as indirect indicators of age. While we acknowledge that wear will also be influenced by behaviour, we expect foundress females to show more extreme measures of wear than first generation females (workers). As wear indicators, we quantified mandibular and wing wear. Mandible wear was calculated as the ratio of the height of the central tooth of the mandible to its width (figure S2). As for size measures, each length was measured twice for both left and right sides, then averaged over all measures. Wing wear was quantified as the number of 'nicks' in the margin of both fore-wings (figure S2).

2.5.3. Density-optical immune assay Protocol

This assay approach for inferring immune efficacy quantifies the capacity for haemolymph to suppress bacterial growth, as measured by changes in optical density. This method accounts for variation in the turbidity of haemolymph resulting from the extraction process by using the change in absorbance over time. It also employs a modified bacteria in which pUC19, a plasmid containing

ampicillin resistance gene, has been inserted. Bacteria can thus be cultured in a medium containing ampicillin to avoid contaminating effects of microorganisms naturally present in subjects. The method is highly sensitive and thus can be used for species in which only small amount of haemolymph can be extracted, for low concentration (diluted) haemolymph, and without immune priming (injecting pathogen into live organisms to increase antimicrobial components).

Extraction of haemolymph

For small bees (*Ceratina*) we first detached antennae, then placed the bee upside down in a 200 µl tube with a small hole on the bottom. This tube was then placed in a 1.5 ml tube containing 10 µl of collection buffer (table S2) and centrifuged at 4500 rpm, for 5 minutes, at 4°C, to extract haemolymph. All samples were then kept on ice during subsequent experimental duration.

Bacterial preparation

Escherichia coli competent cells were used for insertion of pUC19 plasmids and cultured on an LB plate contain ampicillin (table S3). Cloned *E. coli* were then cultured in an LB broth (table S4). Bacterial concentration of this broth was quantified via optical density at 600 nm (OD600) (Eppendorf BioPhotometer), and bacteria harvested when in the log phase of growth (OD600 of around 0.6). At this time, the suspension was diluted using additional LB broth to an OD600 of 0.04 for immune testing.

Bacterial challenge

All of the haemolymph/collection buffer mixture for each individual bee was pipetted into a separate well of a 384 well plate. For *Ceratina* bees, the total volume was approximately 11 µl (10 of which was collection buffer). To each well, we added 40 µl of the prepared *E. coli* suspension and mixed this with the haemolymph/collection buffer. Collection buffer was used instead of haemolymph for control samples. These plates were then incubated at room temperature (25°C) for 15 hours. A period of incubation was necessary to allow bacteria to achieve sufficient abundance to permit accurate quantification of further growth using the optical density method. Preliminary testing indicated that 15 hours was suitable for this purpose. Following this, bacteria growth was measured each hour over the next six hours by quantifying optical density using a spectrophotometer (EnSpire Multimode Plate Reader) at 600 nm. The difference in absorbance

values were at each hour relative to the initial values were calculated to determine bacterial growth and account for inter-sample variation in turbidity.

Table S2. Components of collection buffer (Stoepler et al., 2012). Those shown in italics comprise the anticoagulant buffer.

Component	**Quantity**
Fetal Bovine Serum	10%
Grace's Medium	60%
Anticoagulant buffer (@pH 4.5):	30%
NaOH	*98 mM*
NaCl	*186 mM*
EDTA	*1.7 mM*
Citric acid	*41 mM*

Table S3. Components of LB plates (LB agar with ampicillin).

Component	**Quantity**
Tryptone	10 g
NaCl	10 g
Yeast extract	5 g
Ampicillin (concentration100µl/ml)	1 ml
Agar	20 g
H_2O	1 L

Table S4. Components of LB broth (Miller) with ampicillin

Component	**Quantity**
Tryptone	10 g
NaCl	10 g
Yeast extract	5 g
Ampicillin (concentration100µl/ml)	1 ml
H_2O	1 L

2.6. Supplementary Results

2.6.1. Adult characteristics

Both size characters and wear characteristics of bees were highly correlated, but there was no relationship between size and wear (figure S3). Size and wear characteristics also did not vary between social and solitary nests (figure S3, main text).

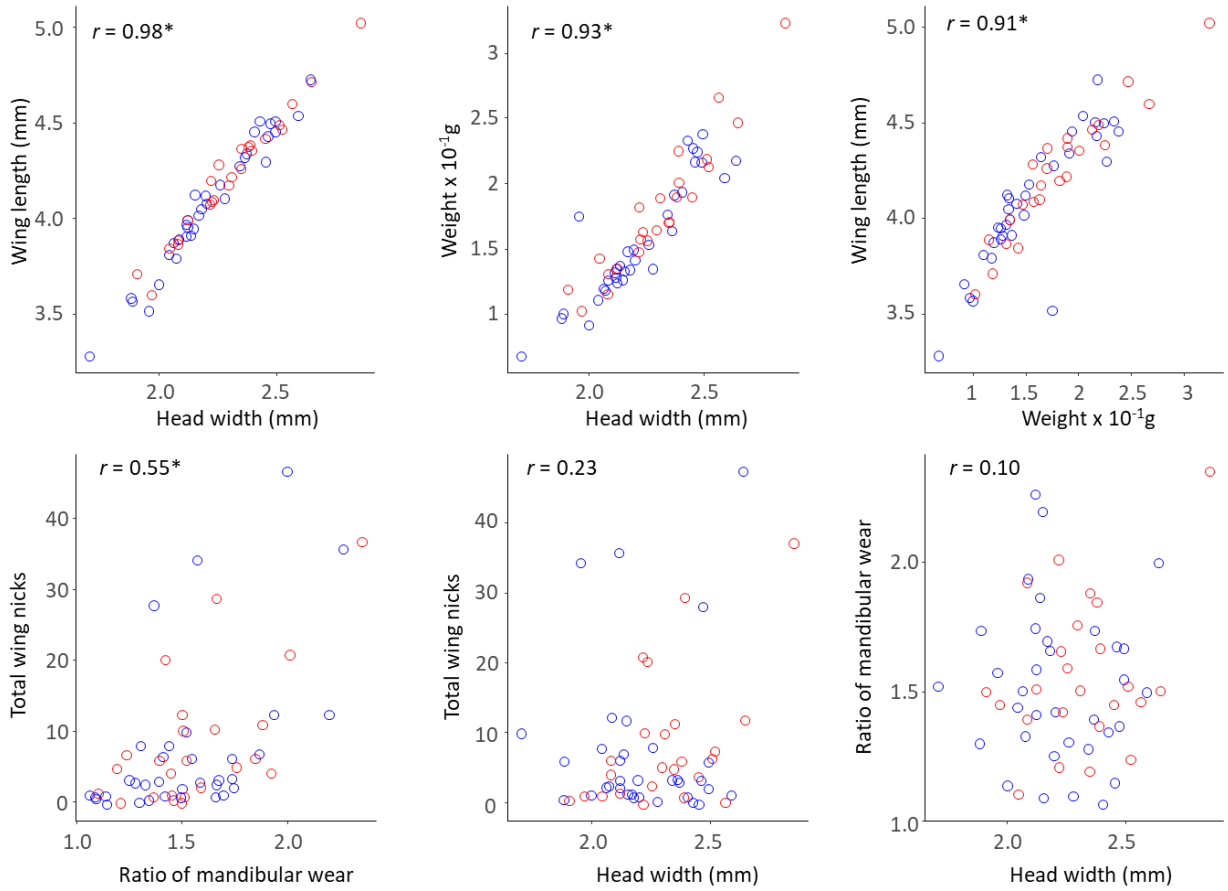

Figure S3. Correlations betweeen measures of wear, weight and size in bees from social (red) and solitary (blue) nests. Pearson's correlation coefficients are shown in each panel, with significant values ($P < 0.05$) indicated by asterisks.

2.6.2. Statistical analysis

To assess the influence of individual and colony characteristics on immune efficacy we used GLM analysis implemented in R 4.0.5 (R Core Team, 2022**)**. Our response variable was log of the change in the absorbance over the 6 hr period. As explanatory factors we included number of group members (including adults, brood, and callows), social status (solitary/social), head width, and

mandibular wear, and the interaction between social status and group size.. Table S5 shows the results of the final model.

Table S5. Results of GLM on absorbance measures from females in social and solitary nests. Analysis of deviance table (Type II tests) of likelihood ratios.

Factor	**Chisq**	***P***
Group members	6.281	0.012
Social status	0.111	0.739
Head width	2.222	0.136
Mandibular wear	2.539	0.111
Group members:social type	0.179	0.672

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Chapter 3.

The influence of social context on regulation of gene expression

3.1. Introduction

3.1.1. Phenotypic plasticity of immune response

Pathogens come in various forms, such as bacteria, viruses, parasites, and fungi. Each type of pathogen may require different immune strategies for effective defense. In response, phenotypic plasticity allows the immune system to adapt and tailor its responses to the specific challenges presented in different environments (Vilcinskas, 2013). Phenotypic plasticity ensures that immune responses are optimized to counter specific threats. This optimization may involve modulating the expression of different immune genes, producing specific types of immune cells or molecules, and activating appropriate immune pathways (Vilcinskas, 2013).

Phenotypic plasticity refers to the capacity of a single genotype to produce various phenotypes in response to changes in the environment (Manfredini et al., 2019). In terms of immune responses, changes in the immune environment can result from changes in pathogen pressure or social context and can lead to plastic changes in the immune system. One such context is changes in social environment, because in group living organisms, there is an increased risk posed by pathogens due to high-density populations and frequent interactions among individuals, which create favorable conditions for the transmission of pathogens (Kappeler et al., 2015; Meunier, 2015).

Immune research is predominantly conducted in laboratories to understand how the immune system combats specific pathogens (Siva-Jothy et al., 2005). For example, studies on silk moth *Hyalophora cecropia* have revealed that attacins and attacin-related proteins are highly effective in fighting Gram-negative bacteria by inhibiting the synthesis of bacterial major outer membrane proteins. Similarly, Metchnikowin peptides found in *Drosophila* have shown effectiveness against both Gram-positive bacteria and fungi (Rosales, 2017). This approach proves valuable in exploring how pathogens influence the specific immune responses. However, using consistent environmental conditions, partly to control confounding factors and partly due to complex research methods, may hinder a comprehensive understanding of how environmental changes impact phenotypic plasticity of immunity (Siva-Jothy et al., 2005).

On the other hand, the phenotypic plasticity of immune responses has been found in studies examining the influence of social context on personal immunity. For example, in the Australian plague locust, *Chortoicetes terminifera*, individuals that were isolated have higher levels of personal immunity compared with group-living individuals (Miller & Simpson, 2010). In contrast,

in the desert locust *Schistocerca gregaria*, individuals in the gregarious phase exhibited higher antimicrobial activities and demonstrated better survival during infections compared to individuals in the solitary phase (Wilson et al., 2002). These experiments imply that there is phenotypic plasticity in immune responses under varying social conditions (social vs. solitary). The plasticity of immune responses has also been observed in highly social species, such as bumble bees. The experiment conducted by Ruiz-Gonzalez et al. in 2009 demonstrated rapid changes in immune responses among adult individuals of these bees in adaptation to shifts in their social environment. By comparing antibacterial activity (Inhibition Zone) and phenoloxidase (PO) activity between isolated individuals and individuals raised in a social environment (alongside four nestmates), these two metrics demonstrated differences between the two groups based on the social context in worker bees in the experiment, despite sharing a long-term social context (all individuals share the same colony) (Ruiz-González et al., 2009). However, the studies have not yielded any data on the mechanism basis of this plasticity, nor have they explained how the phenotypic plasticity of immune responses can rapidly adapt to changes in the social context.

To address the knowledge gap of these mechanistic basis, gene expression analysis is a powerful tool for elucidating phenotype plasticity because it provides insights into how genes are activated or deactivated in response to various environmental factors and stimuli. Therefore, gene expression studies will be conducted in this chapter to explore the phenotypic plasticity of immune responses and how different social contexts influence the expression of the phenotype plasticity.

3.1.2. Study model and aims

Facultatively social bees are ideal research models to study the impact of social context on the phenotypic plasticity of immune responses. These bees are socially polymorphic, displaying both solitary and social forms within a species. This unique characteristic enables us to use natural variation in the social environment and evaluate phenotypic plasticity with the same genome.

Fat bodies in insects are multifunctional organs distributed throughout their bodies, with roles in energy storage, metabolism, and immunity. They are major sites for producing and releasing antimicrobial peptides (Hoffmann, 2003), which play a critical role in the humoral immune response. These peptides are generated by immune genes induced during microbial infections and are predominantly synthesized in the fat bodies before being released into the hemolymph (Hoffmann J. A., 1995; Nakatogawa et al., 2009; Tsakas & Marmaras, 2010)

Therefore, is this study, I use the fat bodies of a facultatively social bee to examine immune plasticity via transcriptomic analysis with two main purposes: i) to elucidate whether social context influences gene expression, and ii) to understand how the social context impacts the phenotypic plasticity of immune responses.

3.2. Methods

3.2.1. Sample collection

The halictine bee *Lasioglossom baleicum* (Fig 1A) displays social polymorphism (Cronin & Hirata, 2003) and constructs its nests underground (Fig 1D). The nest is constructed by a singular unbranched burrow, typically vertical but occasionally slanted or curved; brood are nurtured in individual cells which are arranged in a brood-cell cluster (Figure 1B) and hold a central position within the burrow axis and are usually positioned midway or toward the lower portion of the burrow. The nest can accommodate either a single female, identified as the solitary form, or multiple (usually two) females, identified as the social form. The social behavior is facultative in this species, and both social and solitary nests can coexist within the same place.

Lasioglossom baleicum was collected at Nishioka Forest Park, located in southern Sapporo, Hokkaido (150 m, 141°35'E, 43°00'N) where both solitary and social forms of the species were recorded (Cronin & Hirata, 2003). The collection period was from July 27th to August 1st, 2022. Excavations were carried out early in the morning or during inactive periods when all occupants are likely inside. The excavation process involves gently scraping away the surface soil to reveal the nest burrows and then carefully following the tunnels for further excavation. The social status of collected bees was classified by the number of adult females in the nest. Individuals from the same nest were kept in separate 1.5 ml plastic tubes at cool temperature and in darkness, until bacterial challenges, which were conducted with one hour of excavation. The total sample size was 82 individuals, of which, 60 individuals were from social nests and 22 individuals from solitary nests. All individuals were used for two treatments: bacterial treatment and social context treatment as described below.

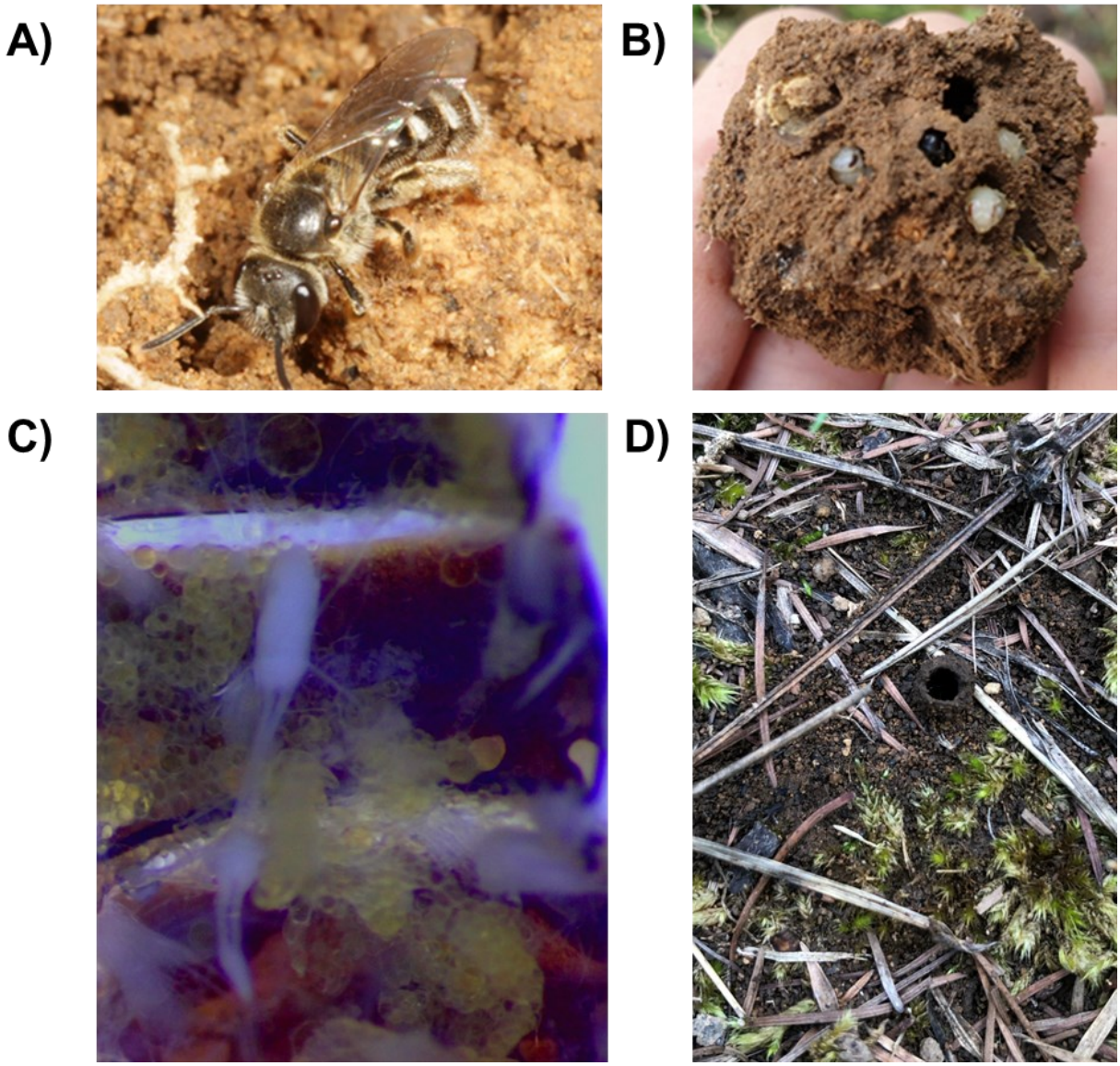

Figure 1. *Lasioglosum baleicum*. A) Bee on the ground; B) Brood chamber, each brood occupied a cell in the chamber. C) fat bodies (yellow tissue) attached to dorsal segments. D) the entrance of a bee nest on the ground.

3.2.2. Experimental design

The experiment used individuals from social and solitary colonies (as defined above), and the natural social status was considered as the 'long-term' social context (Figure 2A). To elucidate the influence of social context on gene expression regulation, I induced immune responses through bacterial treatment (Figure 2B) with injected bacteria as treated individual and its control as untreated individuals. The social context was organized by social text treatment (Figure 2C). The

individuals were treated in this treatment as solitary which contains only solitary individuals, *isolated social* which changed the social context from social to solitary of social individuals, and *paired social* which are social individuals and were paired to reappear social context after bacterial treatment. The overall treatments were as follows:

("Bacterial treated vs "Bacterial untreated") x ("*Paired social*" vs "*Isolated social*" vs "*Solitary*")

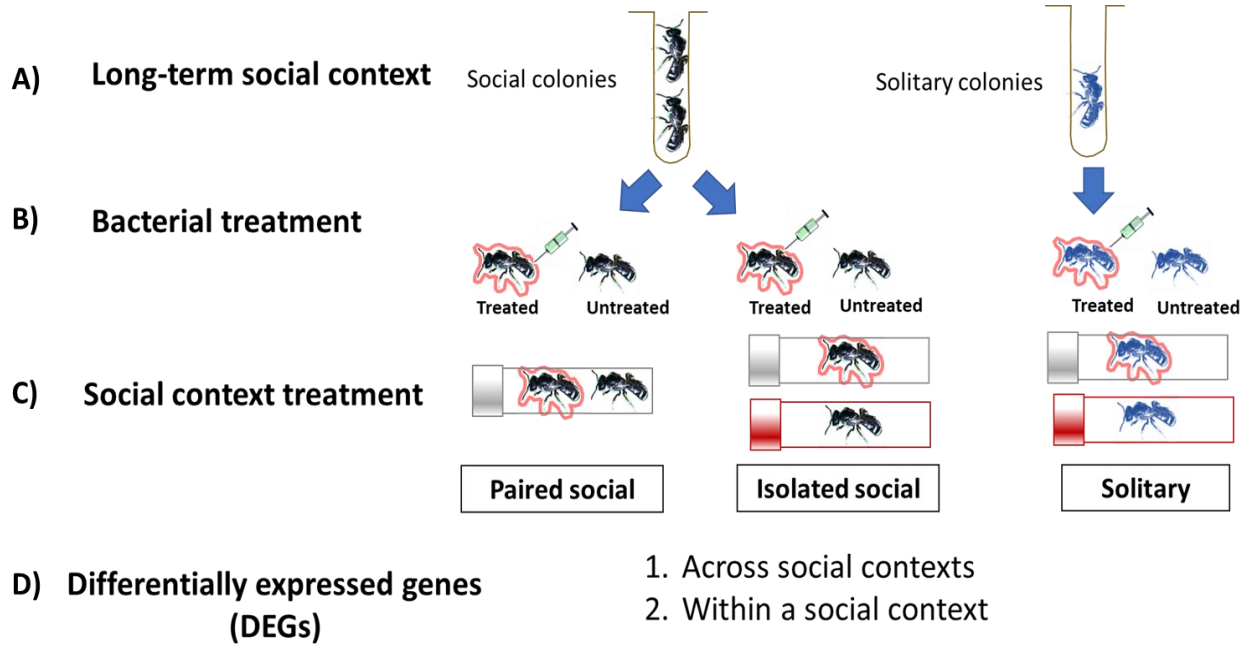

Figure 2. Experimental design. A) original social status of collected bees , and terms of long-term social context . B) After collected, Treated bees were injected by *Escherichia coli* to trigger immune responses. Control individuals were handled in the same way but received no injection. C) bees were separated into treatment groups (see below). D) the influence of gene expression was assessed by differentially expressed genes of across social context and within a social context comparison.

Bacterial treatment

Bacterial preparation

I used dead *Escherichia coli* to activate the immune response and prevent bacterial replication or bacterial transmission through contact between individuals in the colony. *E. coli* were cultured in an LB medium then measured concentration of this broth via optical density at 600 nm (OD600) (Eppendorf BioPhotometer). *E. coli* was harvested at OD600 of 0.6, then heat-killed at 90°C for

10 minutes. The bacterial suspension was pipetted into plastic tubes with a volume of 100µl, centrifuged to precipitate the bacterial cells. Afterward, the cells were washed with PBS to eliminate the culturing medium, and finally, the bacteria were lyophilized for preservation in preparation for field experiments.

Bacterial injection

The bees were divided into two groups based on the treatment. Group one was the treated individuals, and within this group, each individual was injected bacteria. Group two was untreated individuals and served as the control for treated group. The individuals in untreated group underwent the same handling procedures, such as being placed on ice for immobilization before injection and being taken out of the storing tube, similar to the treated individuals. This manipulation aimed to separate the immune response-inducing agent from other potential factors such as stress from experimental processing that could affect gene expression (Figure 2B).

To inject bacteria into the individuals in the bacteria-treated group, the lyophilized bacteria were reconstituted with 100µl of distilled water and thoroughly mixed for injection. Each treated individual was placed on ice to immobilize them, and then 18.4nl of the prepared bacterial suspension was injected using the Nanoject II Auto-Nanoliter Injector (Drummond).

Treatment of social context

After bacterial treatment, I changed social context by social treatment to *Paired social*, *Isolated social* and *Solitary* individuals to elucidate the influence of social context on gene expression (Figure 2C).

Paired social individuals

These social individuals from the same nest were kept together in a plastic tube after bacterial treatment. The tube thus contained both treated and untreated individuals. The treated individuals were controlled by untreated individuals within the same tube. The treatment simulated the effect of *long-term* social context to demonstrate gene expression in a social context and in response with bacterial treatment.

Isolated social individuals

These social individuals were collected from the same colony. However, in this treatment, the social context is changed from social to solitary instead of maintaining the *long-term* social

environment. After injecting bacteria, the treated and control individuals are kept into two separate plastic tubes: one tube for the treated individual and the other for the untreated individual. This alteration represents a shift in the social context and allows for the potential expression of phenotypic plasticity. In this experimental setup, the untreated individual serves as the control for the treated one. The treatment is carried out to evaluate the influence of environmental changes on gene expression. The changes in gene expression can be due to social isolation (social context treatment) but can also result from immune responses in different social contexts (social context treatment x bacterial treatment). Comparisons across social contexts and within social contexts will discuss these possibilities.

Solitary individuals

The individuals used in this treatment are solitary individuals. They are kept as their *long-term* social context. Both the treated and untreated individuals (from different solitary nests) are retained in separate test tubes after the bacterial treatment. Each tube represents a solitary environment, allowing for the assessment of gene expression patterns in a *long-term* solitary environment.

After the treatments, bees were kept in a cool, dark environment for 6 hours to allow the organisms to respond to the presence of pathogens and regulate gene expression accordingly. They were then flash frozen in liquid nitrogen to preserve condition for later RNA extraction.

3.2.3. RNA extraction

Fat bodies were collected from bee abdomens following dissection (Figure 1C) using 400µl RNA-later ICE, which allows us to process flash-frozen samples and preserve the RNA integrity. I then extracted mRNA from tissue of the 2nd to 4th dorsal cuticle segment, which accompanied fat body tissues, using a Dynabeads kit following the kit intructions. I constructed Illumina libraries for each of the samples using the NEBNext Ultra II directional kit and library quantify control using TapeStation. Subsequently, the samples were sequenced and mapped to the *Lasioglossum baleicum* genome, and counts for different mapped reads were obtained. These RNA-Seq count data were used for subsequent data analysis.

3.2.4. Data analysis

All data analysis were used in Rstudio (version 2023.02.3). R packages used in these analyses are described below.

Testing effect of bacterial treatment between treated individuals and its control

The experiment used dead bacteria to prevent bacterial replication in the organisms' bodies and injected a small amount of agent (approximate 250 bacteria cells) (Yap & Trau, 2019) to trigger immune responses and thus expected that there was no bacteria transmission (e.g., through trophallaxis) which can lead to responses in control individuals of the *paired social* treatment. Additionally, the study also utilized a PCA plot to examine whether untreated-*paired social* samples were exposed to bacteria from treated-*paired social* samples when both groups were kept in the same test tube. The main objective of this analysis was to assess the potential transmission of disease between the treated and untreated pairs.

PCA plots were used to investigate differences across treatments. These analyses were done separately for treatment and control individuals. The individuals used for PCA plot were analyzed separately in two groups: control and treated, with each of these groups consisting of individuals from three different social context treatments. Analyzing *paired social*, *isolated social*, and solitary within the control group may reveal whether there is an influence of signal exchange/disease transmission on gene expression activation. On the other hand, comparing them within the treated group could indicate whether there are any differences in their gene expression among social contexts.

The PCA plot (generated using DESeq2 (Love et al., 2014) and ggplot2 package (Wickham, 2016)) provides an overview of the gene expression for each individual. This analysis employs rlog transformed data to reduce the influence of extreme values or outliers.

Identification of differentially expressed genes (DEGs)

The impact of the environment on gene expression triggered by the bacteria was compared based on differentially expressed genes (DEGs) across social contexts and within the same social context. I used DESeq2 package to identify DEGs from RNA-Seq data for all of comparisons. The gene expression was normalized by DESeq2 - normalized counts using the median of ratios method (Michael et al., 2023). The significance of DEGs was tested with adjustment of p-values for repeated testing following the Benjamini and Hochberg method. Upregulated DEGs were determined by log2 (fold change) > 0, while downregulated DEGs were determined by log2 (fold change) < 0.

Enrichment and analysis of DEGs

The GO ID was assigned for all of *Lasioglossum baleicum* genes using Trininotate (Bryant et al., 2017) based on genome sequencing, and DEGs were identified by DESeq2 among group comparison. Then, topGO (Alexa & Rahnenführer, 2007) was used to specify the potential roles of the DEGs. The predictions were explored by using the 'weight01' algorithm with Fisher's Exact Test. The results were focused for "Biological Process" (BP) terms with top 20 prediction and vizualized with ggplot2 (Wickham, 2016).

DEGs across social context

To assess gene expression across different social contexts, DEGs were identified by comparing three groups: *paired social*, *isolated social* and *solitary* . The *paired social* group consisted of 30 individuals, comprising 15 treated social individuals and 15 untreated social individuals. Similarly, the *isolated social* group included 15 treated social individuals and 15 untreated social individuals. The *solitary* group contained 11 treated *solitary* individuals and 11 untreated *solitary* individuals.

To visualize the differential expressed genes across social context, I used pheatmap function from pheatmap package (Kolde, 2022).

DEGs within a social context

To evaluate gene expression under the influence of bacterial treatment, pairs of control and treated individuals from the same social context were compared to identify DEGs. Subsequently, the identified DEGs were compared across different social contexts to find DEGs in response to pathogens in varying contexts.

A Venn Diagram (from ggVennDiagram package (Gao et al., 2021)) was used to visualize shared and unique genes expressed in each context, including both upregulated DEGs, downregulated DEGs, and all DEGs. Meanwhile, a Volcano plot (from EnhancedVolcano package (Blighe et al., 2023)) was utilized to display the expression of common genes among the three groups with different social contexts.

3.3. Results

3.3.1. Testing effect of bacterial treatment between treated individuals and its control

The gene expression database comprised data from 41 individuals in the untreated group and 41 individuals in the treated group. The PCA plot revealed that within the control group, there were no differences in gene expression between *paired social*, *isolated social*, and *solitary* individuals. However, in the treated group, *paired social* individuals tended to have different gene expression patterns compared to the other two groups. This suggests that no immunological effects occurred in the untreated-*paired social* individuals triggered by treated-*paired social* individuals. Any indications of transmission in untreated-*paired social* individuals should result in clustering that separates from the other two groups, possibly resembling the pattern observed in the treatment group.

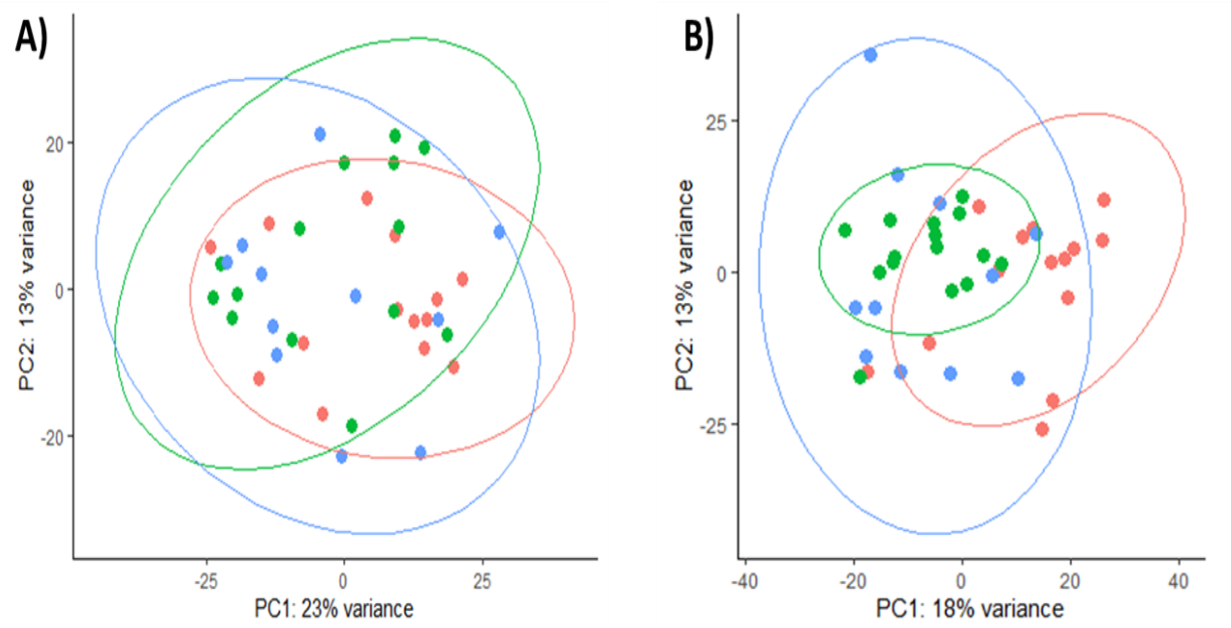

Figure 3. Principle Component Annalysis (PCA) plot of 82 individuals to summarized data structure in. A) Comparison among groups in untreated individuals and B) treated individuals. The blue dots are *solitary* individuals, green dots are *isolated social* individual and red dots are *paired social*. The PCA using rlog data.

3.3.2. DEGs across social contexts

In the comparative analysis of gene expression among the three groups (*paired social*, *isolated social*, and *solitary*), 61 DEGs were identified with a significance level of FDR <0.05. These DEGs were categorized into two gene clusters, showing three distinct patterns of gene expression between the groups (Figure 4).

In the *paired social* group and the *solitary* group, opposite patterns were observed. Genes of cluster 1 showed upregulation in the *paired social* group but downregulation in the *solitary* group, similarly, genes of cluster 2 exhibited downregulation in the *paired social* group but upregulation in the *solitary* group. Meanwhile, the *isolated social* group displayed a combination of upregulated and downregulated DEGs in both cluster 1 and cluster 2.

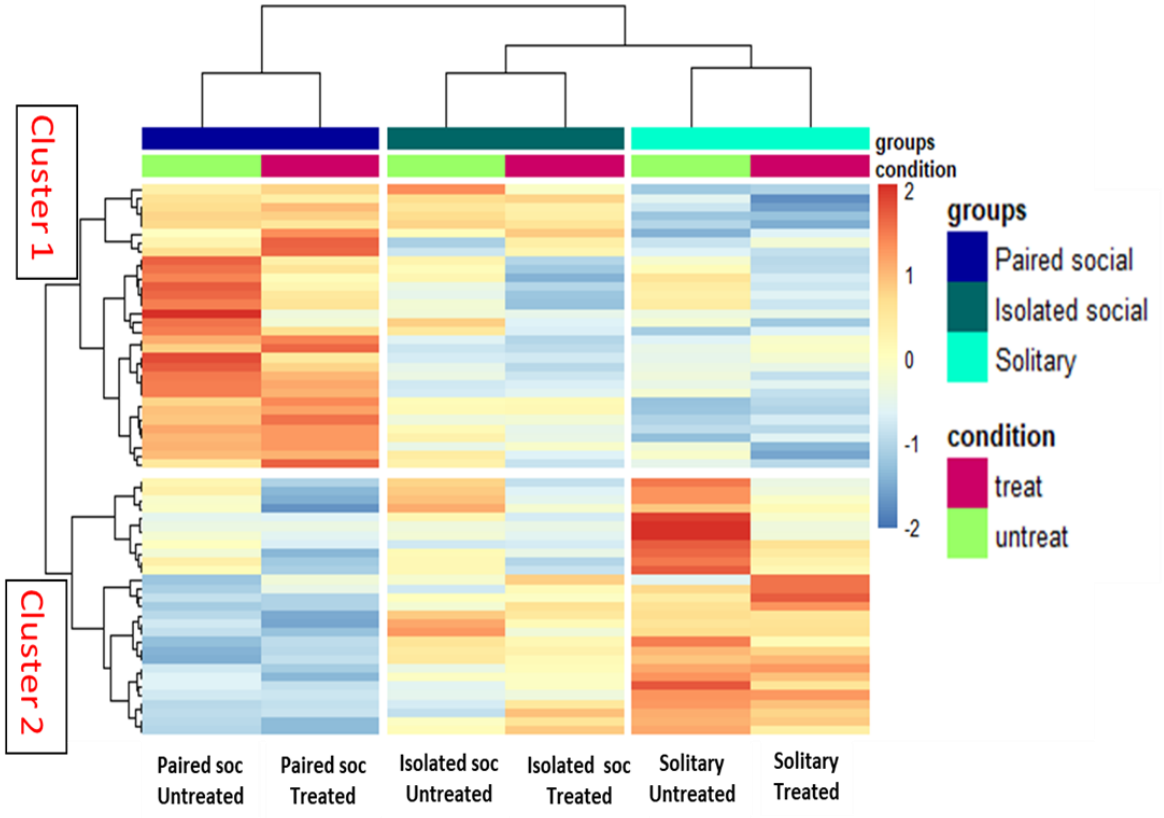

Figure 4. Heatmap of DEGs (FDR < 0.05; N = 61) across groups and condition. Top dendogram indicates relationship between groups and dendogram in the left indicates DEGs with two distinct clusters (cluster 1 and cluster 2). Bar scale from -2 to 2 indicates gene expression level, upregulation is greater than zero and represented by gradient of orange color; downregulated is smaller than zero and represented by gradient of blue color.

3.3.3. DEGs within each social context

DEGs were identified for comparisons between treated individuals and untreated individuals within the same social context separately for each of the three groups *paired social*, *isolated social*, and *solitary* (see 2.4. DEGs within a social context). These DEGs were categorized into three groups: upregulated, downregulated, and all DEGs, as depicted in the Venn Diagram (Figure 5). A

notable distinction is observed between *paired social* individuals and the other two groups. Both the *isolated social* and *solitary* groups share certain genes in both the upregulated (15 DEGs) and downregulated (33 DEGs) categories. However, they have no common DEGs with the *paired social* group in the upregulated DEGs (Figure 5A), and only 1 gene in common with each other group in the downregulated DEGs (Figure 5B).

In the comparison of all DEGs, the number of DEGs in the *paired social* group was much larger than the other two groups (349 DEGs compared to 198 DEGs in *isolated social* and 189 DEGs in *solitary*) (Figure 5C). Among these, 24 DEGs were shared among all three groups of individuals. The expression of these 24 DEGs is visually represented in the Volcano plot (Figure 6).

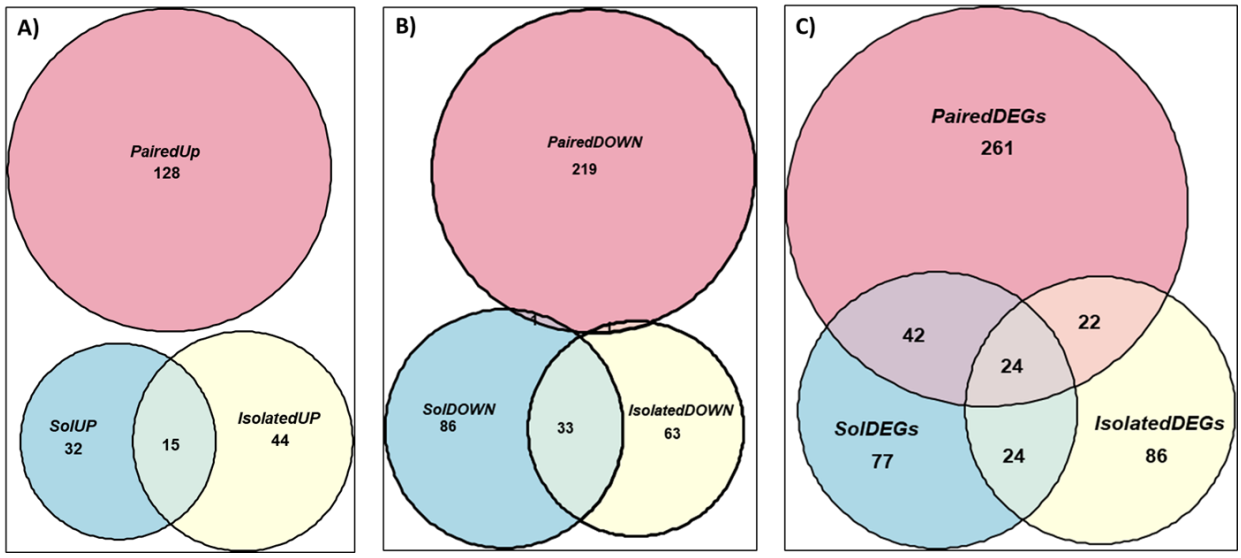

Figure 5. Venn diagram of common and specific DEGs among groups (DEGs from treated and untreated comparison within group; $p<0.05$, $|\log2FoldChange| >1$). Pink indicates *paired social* individuals; blue indicates *solitary* individuals and yellow indicates *isolated social* individuals. Numbers in each circle indicate the number of DEGs. A) Up regulated DEGs. B) Down regulated DEGs. C) All DEGs.

The 24 shared DEGs were distinctly divided into two distinct gene clusters: cluster one, consisting of 7 genes (marked by the yellow ellipse in Figure 6), and cluster two, containing 17 genes (marked by the purple ellipse in Figure 7). In DEGs cluster one, the expression was found to be upregulated in *isolated social* and *solitary* individuals but downregulated in *paired social* individuals; and opposing patterns in DEGs cluster two. Interestingly, the pattern of gene expression in these 24

shared DEGs to bacterial treatment in *isolated social* individuals resembled that observed in *solitary* individuals and was opposite to that of *paired social* individuals.

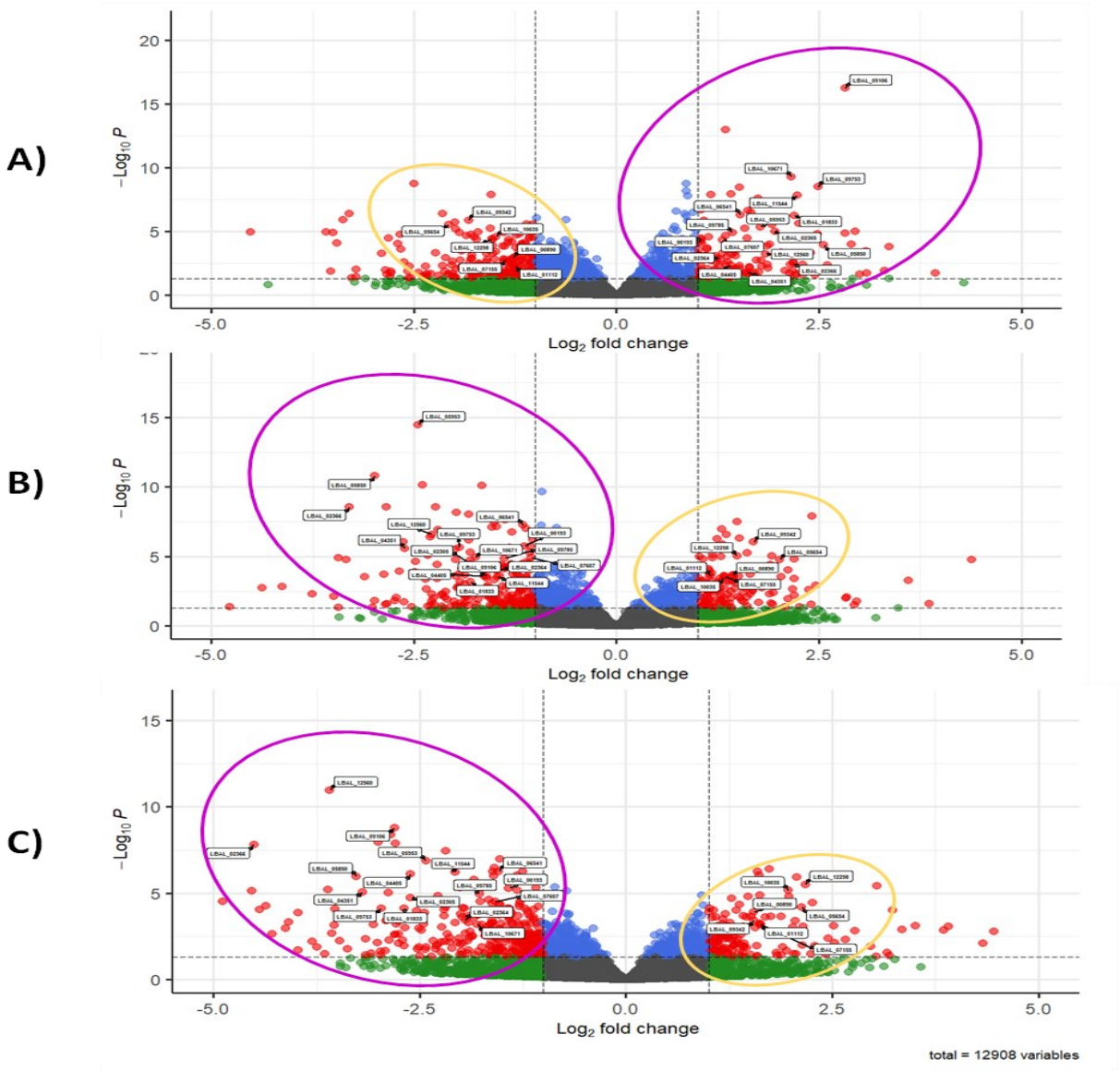

Figure 6. 24 common DEGs among three groups (DEGs from treated and untreated comparison within group; $p<0.05$, $|log2FoldChange| >1$). The yellow (cluster 1) and purple (cluster 2) ellipse indicate gene clusters, in which the same color means the same gene ID cluster. A) *paired social* group. B) *isolated social* group. C) *solitary* group.

The expression of the 24 shared DEGs showed variation under different social context treatments: social (*paired social*, Figure 6A) to solitary (*isolated social* and *solitary*, Figure 6B&C) could indicate the regulation of biological processes in response to bacterial challenge. To achieve a more

comprehensive understanding of these biological processes, the 24 shared DEGs were used as a gene set of interest and subjected to enrichment analysis to categorize their potential biological processes based on Gene Ontology (GO) terms. This analysis was used to predict what biological processes this gene set might be involved in. Figure 7 displays the top 20 best-fit predictions related to biological process terms shared by all 24 DEGs. The predictions associate with metabolic process, cellular process and homeostatic process. Among them, biological process of neutrophil degranulation (GO:0043312) and positive regulation of antifungal peptide biosynthetic process (GO:0006967) were immune system processes (GO:0002376). This suggests that the 24 shared DEGs identified from comparison of treated and untreated individuals are likely to be involved in the immune response process.

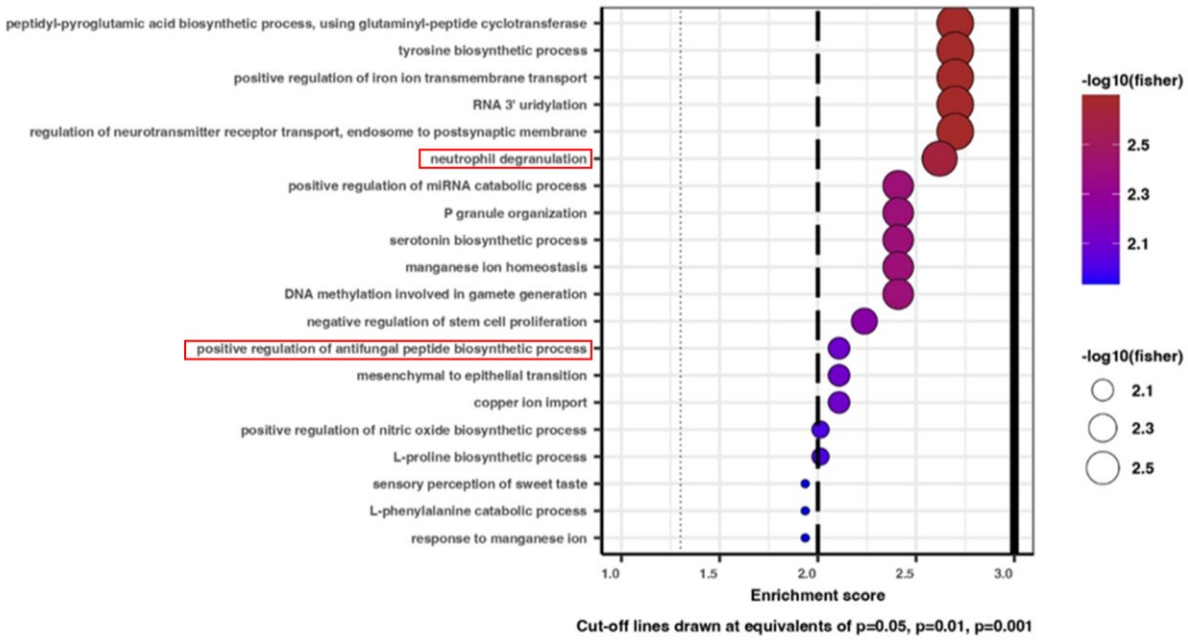

Figure 7. GO enrichment, Biological process terms of 24 shared DEGs, the top 20 best-fit predictions, The red boxes highlight the terms related to immune response processes (GO:0006955). Circle diameter and color is proportional to a -log10 p-value from a Fisher Exact Test for enrichment of each GO term. Three cut-off lines are shown, for $p<0.05$ (dotted), $p<0.01$ (dashed) and $p<0.001$ (solid).

3.4. Discussion

In the comparison among untreated individuals in *paired social*, *isolated social* and *solitary* groups, there was no evidence of an immunotransmission effect between treated and control individuals.

There was no difference between untereated individuals in the three groups, while the gene expression of each individual in the treatment group tended to be different between the *paired social* group and the rest in the PCA analysis (Figure 3). This finding rules out the possibility of transmission effects between treated and untreated individuals, where the immune system of untreated-*paired social* individuals could be triggered by pathogen transmission from treated-*paired social* individuals when living in the same plastic tube, at least over the short term exposure of the experiment (6 hours).

The comparison between treated and untreated individuals within the same social context identified specific DEGs involved in the response against pathogens in each social context. This comparison eliminates the influence of the social context treatment (compared in a social context) and focuses on the effect of bacterial treatment on gene expression (between treated and untreated individuals). One notable observation is the marked difference in the number of DEGs between *paired social* individuals and the other two groups. *Paired social* individuals exhibited a higher number of DEGs, with 349 identified, whereas *isolated social* and *solitary* individuals had a lower and approximately equal number of DEGs, with 198 and 189, respectively (Figure 5C). This suggests that social individuals (*paired social*) may employ more extensive change in gene expression to cope with the same immune threat compared to solitary individuals (*isolated social* and *solitary)* in the social context treatment. The increased number of DEGs in the *paired social* group implies the involvement of a more complex and extensive immune response to combat pathogens within a social context.

Interestingly, despite bees in different social contexts being challenged with the same pathogens and receiving the same amount of injection, they show different responses, which could be caused by different pathways being triggered. Specifically, in the *paired social* individuals, 128 DEGs were found to be upregulated, none of which were shared with the upregulated genes in *isolated social* and *solitary* individuals (Figure 5A). Similarly, for the downregulated genes, there were no common DEGs among the three groups; *paired social* individuals shared only 1 gene with the *solitary* group and another 1 gene with the *isolated social* group (Figure 5B). These findings highlight the distinct gene expression patterns in response to the same pathogen challenge, depending on the social context of the individuals. Utilizing a larger number of genes in the immune response may require a higher investment of energy for the regulation of gene expression.

This observation implies that social individuals may allocate more resources and attention to combat pathogens compared to *solitary* and *isolated social* individuals.

On the other hand, *isolated social* individuals, despite being part of a long-term social context, exhibit a response more similar to *solitary* individuals than to *paired social* individuals. There is a higher number of shared genes between *isolated social* and *solitary* individuals compared to the shared genes between and *paired social* individuals. Specifically, they share 15 upregulated DEGs in common and 33 downregulated DEGs, and only one shared DEG with *paired social* in the downregulated category (Figure 5A & B). This suggests that *isolated social* individuals and *solitary* individuals display a closer resemblance in their gene expression responses than *isolated social* individuals do with *paired social* individuals.

In addition, among the 24 DEGs shared among all three groups, gene expression patterns also exhibited similarity between *isolated social* and *solitary* individuals when compared to *paired social* individuals (Figure 6). Within these shared genes, two sets were identified: one set of seven DEGs and another set of 17 DEGs, both showing opposing patterns in gene expression regulation. The group of 17 genes displayed upregulation in *paired social* individuals but downregulation in *isolated social* and *solitary* individuals. Conversely, the group of seven DEGs showed the opposite pattern, with upregulation in *isolated social* and *solitary* individuals but downregulation in *paired social* individuals. The change in gene expression of *isolated social* individuals, which is closer to the regulatory mechanism of gene expression in *isolated social* individuals than *paired social* individuals, indicates a rapid adaptation to the current social context (social context in *paired social* individuals vs solitary context in *isolated social* and *solitary* individuals). The potential biological processes associated with the set of 24 shared DEGs might be related to immune responses (Figure 7). This highlights the phenotypic plasticity of immune responses to adapt to the current social context. This implies that short term context is more important than long term context with regard to implementation of immune response in these bees.

In the comparison across different social contexts (Figure 3), *paired social* and *solitary* individuals exhibit opposite patterns of gene expression regulation, while *isolated social* individuals show intermediate expression levels. These findings strongly suggest that gene expression is closely linked to the specific social context. *Isolated social* individuals are influenced by both their original social context and their newly enforced solitary contexts. They have a long-term history of social

interactions and exposure to the social environment, while the solitary context is established for a shorter period, during the testing condition. Therefore, one might expect the gene expression patterns of *isolated social* individuals to be more similar to that of *paired social* individuals rather than *solitary* individuals. However, the results indicate that the gene expression patterns of *isolated social* individuals are closer to those of *solitary* individuals than *paired social* individuals (dendrogram of groups in Figure 3). This observation could be related to the characteristics of socially polymorphic bees, such as *Lasioglossum baleicum*. These bees can experience both solitary and social forms throughout their life cycles, and thus the ability to rapidly change in gene expression could help them adapt to the current social context. Therefore, this fluid transition between solitary and social forms may explain the observed patterns of gene expression in *isolated social* individuals, highlighting their flexibility in responding to different social environments.

Overall, the study highlights that gene expression patterns and immune responses are readily influenced by social context and exhibit plasticity in response to adapt with different social contexts. This phenotypic plasticity helps organisms adapt to changes in pathogen pressure caused by changing social context. In terms of immunological research, besides demonstrating the plasticity of the innate immune response, further studies on assessing the influence of social context on the expression of immune behaviors such as grooming will provide a comprehensive elucidation of the phenotypic plasticity of immune responses.

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Chapter 4.

The adaptive strategies between host and parasite in eusocial species.

4.1. Introduction

4.1.1. Host – parasite structure/ relationship

One of the most influential challenges faced by immune systems is that posed by parasites. The relationship between a host and a parasite is a dynamic interplay in which the parasites exert selective pressure on hosts, prompting a range of adaptations in hosts that minimize the negative effects of infection. In response, the parasites themselves adapt to overcome these host defenses, resulting in a process known as host-parasite coevolution (Ebert & Fields, 2020). The continuous interaction between hosts and parasites leads to reciprocal selective pressures, driving the evolution of adaptations and counter-adaptations in a coevolutionary process (Ebert & Fields, 2020). Over time, this process can shape the genetic makeup of both host and parasite populations (Ebert & Fields, 2020). It can manifest as arms race dynamics (ARD), characterized by a constant competition for increasing resistance and infectivity, which can also lead to a reduction in genetic diversity. Alternatively, fluctuating selection dynamics (FSD) can occur, where parasite genotypes specialize in particular host genotypes (Quigley et al., 2012). This specialization has the potential to maintain significant genetic diversity in both host and parasite populations across different locations and over time (Frank, 1993; Lion & Boots, 2010). Overall, the host-parasite co-evolutionary process has far-reaching implications for the genetic structure and diversity of host and parasite populations. It encompasses both competitive dynamics and specialized interactions, contributing to the intricate interplay between hosts and parasites in various ecological contexts.

4.1.2. Host shifts

Parasite host shift is an ideal model to study host-parasite relationships because the model provides a valuable framework for elucidating the mechanisms and consequences of host-parasite interactions, shedding light on the dynamics of co-evolution. Host-shift is a phenomenon in which a parasitic organism transits from its original host species to a new host species. The switch in hosts can be a significant event in the life of the parasite, leading to potential adaptations and changes in its evolutionary trajectory (D'Bastiani et al., 2023). The parasite may face new challenges and opportunities in the new host's environment, which can drive adaptations and speciation over time. On the other hand, the new host may experience new challenges in its interactions with the parasite, potentially influencing its evolutionary path. These events are fundamental to understanding the dynamics of host-parasite interactions, coevolution, and the

spread of diseases or the establishment of host-parasite relationships in various ecosystems (Thompson, 1994).

A successful host shift involves several steps. First, the parasite must encounter and be exposed to a new host species. Subsequently, it must undergo ecological or evolutionary adaptations to effectively infect the new host. Finally, the parasite needs to establish a self-sustaining population within the new host (Poullain & Nuismer, 2012). The outcome of a host shift is influenced by various factors, including the life history and genetic characteristics of both the parasite and the new host (Roberts et al., 2015). To shed light on the evolutionary processes involved in parasite host shifts, studying the population genetics of parasites provides a means to explore the evolutionary dynamics behind host shifts (Wilson et al., 2005). It offers a deeper understanding of the underlying mechanisms and can help identify the key factors that shape these events. Ultimately, this knowledge can contribute to our ability to predict and mitigate the impacts of emerging diseases resulting from host shifts.

4.1.3. Study model and aims

I investigate the host-parasite relationship using honeybees, specifically *Apis cerana* and *Apis mellifera*, as hosts, and *Varroa* mites as parasites. Both honeybee species are affected by infestations of *Varroa* mites. *V. destructor* was originally known as a parasite of *A. cerana* in Asia (Beaurepaire et al., 2015; Navajas et al., 2010). However, with the introduction of *A. mellifera*, a native bee species in Europe, Africa, and the Middle East, to various sites in Asia for apicultural purposes, there was an opportunity for the mites to switch hosts from *A. cerana* to *A. mellifera* (Beaurepaire et al., 2015; Roberts et al., 2015).

Previous research has revealed that *V. destructor* has successfully shifted its infestation from *A. cerana* to *A. mellifera*. The initial recorded event occurred in Japan in 1957, following 80 years of Western honeybee introduction, and a second host switch event took place in the far east of the former Soviet Union, with *Varroa* mites infesting the bees from Korea (Beaurepaire et al., 2015). The susceptibility to *Varroa* mites varies between the two bee species (Traynor et al., 2020). *A. cerana* is known to possess the ability to control *Varroa* mite infestations because they appear to experience fewer negative consequences from parasitization by mites (Lin et al. 2016). The resistance of *A. cerrana* to *Varroa* mites might be due to grooming behavior. This hypothesis suggests that *A. cerana* is more sensitive to the scent of *Varroa* mites than *A. mellifera*, leading to

more effective direct removal of mites in *A. cerana* compared to *A. mellifera* (Peng et al. 1987; Fries et al., 1996). This bee's resistance is also explained by proposing the influence of the social apoptosis phenomenon. According to this hypothesis, the removal of brood infected with *Varroa* mites can be triggered by the scent of pupae that have been damaged due to exposure to *Varroa* mites rather than mites' scent. Research by Page et al. (2016) and Lin et al. (2018) revealed that worker pupae of *A. cerana* were more vulnerable to injuries and infestations of *V. destructor* compared to *A. mellifera* pupae. This heightened susceptibility led to developmental delays and mortality in *A. cerana* pupae. Consequently, this unfavorable environment hindered successful mite reproduction and also signaled worker bees to remove the infested pupae. Meanwhile, *A. mellifera* is highly vulnerable to this parasite. The shift in host from *A. cerana* to *A. mellifera* has posed a severe threat to *A. mellifera* populations. For instance, wild *A. mellifera* populations in the Northern hemisphere was infected by *Varroa* mites, leading to the majority of *A. mellifera* populations being unable to survive, and significant losses of managed colonies worldwide and imposing substantial economic and societal costs (Dietemann et al., 2019).

By using the same species of parasite on two host species with differing sensitivities, this study aims to gain valuable insights into genetic evidence for the dynamics of coevolution, in particularly, the adaptive strategies of parasite to a new host (*A. mellifera*) and original host (*A. cerana*). The findings of this study will be contributed to the understanding of how host-parasite relationship evolve in eusocial context.

4.2. Method

4.2.1. Sample collection

The specimens were collected from both southern and northern Vietnam, with 63 individuals of *Varroa* mites collected from the host *A. cerana* and 46 individuals from *A. mellifera*. The sampling areas are shown in Figure 2A.

In the North, the specimens were collected from nine sampling sites in two provinces Hoa Binh and Hanoi: Hanoi Ba Vi Yen Bai (BVYB), Hanoi Ba Vi Van Hoa (BVVH1, BVVH2), Hoa Binh Ky Son Mong Hoa (KSMH1, KSMH2, KSMH3), Hoa Binh Thi Tran Luong Son (TTLS), Hanoi Thach That Thanh Hoa (TTTH1, TTTH2), Hanoi Tu Liem My Dinh (TLMD), Hanoi Thanh Oai Phuong Trung (TOPT), Hanoi Tu Liem Thuy Phuong (TLTP) and Hanoi Hoai Duc Cat Que (HDCQ) (Appendix chapter 4)

In the South, the specimens were collected from 26 sampling sites in seven provinces Binh Dinh, Ben Tre, Lam Dong, Dong Nai, Tien Giang, Gia Lai, Sai Gon: Binh Dinh Hoai Nhon Hoai Duc (BD10, BD11, BD8), Binh Dinh Phu My My Chau (BD2,BD5), Ben Tre Cho Lach Long Thoi (BT1, BT2), Ben Tre Mo Cay Nam Dinh Thuy (BT3), Gia Lai Ia Ko Chu Se (GL2, GL3, GL4,GL5), Lam Dong Duc Trong Ninh Gia (LD2, LD3), Lam Dong Lam Ha Tan Van (LD5), Sai Gon Quan 9 (SG1), Dong Nai Thong Nhat (SG2, SG5), Dong Nai Long Khanh (SG3), Dong Nai Cam My (SG4), Tien Giang Cho Gao (TG1, TG2,TG3), Tien Giang My Tho (TG4), Tien Giang Chau Thanh (TG6, TG7). (Appendix chapter 4)

The *A. mellifera* and *A. cerana* co-occur throughout their range in Vietnam. Figure 2B and 2C illustrates the intermingling of sampling points representing both *A. mellifera* and *A. cerana* apiaries. Mites were collected from brood cells after uncapping them (Figure 1C) and stored in 95% EtOH at−20 °C for DNA sequencing.

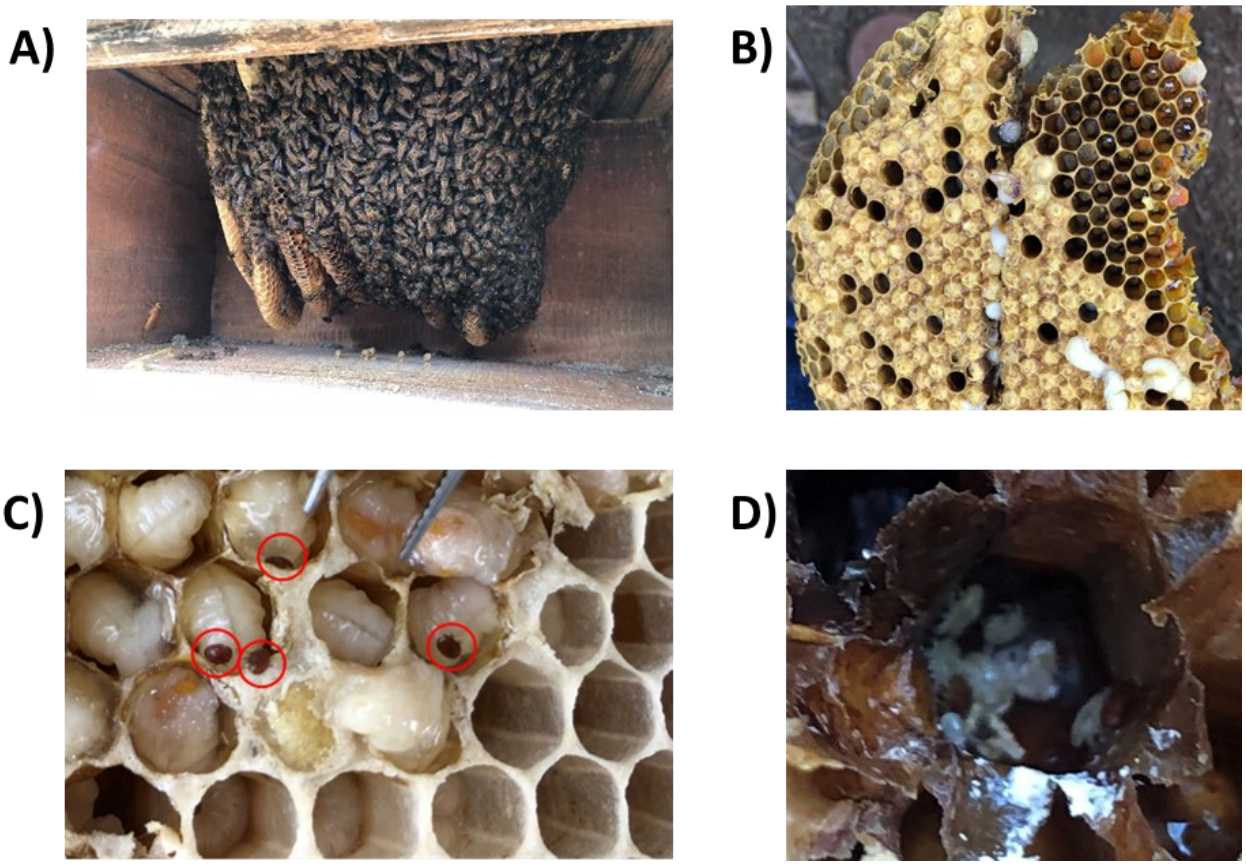

Figure 1. Collecting samples from honeybee hive frames. (A) Honeybee hive frames; (B) Brood area of honeycomb; (C) Mites in uncapped brood cells, mites indicated by red circles; (D) Group of immature *Varroa* mites in an uncapped brood cell.

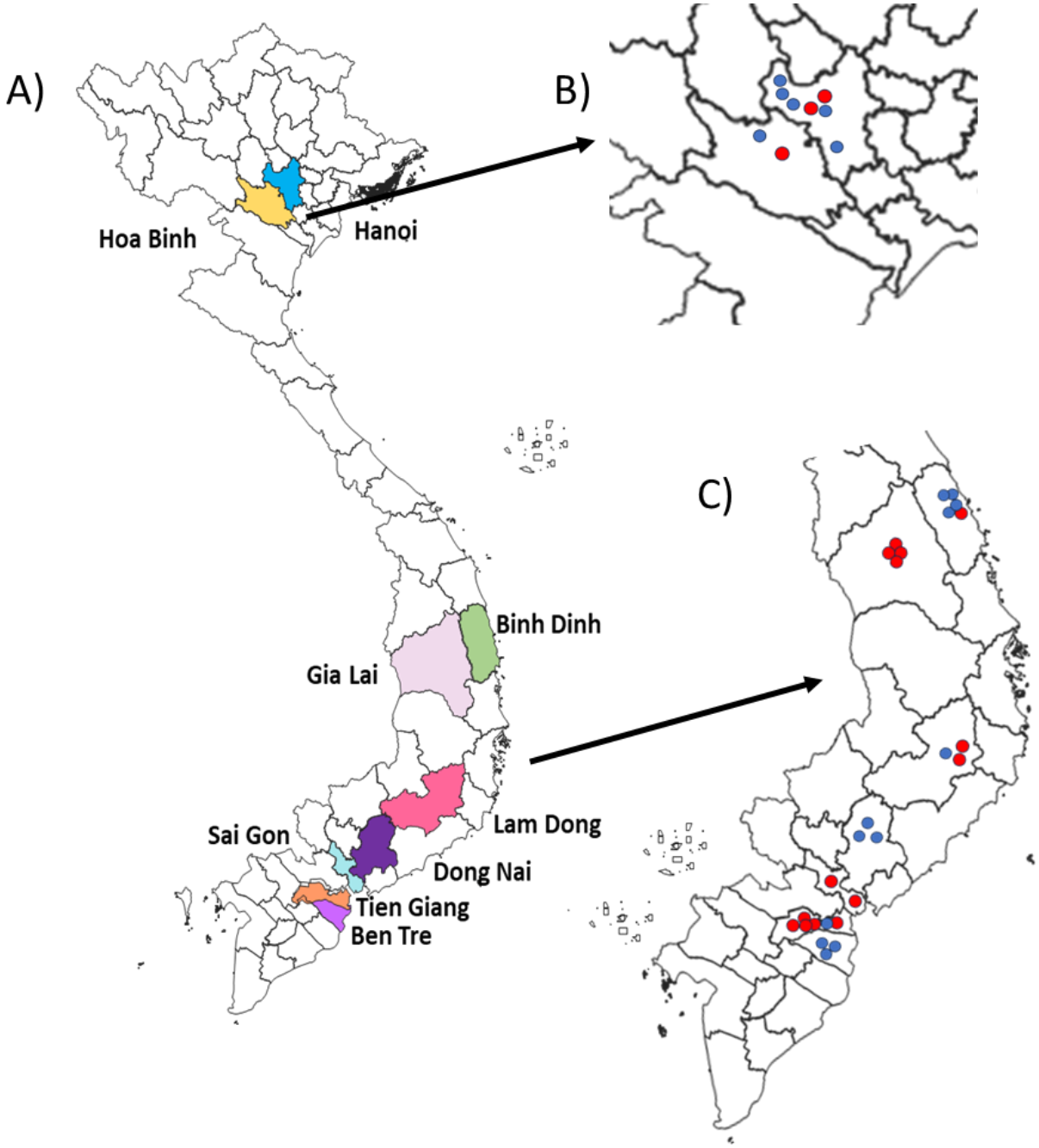

Figure 2. Collecting site map. (A) Collection map by location, marked different colors for different provinces; (B) and (C) Mixing *A. cerana* (blue dots) and *A. mellifera* (red dots) of apiaries, in (B) Northern Vietnam, (C) Southern Vietnam.

4.2.2. DNA sequencing

Extracting DNA of Varroa mites

DNA was extracted from the whole body of single mite using a Maxwell RSC Blood DNA Kit (Promega, USA), then incubated tissue with lysis buffer and proteinase for 2 hours following the kit's protocol. DNA analysis was conducted by Maxwell RSC Instrument (Promega, USA), accompanied by a QuantiFluor dsDNA System (Promega, USA) for DNA quantification.

Preparing RAD-Seq (Restriction-site associated DNA) library

The Multiplex Shotgun Genotyping (MSG) protocol originally described by Andolfatto et al., 2011, was used to generate SNPs across the entire genome. However, certain modifications were made in-house to enable high-throughput DNA sequencing library preparation. Total 25 ng DNA/individual for preparing RAD-seq library. The sample DNA was digested by restriction enzyme MseI and ligated by T4 ligase, then mixed all samples to select six of DNA in Pippin Prep (Sage Science, USA) with a 1.5% agarose gel cassette (size range from 350 to 450 bp, Sage Science, USA). The selected size DNA fragments was amplified by using Q5 High-Fidelity PCR Kit (total 50 µl, NEB, USA) with two forward primers, FC1.13 (5'-CAA GCA GAA GAC GGC ATA CGA GAT CAG ATC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T-3') and FC1.14 (5'-CAA GCA GAA GAC GGC ATA CGA GAT ACT TGA GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T-3'), and one reverse primer FC2 (5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC

4.2.3. Data analysis

Filtering

RAD-seq data of *Varroa* mites were demultiplexed using the paired-end Genotyping-By-Sequencing (PairgbS) approach without allowing barcode mismatch. I used iPyRAD v0.9.10 (Eaton and Overcast 2016) following its protocol and default setup for parameters, then mapping gene with *Varroa destructor* references (GenBank assembly accession: CA_002443255.1). The VCF file from iPyRAD was filtered for loci which were at least 70% complete and then individuals that were at least 95% complete.

Varroa mite population genetic structure

A discriminant analysis of principal components (DAPC) was used for the analysis population structure of the filtered data above (adegenet package of R). I assumed there were two populations are *Varroa* mites, from *A. cerana* and *A. mellifera*. The probability of population assignment to populations defined by collection sites was visualized by compoplot (genotype composition plot).

The genetic diversity of *Varroa* mites between two hosts was evaluated using the following metrics calculated from the SNP data:

Observed Heterozygosity (Ho): Observed heterozygosity measures the proportion of heterozygous individuals observed in the population. It is calculated based on the actual genotypes of individuals.

Expected Heterozygosity (He): Expected heterozygosity is the probability that two alleles chosen at random from the population are different. It considers both the number of alleles and their frequencies and provides an estimate of the expected genetic diversity in a population under the assumption of Hardy-Weinberg equilibrium.

Heterozygosity indicates the level of genetic variation within the population. Higher heterozygosity suggests greater genetic diversity.

Number of alleles: an essential indicator for understanding genetic variation and diversity within a population. A higher number of alleles at a specific genetic locus within a population generally indicates greater genetic diversity and variability at that particular locus.

Allelic richness: directly reflects the level of genetic diversity within a population. A higher allelic richness indicates a greater number of alleles, representing a more diverse gene pool.

Varroa mite genetic evolution

The analysis used the same database with DAPC analysis but was transformed into haploid from diploid to haploid form by PGDSpider and converted to NEXUS format for phylogenetic analysis. Phylogenetic relationships between *Varroa* populations were reconstructed using a Bayesian approach in BEAST 1.10.4 (Drummond et al., 2012). The HKY model and Yule process were used with 50 million iterations sampling every 1000th iteration. Log files from BEAST runs were explored in TRACER 1.7.1 (Rambaut et al., 2014) to determine suitable burn in (10% was used in all cases) and to confirm effective samples sizes were > 200. Maximum credibility trees were generated from BEAST output using the software TreeAnnotator 1.10.4, discarding the first 10% of all trees.

F_{ST} was used for assessing genetic differentiation among populations. The statistical value is a measure of population structure and quantifies the proportion of genetic variation that is due to differences between populations. The populations assigned for F_{ST} statistic was based on the clusters in the phylogenetic tree.

F_{ST} values fall between 0 and 1, indicating varying degrees of genetic differentiation among populations. A higher F_{ST} value suggests that the populations are more distinct and have limited gene flow, while a lower F_{ST}value indicates more genetic similarity and greater gene flow between populations.

F_{ST} = 1 This indicates complete genetic differentiation, meaning there is no gene flow between populations, and each population is genetically unique.

F_{ST} = 0: This indicates that there is no genetic differentiation among populations, meaning all populations are genetically identical, and there is a free flow of genes between them.

4.3. Results

4.3.1. *Varroa* mite population structure

The analysis was conducted on 84 mite individuals of both *A. cerana* and *A. mellifera* host with 4287 loci after filtering. The compoplot for DAPC shows that the mites in *A. mellifera* show evidence of admixture, with no spatial genetic structuring of populations, which indicates they are genetically similar and implies a recent shared origin or that gene flow is occurring across locations. On the other hand, *Varroa* on *A. cerana* form distinct populations with genetic clusters by location (Figure 3). The mites parasitizing *A. cerana* also show higher genetic diversity in all indicators compared with those in *A. mellifera* (Table 1.)

Table 1. Genetic diversity of *Varroa* mites, the comparison in different host.

Indicators	Ho	Hs	Number of alleles	Allelic richness
***A. mellifera* host**	0.0058	0.0095	4803	1.1083
***A. cerana* host**	0.0119	0.2374	7848	1.8146

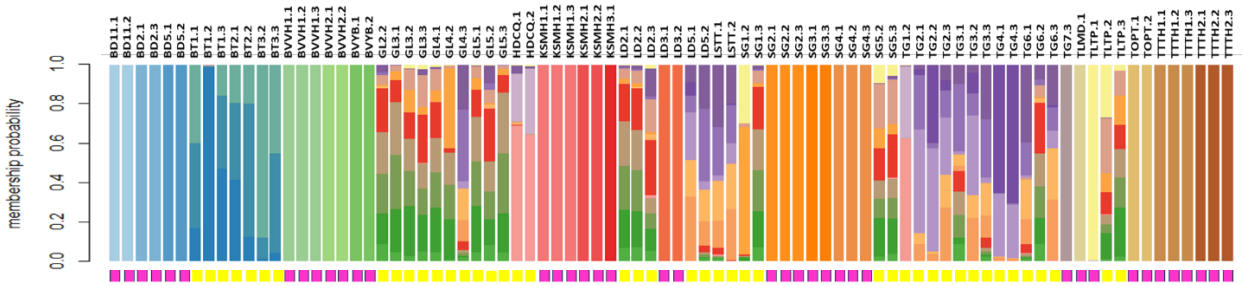

Figure 3. Discriminant analysis of principal components (DAPC) compoplot for *Varroa* mite population genetic structure. The top bar represented the name of individual samples, the small bar at the bottom represents different hosts of *Varroa* mites, with pink representing *A. cerana* and yellow for *A. mellifera.*

4.3.2. *Varroa* mite genetic evolution

The evolutionary relationship among mites reflects that mites were strongly divided by host and, furthermore, that mites hosted on *A. cerana* formed two distinct clusters (Figure 4). At the same time, there was no indication of barriers by location as the mixing location on L bar of Figure 4. There are two clusters of mites that belongs host *A. cerana*, in which one cluster is closer with those in *A. mellifera* than the other cluster. The F_{ST} among three mite clusters C1, C2, M (Figure 4) indicate very high differentiation, approximating 1 (Table 2). The F_{ST} indicates that each population, corresponding to each cluster in Figure 4, is genetically unique with almost no gene flow between the three clusters.

Table 2. F_{ST}-statistics among the three mites' clusters (C1, C2, M) based on groups inferred from the phylogenetic tree.

	C1	C2	M
C1	-	0.9360	0.9701
C2	0.9360	-	0.8637
M	0.9701	0.8640	-

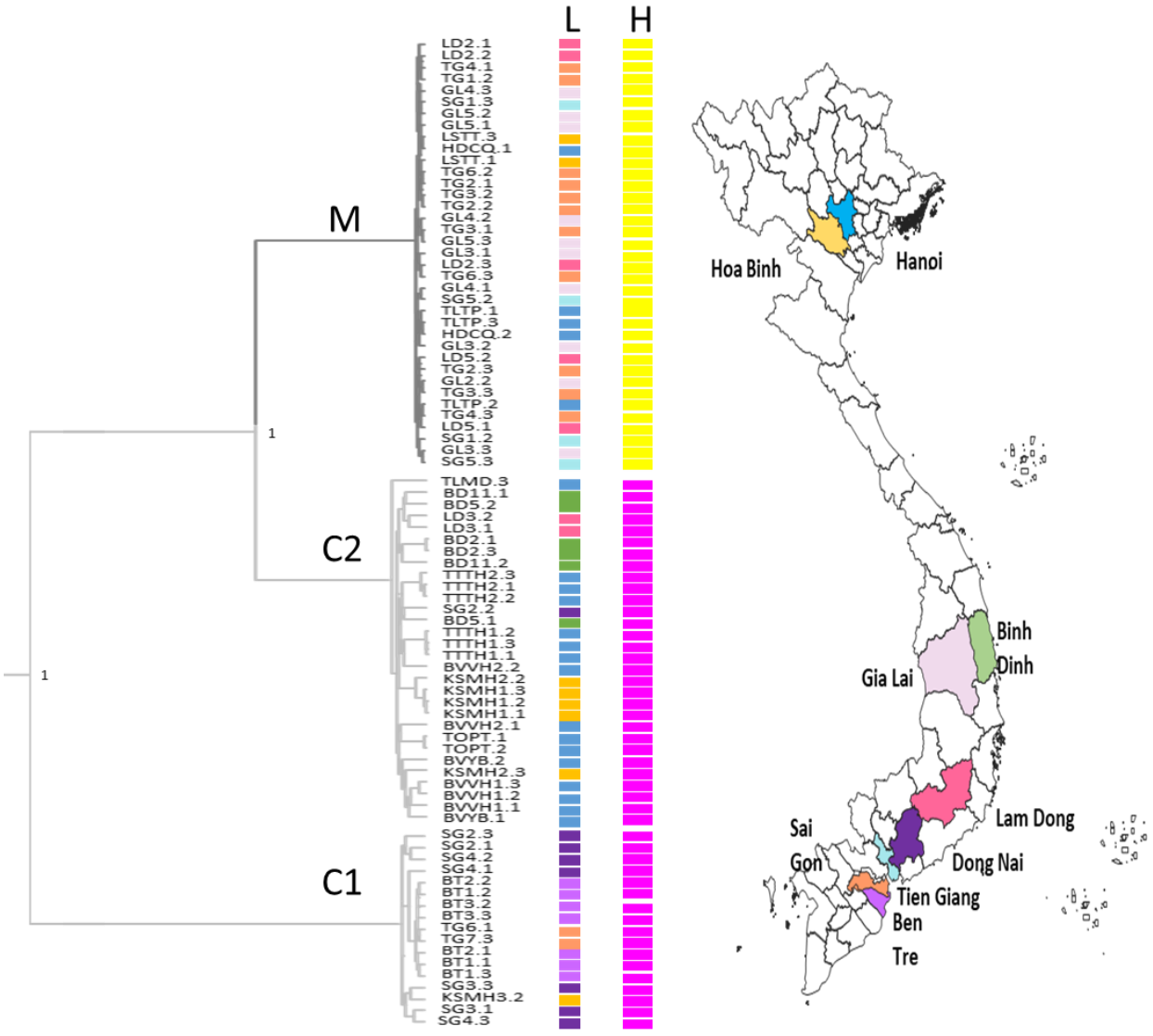

Figure 4. Phylogenetic tree of mites generated from 4287 SNP makers from RAD-seq.

L bar displays collecting sample sites, with color relative to collecting map of Vietnam.

H bar shows the host of *Varroa* mites, yellow represents *A. mellifera*, and pink represents *A. cerana*. The posterior probability values of the analysis are indicated on the nodes by small numbers. C1, C2 and M are mite clusters.

4.4. Discussion

The results regarding the *Varroa* mite genetic evolution demonstrate a clear clustering of these types according to hosts, and this clustering is not influenced by sampling location. *Varroa* mites are known to be parasites that jumped from *A. cerana* to *A. mellifera* hosts (Beaurepaire et al., 2015; Dietemann et al., 2019). Additionally, since honeybee species are often kept in close proximity to each other, such as in the same apiary, the mite is in constant and direct contact with both host species (Beaurepaire et al., 2015). Thus, there is a high possibility that mites on different hosts interact and share genes. This interaction includes not only the potential for mites to spill back from *A. mellifera* to *A. cerana* or to spill over from *A. cerana* to *A. mellifera* but also the possibility of hybridization between mites in *A. mellifera* hosts and those in *A. cerana* hosts (Beaurepaire et al., 2015).

In this context, the disease pressure on both *A. cerana* and *A. mellifera* will significantly increase due to the expected changes in the mite genetic structure resulting from hybridization and interaction between hosts (Beaurepaire et al., 2015; Dietemann et al., 2019). Although studies on mite populations in Thailand or the Philippines which used mitochondrial DNA and microsatellite DNA show a high chance of changes in host specificity, raising the possibility of host-shifted *Varroa destructor* haplotypes spilling back to *A. cerana* and hybridizing with *Varroa* mites (Beaurepaire et al., 2015; Dietemann et al., 2019), my research, from a RAD markers aspect, interestingly presents a different scenario in the adaptive strategies of the host-parasite relationship, indicating strong host specificity. The study does not find evidence of host mixing within any mite clusters, even when they were located in the same apiary or in close proximity. Furthermore, despite employing the same RAD seq approach, my study yielded contrasting results compared to the research conducted by Chiu et al. in 2023. Their study, which involved samples from North Vietnam and Taiwan, suggested the possibility of hybridization occurring between mite populations in Vietnam (Chiu et al., 2023). In contrast, my research, which added a large number of specimens collected in southern Vietnam, highlights that there is no gene flow between the mites found in both hosts within Vietnam. The analysis revealed three distinct clusters of mites, strongly segregated by host species (Figure 4), and an F_{ST} value close to 1 (Table 2). This suggests that there is no interaction or hybridization between both hosts occurring in the observed populations in Vietnam.

Although no evidence was found for host mixing among mite populations, the relationship among three clusters of mites showed that one cluster, which originated from mites parasitizing *A. cerana*, was closer to the mites on the host *A. mellifera* than to the other host *A. cerana*. This branching pattern aligns with the hypothesis that not all *Varroa* mites can successfully switch hosts. Mitochondrial data provided evidence of two successful host switches, corresponding to the J1 mitotype recorded in Japan and the K1 mitotype recorded in South Korea (Anderson & Trueman, 2000). Therefore, the proximity of two clusters from different hosts may indicate the presence of the same gene lineage, with one branch remaining with the original host and another branch switching to a new host.

The differences in coevolutionary strategies between *Varroa* mites and honeybees in different geographical regions not only indicate the driving forces behind the evolutionary processes, which can be based on the continuous variations in gene types causing new pressures for the host's adaptation, but also depend on specific situations between the host and the parasite. These variations generate adaptive strategies, such as host specificity, as observed in the *Varroa* mite population in Vietnam.

The *Varroa* mite population structure showing mixing of genotypes among mite individuals in the host *A. mellifera* is consistent with the ARD hypothesis (Figure 3), which posits that both the parasite and host species continually accumulate adaptive mutations (Gandon et al., 2008). Simultaneously, along with a lower genetic diversity (approximately 2 times less compared to the host *A. cerana*) (Table 2), directional selection for increased resistance and infectivity may potentially lead to the loss of certain alleles over time, then reduce in diversity within the host-parasite populations (Quigley et al., 2012). On the other hand, the higher genetic diversity of *Varroa* populations found in *A. cerana* host is in line with the specificity by host of the FSD hypothesis, potentially facilitates the maintenance of substantial genetic diversity within both the host and parasite populations (Quigley et al., 2012). In this scenario, the *Varroa* mite population structure supports the hypothesis that ARD plays a role in the adaptation of a novel parasite to its host (Hall et al., 2011) while FSD can maintain stable genetic diversity within populations (Buckling & Rainey, 2002; Hamilton et al., 1990). ARD is likely to be short-lived, and coevolution may either cease or transition towards FSD (Hall et al., 2011; Lenski, 1984; Sasaki, 2000).

In other hand, the mites have been observed shifting hosts from *A. cerana* to *A. mellifera*, but this transition has been successful in only two mitotypes of mites (J1 and K1 mitotypes) (Anderson & Trueman, 2000). This indicates that only a limited number of mite genotypes were capable of successfully adapting to the new host, suggesting that the initial genetic diversity of the mite population in *A. mellifera* was lower compared to *A. cerana*. As a result, the low genetic diversity of *Varroa* mites found in host *A. mellifera* could be attributed to a kind of founder effect, in which the new host population started with a small group from the old population, leading to lower genetic diversity.

Overall, no evidence of hybridization was found between mite populations of different hosts, suggesting host specificity in Vietnam. Additionally, the mite population on host species *A. cerana* exhibited higher gene diversity and showed spatial genetic patterns. These findings indicate that the mites infesting *A. cerana* conform to the Fluctuating Selection Dynamics (FSD) model, while the mites on host *A. mellifera* align with the Arms Race Dynamics (ARD) model of host-parasite evolution. However, this low genetic diversity is also possible because the population of *A. mellifera* mites is a young population, and likely formed from a small number of individuals that successfully switched hosts. Besides, it is important to consider that *A. cerana* is a native bee species adapted to the local climatic conditions in Vietnam, which could explain the higher genetic diversity observed in these bees compared to the introduced *A. mellifera*. On one hand, *A. mellifera* does not have a wild population to interbreed with like *A. cerana* due to its introduction. On the other hand, *A. mellifera* has adapted to a semi-artificial environment in Vietnam, with beekeepers providing disease control and supplementary feeding during adverse conditions. All these factors could lead to lower genetic diversity in *A. mellifera*. This suggests another interesting possibility in that low genetic diversity in mites is linked to low genetic diversity in the host *A. mellifera*, as less diverse hosts may select for less diverse parasites. To gain a more comprehensive understanding of this co-evolutionary relationship, future studies that consider both the host and parasite aspects in conjunction would provide valuable insights. Combining data from both hosts and parasites will offer a broader perspective on the intricate dynamics of co-evolution between them.

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Chapter 5.
General discussion

5.1. General discussion

"How does social context influence immune efficacy?"

The study on *Ceratina okinawana* bees revealed that social nesting bees had higher antibacterial activity compared to solitary nesters. However, this difference in immune efficacy could be explained by Density-dependent prophylactic effects rather than the influence of social status per se. The findings imply that personal immunity increases in response to the higher infection risk in larger groups. The reliance on personal immunity across different social contexts suggests that social immunity may have evolved as a secondary adaptation to group-living (Eusocial framework), rather than being an ancestral trait that facilitated the evolution of sociality (Group-living framework).

To disentangle the Eusocial framework from the and Group-living framework, this study provides a new perspective on the early stages in social evolution from an immune perspective and takes advantage of the unique opportunities offered by facultatively social model systems. With limited evidence on the role of social immunity in non-eusocial models, the Eusocial-framework model faces challenges in explaining the emergence of social immunity in non-eusocial models. Meanwhile, the presence of social immunity in non-eusocial species raises questions about its applicability to these models. My results indicate a reliance on personal immune systems during the transition from solitary to social behavior in this species, suggesting that the emergence of social immunity does not diminish individual defenses against pathogens but rather complements them. The trade-off between investment on personal immunity may occur through different processes such as reproductive instead of social immunity.

To implement this study, I developed a novel immune assay to evaluate immunological activity of haemolymph, adding to the array of traditional immunoassays such as Inhibition Zone (Kirby-Bauer Test, Antimicrobial Susceptibility Test, Disk Diffusion Test, or the Agar Diffusion Test) or microdilution (Minimal Inhibitory Concentration, MIC). This novel assay approach for inferring immune efficacy quantifies the capacity for haemolymph to suppress bacterial growth, as measured by changes in optical density. This method accounts for variation in the turbidity of haemolymph resulting from the extraction process by using the change in absorbance over time. It also employs a modified bacteria in which pUC19, a plasmid containing ampicillin resistance gene, has been inserted. Bacteria can thus be cultured in a medium containing ampicillin to avoid

contaminating effects of microorganisms naturally present in subjects. The method is highly sensitive and thus can be used for species in which only small amount of haemolymph can be extracted, for low concentration (diluted) haemolymph, and without immune priming (injecting pathogen into live organisms to increase antimicrobial components).

"Does social context influence the mechanistic basis of the immune response to pathogen challenge? The study on *Lasioglossom baleicum* bees found that gene expression and immune responses varied depending on the social context of these bees. *Paired social* individuals showed a higher number of differentially expressed genes (DEGs) compared to *isolated social* and *solitary* individuals, suggesting a more extensive immune response in social individuals to cope with the same threat. Remarkably, despite bees from different social contexts being challenged with the same pathogens, they exhibited distinct gene expression patterns, indicating different pathways being triggered. Individuals in current social context (*paired social*) demonstrated a more extensive pattern of gene expression and attention to combat pathogens compared to individuals in current solitary context (*isolated social* and *solitary).* Additionally, *isolated social* individuals displayed a response more similar to *solitary* individuals than to *paired social* individuals, suggesting an ability to rapidly adapt to the current social context. The study highlights that gene expression patterns and immune responses are influenced by social context and exhibit plasticity in response to adapt with different social contexts. On one hand, Density-dependent prophylaxis may be a form of phenotypic plasticity that benefits to adapt with changing social context. Other the other hand, gene-expression and immune response showed flexibility in responding to different social environments. As transitions from solitary to social life are likely to progress through a facultative stage, phenotypic plasticity offers flexibility at this stage. Continued investigations into socially polymorphic species will aid in establishing the generality of these patterns.

Notably, the study was provided new findings on phenotypic plasticity of immune responses. Most immune research is conducted in controlled laboratory conditions to understand specific pathogen responses. However, this approach may limit the understanding of how environmental changes impact the plasticity of immunity. Meanwhile, studies on changes in immune expression in different social contexts demonstrate the flexibility of the immune system. However, these studies do not provide any data on the mechanisms behind this phenotypic plasticity of immune responses. My research is the first to show how varied social context influence the gene expression and

phenotypic plasticity of immune responses within a species and provide insights into its' mechanistic basis.

Together, this work provides a new perspective on the early stages of social evolution and takes advantage of the unique opportunities offered by facultatively social model systems. The diversity in social organization of these bees is facilitated by behavioral plasticity (Kocher et al., 2018). However, this study shows that immune plasticity also plays an important role in responding to the transition between solitary and social lifestyles. These findings of phenotypic plasticity in immune responses, observed in both studies of immune mechanisms (gene expression) and immune effectiveness (bacterial inhibition), contribute to elucidating the relationship between immunological and ecological/evolutionary perspectives (social evolution).

"How do host-parasite relationships evolve in a eusocial context?"

The study on Varroa mite genetic evolution in Vietnam revealed clear clustering of mite types according to their host species, indicating that this parasite has adapted to specific host species. In contrast to previous studies in Thailand, the Philippines, Taiwan, and the North of Vietnam, the study showed no evidence of hybridization between mite populations from the two host species, suggesting different host adaptation scenarios for Varroa mites in different geographical regions.

The low genetic diversity and mixing of genotypes of mites in A. mellifera host supporting the Arms Race Dynamics (ARD) model of ongoing coevolution with host species. On the other hand, mites in *A. cerana* hosts exhibited higher genetic diversity, supporting the Fluctuating Selection Dynamics (FSD) model, which maintains stable genetic diversity within populations. The low genetic diversity in *Varroa* mites on *A. mellifera* hosts also may be attributed to a founder effect, as only a limited number of mite genotypes successfully adapted to the new host. This low genetic diversity might also be linked to the lower genetic diversity observed in introduced *A. mellifera* compared to native *A. cerana* bees in Vietnam.

Overall, the study highlights the host specificity of *Varroa* mites in Vietnam and provides insights into the complex dynamics of coevolution between mites and honeybee hosts. The study revealed different coevolutionary strategies between *Varroa* mites and honeybees in different geographic regions (Philippines, Taiwan, Thailand and Vietnam). Further research combining data from both hosts and parasites would offer a comprehensive understanding of this co-evolutionary relationship (see Future work).

5.2. Future works

The links between social evolution and immunity

The studies outlined herein were primarily conducted to directly assess personal immunity in different social environments. We can hypothesize that individual immunity plays a key role in the solitary-social transition. However, there is no direct evidence yet of the trade-off between personal immunity and social immunity. Therefore, future studies should focus on exploring the relationship between personal immunity and social immunity. One suitable candidate for such studies is *Ceratina okinawana.* In this species, social immune behaviors can be easily observed in the laboratory in artificial nests with a transparent plastic sheet covering the top for observation, and density can be adjusted manually based on the number of individuals present in the nest, facilitating observations under controlled laboratory conditions. Thus, observing the immune behavior of individuals before and after exposure to pathogens and under different population densities in these experimental conditions could provide evidence of the relationship between personal immunity and social immunity. Additionally, the relationship between social immune behaviors and group size (by changing density of individual in experiment condition) also gives a look on the cost and benefits of the social immune behaviours.

The presence of social immune behaviors in the *Cerana okinawana* model has been observed in pilot studies in the laboratory, such as removal of corpses. However, my results indicate that they rely on personal immunity under different social conditions. One possible explanation for this case is the use of a single immune system, personal immunity, which provides flexibility for the transition between solitary and social lifestyles. However, another possibility that the study also suggested is that the emergence of social immunity in the early stages of social evolution may complement individual immunity. In this context, individual immunity can be expected to trade off with other processes, such as reproduction. Therefore, future studies can evaluate the relationship between personal immunity and reproductive ability by comparing the personal immunity of adult offspring at different brood stages and during different breeding seasons. I believe that considering additional influences on personal immunity, such as social immunity and reproductive capacity, will help elucidate the relationship between immune evolution and social evolution.

In my work on gene expression related to immunity, the characteristic differentially expressed genes (DEGs) for each context provide insights into the variations in gene expression in a single social context (*paired social* vs *isolated social* vs *solitary*). Therefore, identifying specific DEGs in each group will help illuminate distinct adaptive mechanisms for each social context, indicating which genes are responsible for rapid adaptation and which genes are specific to particular social contexts.

On the other hand, the mechanisms demonstrating the plasticity of immune responses discovered in this study are related to personal immunity. Therefore, the phenotypic plasticity of immunity should also be further considered in the context of social immunity. These social immune behaviors can be investigated through brain transcriptome. The relationship between individual immunity and social immunity will contribute to a deeper understanding of immune plasticity.

Evolution of host-parasite relationships.

Although my study has shown higher gene pool diversity of mites in host *A. cerana* compared to those in host *A. mellifera*, the factors influencing the gene pool diversity of mites in the two hosts have not been elucidated. Therefore, studies on both the host and the parasite aspects are necessary to explain the evolutionary scenarios between the host and the parasite.

Further studies could examine the genetic diversity between *A. mellifera* and *A. cerana* to explain the low genetic diversity of *Varroa* mites between these two hosts. If the low genetic diversity of *A. mellifera* leads to the low genetic diversity of the mites, crossbreeding *A. mellifera* populations from different regions to increase the genetic diversity of the gene pool may be a solution to help *A. mellifera* resist *Varroa* mite infestations and promote the transition from ARS to FSD more rapidly. This would be beneficial in minimizing losses for agriculture.

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Appendix chapter 2

Table 1. *Ceratina okinawana* demographic data

The data used for assessing the influence of group size on immune efficacy, and comparing total brood between social and solitary nests.

Nest	Type	Female Adult	Female Callow	Male Adult	Male Callow	Egg	Larvae S	Larvae M	Larvae L	Pre-Pupa	Male Pupa	Female Pupae	Pollen Ball
TK03.19	solitary	1	0	0	0	0	1	0	0	0	0	0	1
TK03.24	solitary	1	0	0	0	0	0	0	0	1	0	1	0
TK03.25	solitary	1	0	0	0	0	0	0	0	0	0	0	0
TK01.06	solitary	1	0	0	0	1	1	1	0	0	0	0	0
TK06.02	solitary	1	0	0	0	0	0	0	0	0	0	0	1
TK03.20	solitary	1	0	0	0	0	0	0	0	0	0	0	0
TK03.26	solitary	1	0	0	0	0	0	1	0	1	0	0	0
TK06.05	solitary	1	0	0	0	0	0	0	0	0	0	0	0
TK07.04	solitary	1	0	0	0	0	0	0	0	0	0	0	0
TK04.04	solitary	1	0	0	0	0	0	1	0	2	0	0	0
TK03.09	solitary	1	0	0	0	0	0	0	0	0	1	2	0
TK08.04	solitary	1	0	0	0	0	1	0	0	0	0	0	0
TK03.05	solitary	1	0	0	0	0	0	0	0	0	0	0	0
TK01.07	solitary	1	0	0	0	0	0	0	0	0	0	2	0
TK08.06	solitary	1	0	0	0	0	1	0	0	0	0	0	0
TK05.10	solitary	1	0	0	0	0	0	0	0	1	0	1	0
TK02.04	solitary	1	0	0	0	0	0	0	0	1	2	3	0
TK06.03	solitary	1	0	0	0	0	0	2	0	0	0	0	0
TK05.08	solitary	1	0	0	0	0	0	0	0	0	0	0	0

Nest	Type	Female Adult	Female Callow	Male Adult	Male Callow	Egg	Larvae S	Larvae M	Larvae L	Pre-Pupa	Male Pupa	Female Pupae	Pollen Ball
TK03.12	solitary	1	0	0	0	0	0	0	0	0	0	0	0
TK08.11	solitary	1	0	0	0	0	0	1	0	2	0	0	0
TK04.11	solitary	1	0	0	0	0	1	1	0	0	0	0	1
TK04.15	solitary	1	0	0	0	0	0	1	0	0	0	0	0
TK05.03	solitary	1	0	0	0	0	0	1	0	0	0	0	0
TK05.01	solitary	1	0	0	0	0	0	0	0	0	0	0	0
TK05.05	solitary	1	0	0	0	0	0	0	0	0	0	0	1
TK02.06	solitary	1	0	0	0	0	1	2	0	1	0	0	0
TK04.03	social	4	0	1	1	0	0	0	0	0	0	0	0
TK04.08	social	4	0	4	0	0	0	0	0	0	0	0	0
TK07.09	social	2	1	2	0	0	0	0	0	0	0	0	0
TK04.01	social	4	2	0	0	0	0	0	0	0	0	0	0
TK04.13	social	3	0	0	0	0	0	1	1	0	0	0	1
TK06.04	social	4	0	0	0	0	0	0	0	1	0	0	1
TK04.05	social	3	0	1	0	0	1	0	0	0	0	0	0
TK03.17	social	3	0	2	0	0	0	2	0	0	0	0	0
TK02.05	social	2	0	0	0	1	0	0	0	1	0	0	2
TK03.02	solitary	1	0	1	1	0	0	0	0	0	2	0	0
TK03.14	social	3	0	0	1	0	0	0	0	0	0	0	1
TK05.07	social	3	0	1	0	0	0	0	0	0	0	0	0
TK04.10	social	3	0	0	0	0	0	0	0	0	0	0	0
TK03.21	social	2	0	2	0	0	0	0	0	0	0	0	0
TK08.10	social	2	0	1	0	0	0	1	0	0	0	0	0
TK08.05	solitary	1	1	0	1	0	0	0	0	0	0	0	0

Nest	Type	Female Adult	Female Callow	Male Adult	Male Callow	Egg	Larvae S	Larvae M	Larvae L	Pre-Pupa	Male Pupa	Female Pupae	Pollen Ball
TK01.04	social	2	0	0	0	0	0	0	0	0	0	0	2
TK08.08	social	3	0	0	0	0	0	0	0	0	0	0	0
TK01.11	solitary	1	0	1	0	0	0	0	1	1	0	0	0
TK03.11	social	2	0	0	0	0	1	1	1	0	0	0	1
TK03.04	social	2	0	0	0	0	0	0	0	0	0	0	0
TK04.09	social	2	0	0	0	0	0	0	0	0	0	0	0
TK03.06	social	2	0	0	0	0	0	0	0	2	1	0	0
TK03.13	solitary	1	0	0	1	0	0	2	0	0	0	2	0
TK03.18	solitary	1	1	0	0	0	0	1	1	0	1	1	1
TK08.07	social	2	0	0	0	0	0	1	0	0	0	0	1
TK01.10	social	3	1	0	0	0	0	0	0	0	0	0	0
TK01.02	social	2	0	1	0	0	0	0	0	0	0	0	0
TK08.02	social	3	0	1	0	0	0	0	0	0	3	0	0
TK07.03	social	3	2	1	0	0	0	0	0	0	0	0	0
TK07.03	social	3	2	1	0	0	0	0	0	0	0	0	0
TK07.10	solitary	1	0	2	1	0	0	0	0	0	0	0	0
TK09.01	social	2	0	2	0	0	0	0	0	0	0	0	0
TK03.08	solitary	1	0	0	0	0	0	0	0	0	0	0	0
TK07.01	social	2	0	1	0	0	0	0	0	0	0	0	0
TK06.06	social	3	1	1	0	0	0	0	0	0	0	0	0
TK06.06	social	3	1	1	0	0	0	0	0	0	0	0	0

Table 2. *Ceratina okinawana* nest characteristics

The data used for comparing nest length between social and solitary nests.

Nest	Type	Nest Width (cm)	Nest Length (cm)	Nest condition
TK03.19	solitary	3.52	78	New
TK03.24	solitary	4.22	132	New
TK03.25	solitary	3.52	63	New
TK01.06	solitary	3.79	95	New
TK06.02	solitary	3.65	112	New
TK03.20	solitary	3.89	36	New
TK03.26	solitary	3.21	38	New
TK06.05	solitary	3.27	145	Old
TK07.04	solitary	2.76	34	New
TK04.04	solitary	4.31	69	New
TK03.09	solitary	4.25	72	New
TK08.04	solitary	3.8	45	Old
TK03.05	solitary	3.95	70	New
TK01.07	solitary	4.09	125	New
TK08.06	solitary	3.87	58	New
TK05.10	solitary	3.84	95	New
TK02.04	solitary	4.95	192	New
TK06.03	solitary	4.34	99	New
TK05.08	solitary	4.1	130	Old
TK03.12	solitary	4.37	98	Old
TK08.11	solitary	3.66	46	New
TK04.11	solitary	3.76	121	New
TK04.15	solitary	4.23	88	New
TK05.03	solitary	4.52	90	Old
TK05.01	solitary	4.01	35	New
TK05.05	solitary	3.97	125	Old
TK02.06	solitary	3.22	181	New
TK04.03	social	4.9	245	Old
TK04.08	social	3.57	150	Old
TK07.09	social	4.42	259	Old
TK04.01	social	4.24	123	Old
TK04.13	social	4.22	206	New
TK06.04	social	4.11	162	Old
TK04.05	social	4.44	122	New
TK03.17	social	3.87	180	Old/New
TK02.05	social	4.77	152	New

Nest	Type	Nest Width (cm)	Nest Length (cm)	Nest condition
TK03.02	solitary	4.69	154	NA
TK03.14	social	4.31	190	New
TK05.07	social	3.79	140	Old
TK04.10	social	3.95	112	Old
TK03.21	social	4.1	235	Old
TK08.10	social	4.18	130	New
TK08.05	solitary	4.38	87	New
TK01.04	social	4.34	75	Old
TK08.08	social	4.42	92	Old
TK01.11	solitary	3.9	55	New
TK03.11	social	4.05	116	New
TK03.04	social	3.73	87	Old
TK04.09	social	3.79	148	New
TK03.06	social	3.87	85	Old
TK03.13	solitary	4.03	150	New
TK03.18	solitary	3.62	142	NA
TK08.07	social	3.65	62	Old
TK01.10	social	3.66	130	New
TK01.02	social	3.28	55	New
TK08.02	social	4.5	220	Old
TK07.03	social	4.43	84	Old
TK07.03	social	4.43	84	Old
TK07.10	solitary	4.41	76	Old
TK09.01	social	4.09	62	Old
TK03.08	solitary	3.81	48	New
TK07.01	social	4.35	132	Old
TK06.06	social	3.56	192	Old
TK06.06	social	3.56	192	Old

Table 3. *Ceratina okinawana* body size measurement

The data used for assessing the influence of body size on immune efficacy.

Nest	Type	Wing1	Wing2	Wing3	Wing4	Wing5	Wing6	Wing7	Wing8	Head1	Head2	Head3	Weight
TK03.19	solitary	3.928	3.952	4.001	3.918	3.962	3.968	3.986	3.931	2.122	2.113	2.131	1235
TK03.24	solitary	4.344	4.339	4.326	4.285	4.377	4.391	4.331	4.302	2.358	2.376	2.381	1912
TK03.25	solitary	3.565	3.599	3.579	3.591	3.571	3.589	3.61	3.582	1.885	1.885	1.88	969
TK01.06	solitary	4.231	4.305	4.328	4.314	4.232	4.273	4.252	4.273	2.326	2.351	2.347	1765
TK06.02	solitary	4.496	4.513	4.505	4.512	4.466	4.47	4.508	4.492	2.485	2.469	2.464	2242
TK03.20	solitary	3.961	3.99	4.006	3.978	3.924	3.943	3.986	3.963	2.094	2.115	2.137	1321
TK03.26	solitary	3.3	3.297	3.302	3.268	3.251	3.27	3.302	3.269	1.704	1.714	1.698	677
TK06.05	solitary	3.915	3.958	3.975	3.951	3.931	3.954	3.985	3.914	2.167	2.13	2.146	1260
TK07.04	solitary	3.663	3.648	3.633	3.589	3.711	3.677	3.669	3.653	2.006	2.001	1.995	916
TK04.04	solitary	4.164	4.16	4.188	4.162	4.179	4.192	4.199	4.172	2.267	2.261	2.25	1529
TK03.09	solitary	4.295	4.31	4.368	4.343	4.3	4.31	4.332	4.309	2.365	2.353	2.375	1641
TK08.04	solitary	3.882	3.917	3.946	3.934	3.85	3.912	3.942	3.88	2.115	2.13	2.109	1278
TK03.05	solitary	4.105	4.125	4.032	4.028	4.125	4.131	4.067	4.01	2.203	2.209	2.194	1412
TK01.07	solitary	4.068	4.107	4.173	4.124	4.068	4.108	4.161	4.146	2.193	2.204	2.193	1493
TK08.06	solitary	3.864	3.881	3.864	3.861	3.884	3.937	3.832	3.856	2.054	2.07	2.065	1195
TK05.10	solitary	3.778	3.801	3.862	3.828	3.736	3.793	3.877	3.808	2.033	2.048	2.043	1105
TK02.04	solitary	4.433	4.425	4.521	4.45	4.466	4.458	4.449	4.442	2.503	2.491	2.487	2380
TK06.03	solitary	3.472	3.516	3.54	3.519	3.488	3.542	3.533	3.545	1.95	1.971	1.945	1750
TK05.08	solitary	4.287	4.301	4.248	4.331	4.296	4.293	4.292	4.313	2.468	2.453	2.444	2267
TK03.12	solitary	4.395	4.471	4.516	4.451	4.402	4.441	4.498	4.459	2.403	2.403	2.412	1936
TK08.11	solitary	3.545	3.573	3.576	3.582	3.556	3.599	3.562	3.556	1.885	1.884	1.891	1003
TK04.11	solitary	3.966	3.984	4.04	4.042	3.939	3.974	4.036	3.969	2.106	2.124	2.133	1347

Nest	Type	Wing1	Wing2	Wing3	Wing4	Wing5	Wing6	Wing7	Wing8	Head1	Head2	Head3	Weight
TK04.15	solitary	3.768	3.799	3.827	3.805	3.735	3.75	3.849	3.8	2.073	2.083	2.063	1178
TK05.03	solitary	4.495	4.532	4.479	4.526	4.516	4.506	4.49	4.495	2.482	2.498	2.5	2159
TK05.01	solitary	4.049	4.104	4.167	4.123	4.137	4.108	4.063	4.097	2.272	2.281	2.282	1346
TK05.05	solitary	4.062	4.057	4.064	4.026	4.023	4.028	4.085	4.052	2.182	2.172	2.183	1340
TK02.06	solitary	3.969	4.023	4.064	4.008	3.974	4.018	4.054	4.031	2.163	2.166	2.178	1480
TK04.03	social	4.442	4.511	4.437	4.501	4.534	4.486	4.519	4.481	2.521	2.511	2.505	2188
TK04.08	social	3.594	3.633	3.621	3.557	3.594	3.628	3.616	3.593	1.972	1.971	1.96	1022
TK07.09	social	4.433	4.479	4.465	4.45	4.476	4.503	4.479	4.435	2.526	2.526	2.516	2125
TK04.01	social	4.153	4.241	4.224	4.233	4.189	4.231	4.271	4.216	2.308	2.313	2.307	1886
TK04.13	social	4.705	4.752	4.695	4.731	4.735	4.696	4.719	4.685	2.647	2.651	2.646	2467
TK06.04	social	4.392	4.403	4.424	4.412	4.422	4.452	4.433	4.405	2.453	2.459	2.438	1896
TK04.05	social	4.303	4.322	4.397	4.404	4.299	4.361	4.397	4.373	2.385	2.409	2.387	2004
TK03.17	social	4.365	4.422	4.407	4.359	4.365	4.37	4.358	4.345	2.369	2.394	2.375	1898
TK02.05	social	4.037	4.115	4.133	4.129	4.05	4.11	4.116	4.094	2.235	2.23	2.24	1627
TK03.02	solitary	4.464	4.522	4.506	4.511	4.495	4.516	4.522	4.516	2.428	2.438	2.422	2332
TK03.14	social	4.203	4.245	4.304	4.285	4.212	4.27	4.279	4.279	2.347	2.337	2.357	1702
TK05.07	social	3.975	4.018	4.009	3.959	4.004	4.014	4.004	3.969	2.12	2.125	2.12	1350
TK04.10	social	4.219	4.246	4.184	4.163	4.194	4.217	4.193	4.153	2.214	2.224	2.22	1820
TK03.21	social	4.574	4.616	4.609	4.606	4.597	4.596	4.618	4.57	2.573	2.557	2.563	2664
TK08.10	social	4.038	4.064	4.108	4.068	4.044	4.07	4.118	4.089	2.222	2.215	2.211	1473
TK08.05	solitary	4.739	4.758	4.764	4.734	4.65	4.722	4.748	4.681	2.653	2.642	2.632	2176
TK01.04	social	3.702	3.702	3.714	3.682	3.712	3.697	3.792	3.693	1.908	1.903	1.911	1187
TK08.08	social	4.363	4.388	4.412	4.368	4.333	4.382	4.379	4.3	2.351	2.343	2.358	1708
TK01.11	solitary	3.887	3.898	3.943	3.899	3.886	3.893	3.957	3.93	2.125	2.146	2.136	1370
TK03.11	social	3.893	3.901	3.913	3.881	3.874	3.915	3.871	3.861	2.083	2.08	2.084	1153

Nest	Type	Wing1	Wing2	Wing3	Wing4	Wing5	Wing6	Wing7	Wing8	Head1	Head2	Head3	Weight
TK03.04	social	4.417	4.449	4.391	4.376	4.387	4.339	4.356	4.349	2.387	2.387	2.394	2248
TK04.09	social	4.064	4.104	4.11	4.057	4.034	4.088	4.115	4.12	2.226	2.225	2.225	1573
TK03.06	social	4.168	4.189	4.198	4.204	4.148	4.148	4.188	4.146	2.298	2.295	2.297	1642
TK03.13	solitary	4.415	4.469	4.423	4.412	4.414	4.441	4.449	4.422	2.474	2.459	2.458	2167
TK03.18	solitary	4.558	4.552	4.553	4.578	4.525	4.499	4.526	4.5	2.583	2.592	2.599	2041
TK08.07	social	3.825	3.868	3.849	3.839	3.823	3.855	3.875	3.839	2.041	2.048	2.048	1428
TK01.10	social	4.263	4.275	4.334	4.276	4.234	4.271	4.329	4.276	2.256	2.236	2.271	1563
TK01.02	social	3.853	3.869	3.903	3.859	3.839	3.863	3.902	3.849	2.075	2.096	2.079	1311
TK08.02	social	5.008	5.022	4.961	5.045	5.042	5.016	5.047	5.031	2.851	2.863	2.853	3234
TK07.03	social	4.102	4.123	4.11	4.092	4.07	4.098	4.089	4.065	2.15	2.16	2.137	1410
TK07.03	social	3.849	3.901	3.927	3.912	3.877	3.91	3.943	3.934	2.09	2.076	2.067	1179
TK07.10	solitary	3.923	3.977	3.952	3.951	3.934	3.955	3.931	3.908	2.153	2.127	2.167	1162
TK09.01	social	3.742	3.799	3.791	3.777	3.724	3.757	3.805	3.76	2.011	2.016	2.016	1390
TK03.08	solitary	4.142	4.219	4.141	4.126	4.132	4.167	4.125	4.126	2.185	2.178	2.178	1430
TK07.01	social	3.98	4.025	4.012	4.001	3.995	4.012	4.053	4.018	2.103	2.122	2.134	1278
TK06.06	social	4.013	4.069	4.03	4.016	4.001	4.081	4.041	4.029	2.18	2.184	2.193	1479
TK06.06	social	4.323	4.339	4.36	4.349	4.313	4.287	4.323	4.307	2.339	2.339	2.333	1544

Table 4. *Ceratina okinawana* age class

The data used for assessing the influence of bee age class on immune efficacy.

Nest	Type	Mandible width 1	Mandible height 1	Mandible width 2	Mandible height 2	Total nick (wing left)	Total nick (wing right)
TK03.19	solitary	0.094	0.072	0.1	0.066	6	0
TK03.24	solitary	0.104	0.059	0.106	0.062	1	2
TK03.25	solitary	0.09	0.068	0.079	0.062	0	0
TK01.06	solitary	0.107	0.087	0.11	0.083	2	1
TK06.02	solitary	0.102	0.084	0.12	0.079	11	17
TK03.20	solitary	0.087	0.04	0.08	0.034	20	16
TK03.26	solitary	0.069	0.05	0.078	0.047	8	2
TK06.05	solitary	0.092	0.034	0.086	0.051	6	6
TK07.04	solitary	0.087	0.082	0.091	0.075	1	0
TK04.04	solitary	0.106	0.085	0.106	0.078	2	6
TK03.09	solitary	0.114	0.088	0.122	0.082	0	3
TK08.04	solitary	0.098	0.057	0.101	0.057	2	0
TK03.05	solitary	0.1	0.078	0.106	0.068	0	1
TK01.07	solitary	0.101	0.077	0.1	0.084	2	1
TK08.06	solitary	0.101	0.07	0.103	0.066	1	1
TK05.10	solitary	0.086	0.055	0.084	0.064	2	6
TK02.04	solitary	0.124	0.08	0.125	0.081	4	2
TK06.03	solitary	0.099	0.058	0.102	0.071	18	16
TK05.08	solitary	0.113	0.095	0.118	0.107	0	0
TK03.12	solitary	0.103	0.111	0.115	0.096	0	1
TK08.11	solitary	0.089	0.058	0.095	0.049	3	3
TK04.11	solitary	0.087	0.06	0.093	0.054	1	2
TK04.15	solitary	0.087	0.067	0.084	0.062	1	1
TK05.03	solitary	0.113	0.077	0.123	0.066	2	0
TK05.01	solitary	0.11	0.096	0.101	0.097	0	0
TK05.05	solitary	0.102	0.053	0.103	0.074	1	0
TK02.06	solitary	0.097	0.057	0.098	0.058	0	1
TK04.03	social	0.122	0.086	0.125	0.077	3	3
TK04.08	social	0.092	0.061	0.092	0.066	1	0
TK07.09	social	0.112	0.093	0.108	0.085	3	4
TK04.01	social	0.108	0.07	0.113	0.077	5	5
TK04.13	social	0.129	0.072	0.127	0.105	5	7
TK06.04	social	0.11	0.077	0.119	0.081	0	4
TK04.05	social	0.104	0.069	0.104	0.057	16	13

Nest	Type	Mandible width 1	Mandible height 1	Mandible width 2	Mandible height 2	Total nick (wing left)	Total nick (wing right)
TK03.17	social	0.118	0.06	0.107	0.062	1	5
TK02.05	social	0.099	0.073	0.104	0.07	10	10
TK03.02	solitary	0.149	0.097	0.117	0.102	0	0
TK03.14	social	0.111	0.094	0.113	0.094	4	1
TK05.07	social	0.102	0.064	0.097	0.068	0	1
TK04.10	social	0.11	0.09	0.111	0.093	0	0
TK03.21	social	0.129	0.098	0.125	0.078	0	0
TK08.10	social	0.101	0.049	0.1	0.051	10	11
TK08.05	solitary	0.106	0.05	0.105	0.056	25	22
TK01.04	social	0.09	0.077	0.097	0.053	0	0
TK08.08	social	0.103	0.062	0.105	0.05	4	7
TK01.11	solitary	0.095	0.051	0.099	0.053	5	2
TK03.11	social	0.056	0.041	0.054	0.038	4	2
TK03.04	social	0.12	0.086	0.115	0.086	1	0
TK04.09	social	0.105	0.065	0.112	0.066	4	6
TK03.06	social	0.1	0.057	0.111	0.063	1	4
TK03.13	solitary	0.125	0.095	0.118	0.058	1	2
TK03.18	solitary	0.113	0.068	0.109	0.082	0	1
TK08.07	social	0.09	0.085	0.093	0.081	0	1
TK01.10	social	0.109	0.062	0.111	0.078	2	0
TK01.02	social	0.098	0.048	0.092	0.051	1	3
TK08.02	social	0.124	0.053	0.135	0.057	19	18
TK07.03	social	0.11	0.068	0.1	0.074	1	0
TK07.03	social	0.112	0.079	0.096	0.082	1	0
TK07.10	solitary	0.113	0.085	0.104	0.083	0	0
TK09.01	social	0.083	0.077	0.1	0.067	0	0
TK03.08	solitary	0.11	0.065	0.106	0.068	1	3
TK07.01	social	0.11	0.079	0.097	0.089	0	0
TK06.06	social	0.107	0.078	0.11	0.057	0	1
TK06.06	social	0.107	0.1	0.112	0.088	0	0

Table 5. Absorbance value of haemolymph-bacteria suspension at OD600nm

The data indicates immune efficacy of bees following time of challenging with bacteria.

Nest	Type	abs1	abs2	abs3	abs4	abs5	abs6	abs7	abs8	abs9
TK03.19	solitary	117	121	137	148	158	167	166	173	171
TK03.24	solitary	304	310	328	336	345	350	340	357	348
TK03.25	solitary	105	113	118	124	130	135	131	139	135
TK01.06	solitary	88	89	94	100	105	109	109	114	114
TK06.02	solitary	108	112	122	131	140	148	148	154	153
TK03.20	solitary	112	115	125	135	143	151	152	156	156
TK03.26	solitary	115	118	128	135	143	150	151	158	157
TK06.05	solitary	113	117	129	139	149	157	158	164	165
TK07.04	solitary	154	139	142	149	158	166	185	174	189
TK04.04	solitary	141	139	153	156	164	173	174	181	181
TK03.09	solitary	118	119	128	135	144	151	153	157	159
TK08.04	solitary	106	107	116	131	133	142	144	149	151
TK03.05	solitary	105	109	119	127	136	143	145	149	151
TK01.07	solitary	103	107	115	123	130	138	140	144	145
TK08.06	solitary	103	107	117	127	137	149	152	158	160
TK05.10	solitary	105	110	120	128	135	141	141	147	147
TK02.04	solitary	113	117	128	136	144	150	151	156	156
TK06.03	solitary	115	118	130	140	149	158	157	165	163
TK05.08	solitary	667	677	695	703	714	722	704	726	708
TK03.12	solitary	109	110	121	129	136	144	141	150	147
TK08.11	solitary	111	109	119	128	135	142	143	147	149
TK04.11	solitary	102	104	113	122	130	137	136	143	141

Nest	Type	abs1	abs2	abs3	abs4	abs5	abs6	abs7	abs8	abs9
TK04.15	solitary	140	145	158	163	168	173	166	174	167
TK05.03	solitary	114	117	128	134	140	147	146	152	151
TK05.01	solitary	117	123	135	141	146	152	144	155	147
TK05.05	solitary	105	107	118	126	133	140	142	146	147
TK02.06	solitary	107	111	123	132	140	147	147	154	153
TK04.03	social	428	419	429	435	441	447	448	449	448
TK04.08	social	91	93	97	104	110	116	116	121	121
TK07.09	social	133	139	145	151	158	164	159	171	165
TK04.01	social	90	89	92	96	100	103	103	106	107
TK04.13	social	180	172	181	189	196	200	206	206	211
TK06.04	social	173	168	174	180	184	189	194	192	196
TK04.05	social	91	92	99	106	111	115	114	116	115
TK03.17	social	93	94	102	108	114	117	117	121	120
TK02.05	social	93	93	101	107	110	115	117	117	117
TK03.02	solitary	102	102	111	118	124	130	129	136	135
TK03.14	social	98	99	105	113	120	127	128	131	130
TK05.07	social	115	121	140	154	165	179	178	192	189
TK04.10	social	115	119	129	136	144	151	149	156	154
TK03.21	social	508	512	541	546	561	564	551	577	560
TK08.10	social	101	104	113	121	131	138	137	144	143
TK08.05	solitary	102	105	117	125	132	139	138	144	143
TK01.04	social	105	108	117	124	130	136	135	141	140
TK08.08	social	101	102	112	120	125	131	132	137	136
TK01.11	solitary	107	112	124	133	140	148	147	154	152
TK03.11	social	103	106	118	126	134	141	138	146	144

Nest	Type	abs1	abs2	abs3	abs4	abs5	abs6	abs7	abs8	abs9
TK03.04	social	106	106	119	126	133	139	134	141	139
TK04.09	social	96	96	105	110	115	118	116	122	120
TK03.06	social	101	98	103	108	114	117	119	121	122
TK03.13	solitary	81	80	86	90	94	96	94	98	96
TK03.18	solitary	226	226	229	233	235	237	232	236	232
TK08.07	social	95	95	103	109	114	117	116	121	120
TK01.10	social	109	111	123	133	142	146	146	154	153
TK01.02	social	94	93	102	108	114	115	116	123	121
TK08.02	social	94	93	103	111	119	125	126	130	129
TK07.03	social	131	129	140	147	153	158	158	164	162
TK07.03	social	126	127	136	143	150	155	154	161	159
TK07.10	solitary	126	127	137	144	150	155	155	160	158
TK09.01	social	123	123	131	139	146	149	50	156	156
TK03.08	solitary	127	130	137	145	149	155	156	161	161
TK07.01	social	128	129	138	146	153	157	156	162	160
TK06.06	social	123	125	135	142	147	151	151	158	156
TK06.06	social	143	141	151	156	160	161	162	165	164
NA	buffer	104	108	121	129	138	150	149	154	155
NA	buffer	106	109	122	133	136	147	148	153	151
NA	buffer	104	107	120	132	136	148	145	155	152
NA	buffer	108	111	122	140	146	152	151	164	163
NA	buffer	105	108	123	133	140	149	148	157	153
NA	buffer	108	110	124	137	142	150	150	159	154
NA	buffer	103	107	122	134	142	146	149	157	153
NA	buffer	104	107	123	128	138	144	146	153	151

Nest	Type	abs1	abs2	abs3	abs4	abs5	abs6	abs7	abs8	abs9
NA	buffer	103	105	121	127	136	145	148	157	157
NA	buffer	105	110	121	127	140	149	150	159	156

After 15 hours of incubation, at room temperature

Abs1 = recorded absorbance value at 1:41pm
Abs2 = recorded absorbance value at 2:02pm
Abs3 = recorded absorbance value at 3:07pm
Abs4 = recorded absorbance value at 4:08pm
Abs5 = recorded absorbance value at 5:03pm
Abs6 = recorded absorbance value at 6pm
Abs7 = recorded absorbance value at 6:07pm
Abs8 = recorded absorbance value at 6:49pm
Abs9 = recorded absorbance value at 6:51pm

Appendix chapter 3

Table 6. GO ID of 24 shared DEGs from Venn Diagram analysis

These GO IDs used for identifying GO terms of biological process of 24 shared DEGs.

Gene IDs	GO IDs
LBAL_09753	GO:0016021,GO:0016020,GO:0005886,GO:0120200,GO:0005524,GO:0005525,GO:0004383,GO:0042802,GO:0016941,GO:0017046,GO:0001653,GO:0004672,GO:0044877,GO:0006182,GO:0019934,GO:0050908,GO:0007168,GO:0007165
LBAL_07155	GO:0005789,GO:0020037,GO:0005506,GO:0004497,GO:0016705
LBAL_05654	GO:0004016,GO:0005524,GO:0046872,GO:0006171,GO:0035556
LBAL_11544	GO:0035577,GO:0005623,GO:0005694,GO:0005789,GO:0016021,GO:0005887,GO:0016020,GO:0005815,GO:0005635,GO:0005886,GO:0000922,GO:0043312,GO:0007338,GO:0006906,GO:0006903
LBAL_02364	GO:0016324,GO:0005768,GO:0005887,GO:0016020,GO:0005886,GO:0015086,GO:0005375,GO:0005381,GO:0005384,GO:0015293,GO:0055070,GO:0015677,GO:0055072,GO:0034755,GO:0055071,GO:0034761,GO:0010042,GO:0010038,GO:0050916,GO:0055076,GO:0000041,GO:0046718
LBAL_09785	GO:0005737,GO:0005524,GO:0046872,GO:0050265,GO:2000627,GO:0031054,GO:0071076
LBAL_04351	GO:0030424,GO:0005623,GO:0030864,GO:0005737,GO:0005856,GO:0031901,GO:0000137,GO:0005886,GO:0032588,GO:0005096,GO:0045159,GO:0019901,GO:0005198,GO:0007409,GO:0030866,GO:0051294,GO:0006887,GO:0006893,GO:0065003,GO:0032878,GO:0008593,GO:0050708
LBAL_04405	GO:0016811,GO:0006807
LBAL_05850	GO:0005737,GO:0098978,GO:0005641,GO:0005886,GO:0014069,GO:0098685,GO:0042802,GO:0030165,GO:0006886,GO:0008104,GO:0099152,GO:0007165
LBAL_12298	NA
LBAL_10035	GO:0005739,aGO:0004735,GO:0034599,GO:0055129,GO:0006561
LBAL_06541	NA
LBAL_09106	GO:0005576,GO:0004177,GO:0008236

Gene IDs	GO IDs
LBAL_00193	GO:0005737,GO:0005829,GO:0033256,GO:0005654,GO:0005634,GO:0003682,GO:0001228,GO:0000981,GO:0042802,GO:0000977,GO:0034198,GO:0006974,GO:0042742,GO:0050829,GO:0051607,GO:0048813,GO:0007249,GO:0006955,GO:0006954,GO:0045087,GO:2000647,GO:0000122,GO:0038061,GO:0061057,GO:0048935,GO:0006963,GO:0006967,GO:0006964,GO:0002230,GO:0010628,GO:0045089,GO:0045429,GO:0050766,GO:0045944,GO:0045088,GO:0009617,GO:0034097
LBAL_05953	GO:0005506,GO:0004505,GO:0004510,GO:0006726,GO:0006559,GO:0007616,GO:0042427,GO:0006571
LBAL_01833	NA
LBAL_01112	GO:0005634,GO:0003700,GO:0000978,GO:0043565,GO:0007275
LBAL_02305	GO:0016021,GO:0005741,GO:0035755,GO:0004519,GO:0046872,GO:0042803,GO:0043046,GO:0016042,GO:0051321,GO:0008053,GO:0030719,GO:0034587,GO:0007286
LBAL_09342	GO:0005759,GO:0005739,GO:0003995,GO:0050660,GO:0009083,GO:0006631
LBAL_00890	GO:0005829,GO:0003937,GO:0004643,GO:0042803,GO:0006189
LBAL_07607	NA
LBAL_10671	NA
LBAL_02366	GO:0090575,GO:0001228,GO:0046983,GO:0000978,GO:0043583,GO:0042755,GO:0045198,GO:0060231,GO:0048644,GO:0050884,GO:0048339,GO:0036342,GO:1903053,GO:0003016,GO:0048705,GO:0043588,GO:0001756
LBAL_12560	GO:0070062,GO:0005576,GO:1904813,GO:0035580,GO:1904724,GO:0016603,GO:0008270,GO:0006464,GO:0043312,GO:0017186

Appendix chapter 4

Table 7. Collecting sample sites in Northern Vietnam

The information of collecting sample sites of *Varroa* mites

SampleID	Host	Province	Latitude/Longitude
BVYB.1	*Apis cerana*	Ha Noi	21.030774, 105.40804
BVYB.2	*Apis cerana*	Ha Noi	21.030774, 105.40804
BVVH1.1	*Apis cerana*	Ha Noi	21.079834, 105.416906
BVVH1.2	*Apis cerana*	Ha Noi	21.079834, 105.416906
BVVH1.3	*Apis cerana*	Ha Noi	21.079834, 105.416906
BVVH2.1	*Apis cerana*	Ha Noi	21.071059, 105.42992
BVVH2.2	*Apis cerana*	Ha Noi	21.071059, 105.42992
BVVH2.3	*Apis cerana*	Ha Noi	21.071059, 105.42992
KSMH1.1	*Apis cerana*	Nghe An	20.900421, 105.406571
KSMH1.2	*Apis cerana*	Nghe An	20.900421, 105.406571
KSMH1.3	*Apis cerana*	Nghe An	20.900421, 105.406571
KSMH2.1	*Apis cerana*	Nghe An	20.899633, 105.40684
KSMH2.2	*Apis cerana*	Nghe An	20.899633, 105.40684
KSMH2.3	*Apis cerana*	Nghe An	20.899633, 105.40684
KHMH3.1	*Apis cerana*	Nghe An	20.903857, 105.404089
KHMH3.2	*Apis cerana*	Nghe An	20.903857, 105.404089
KHMH3.3	*Apis cerana*	Nghe An	20.903857, 105.404089
TTTH1.1	*Apis cerana*	Ha Noi	21.012589, 105.502336
TTTH1.2	*Apis cerana*	Ha Noi	21.012589, 105.502336
TTTH1.3	*Apis cerana*	Ha Noi	21.012589, 105.502336
TTTH2.1	*Apis cerana*	Ha Noi	20.989346, 105.541019
TTTH2.2	*Apis cerana*	Ha Noi	20.989346, 105.541019
TTTH2.3	*Apis cerana*	Ha Noi	20.989346, 105.541019
TLMD.1	*Apis cerana*	Ha Noi	21.029168, 105.774754
TLMD.2	*Apis cerana*	Ha Noi	21.029168, 105.774754
TLMD.3	*Apis cerana*	Ha Noi	21.029168, 105.774754
TOTP.1	*Apis cerana*	Ha Noi	20.822362, 105.756789
TOTP.2	*Apis cerana*	Ha Noi	20.822362, 105.756789
TOTP.3	*Apis cerana*	Ha Noi	20.822362, 105.756789
LSTT.1	*Apis mellifera*	Hoa Binh	20.871663, 105.496729
LSTT.2	*Apis mellifera*	Hoa Binh	20.871663, 105.496729
HDCQ.1	*Apis mellifera*	Ha Noi	21.045923, 105.675046
HDCQ.2	*Apis mellifera*	Ha Noi	21.045923, 105.675046
HDCQ.3	*Apis mellifera*	Ha Noi	21.045923, 105.675046
TLTP.1	*Apis mellifera*	Ha Noi	21.081353, 105.76987
TLTP.2	*Apis mellifera*	Ha Noi	21.081353, 105.76987
TLTP.3	*Apis mellifera*	Ha Noi	21.081353, 105.76987

Table 8. Collecting sample sites in Southern Vietnam

The information of collecting sample sites of *Varroa* mites

SampleID	Host	Province	Latitude/Longitude
BD10.1	*A. cerana*	Binh Dinh	14.418957,109.045613
BD11.1	*A. cerana*	Binh Dinh	14.418999,109.045585
BD11.2	*A. cerana*	Binh Dinh	14.418999,109.045585
BD2.1	*A. cerana*	Binh Dinh	14.339255,109.049763
BD2.2	*A. cerana*	Binh Dinh	14.339255,109.049763
BD2.3	*A. cerana*	Binh Dinh	14.339255,109.049763
BD5.1	*A. cerana*	Binh Dinh	14.341586,109.047945
BD5.2	*A. cerana*	Binh Dinh	14.341586,109.047945
BD5.3	*A. cerana*	Binh Dinh	14.341586,109.047945
BD8.1	*A. cerana*	Binh Dinh	14.414907,109.045717
BT1.1	*A. cerana*	Ben Tre	10.245511,106.173606
BT1.2	*A. cerana*	Ben Tre	10.245511,106.173606
BT1.3	*A. cerana*	Ben Tre	10.245511,106.173606
BT2.1	*A. cerana*	Ben Tre	10.243394,106.180262
BT2.2	*A. cerana*	Ben Tre	10.243394,106.180262
BT2.3	*A. cerana*	Ben Tre	10.243394,106.180262
BT3.1	*A. cerana*	Ben Tre	10.135324,106.349621
BT3.2	*A. cerana*	Ben Tre	10.135324,106.349621
BT3.3	*A. cerana*	Ben Tre	10.135324,106.349621
GL2.1	*A.mellifera*	Gia Lai	13.645078,108.025661
GL2.2	*A.mellifera*	Gia Lai	13.645078,108.025661
GL2.3	*A.mellifera*	Gia Lai	13.645078,108.025661
GL3.1	*A.mellifera*	Gia Lai	13.612163,108.048117
GL3.2	*A.mellifera*	Gia Lai	13.612163,108.048117
GL3.3	*A.mellifera*	Gia Lai	13.612163,108.048117
GL4.1	*A.mellifera*	Gia Lai	13.612582,108.048615
GL4.2	*A.mellifera*	Gia Lai	13.612582,108.048615
GL4.3	*A.mellifera*	Gia Lai	13.612582,108.048615
GL5.1	*A.mellifera*	Gia Lai	13.612582,108.048615
GL5.2	*A.mellifera*	Gia Lai	13.612582,108.048615
GL5.3	*A.mellifera*	Gia Lai	13.612582,108.048615
LD2.1	*A.mellifera*	Lam Dong	11.659779,108.256218
LD2.2	*A.mellifera*	Lam Dong	11.659779,108.256218
LD2.3	*A.mellifera*	Lam Dong	11.659779,108.256218
LD3.1	*A. cerana*	Lam Dong	11.659792,108.256222
LD3.2	*A. cerana*	Lam Dong	11.659792,108.256222
LD3.3	*A. cerana*	Lam Dong	11.659792,108.256222

SampleID	Host	Province	Latitude/Longitude
LD5.1	*A.mellifera*	Lam Dong	11.804792,108.215266
LD5.2	*A.mellifera*	Lam Dong	11.804792,108.215266
LD5.3	*A.mellifera*	Lam Dong	11.804792,108.215266
SG1.1	*A.mellifera*	Sai Gon	10.86683,106.809822
SG1.2	*A.mellifera*	Sai Gon	10.86683,106.809822
SG1.3	*A.mellifera*	Sai Gon	10.86683,106.809822
SG2.1	*A. cerana*	Dong Nai	10.967941,107.116564
SG2.2	*A. cerana*	Dong Nai	10.967941,107.116564
SG2.3	*A. cerana*	Dong Nai	10.967941,107.116564
SG3.1	*A. cerana*	Dong Nai	10.837227,107.121073
SG3.2	*A. cerana*	Dong Nai	10.837227,107.121073
SG3.3	*A. cerana*	Dong Nai	10.837227,107.121073
SG4.1	*A. cerana*	Dong Nai	10.839696,107.117507
SG4.2	*A. cerana*	Dong Nai	10.839696,107.117507
SG4.3	*A. cerana*	Dong Nai	10.839696,107.117507
SG5.1	*A.mellifera*	Dong Nai	10.964755,107.122453
SG5.2	*A.mellifera*	Dong Nai	10.964755,107.122453
SG5.3	*A.mellifera*	Dong Nai	10.964755,107.122453
TG1.1	*A.mellifera*	Tien Giang	10.394336,106.367293
TG1.2	*A.mellifera*	Tien Giang	10.394336,106.367293
TG2.1	*A.mellifera*	Tien Giang	10.390647,106.428341
TG2.2	*A.mellifera*	Tien Giang	10.390647,106.428341
TG2.3	*A.mellifera*	Tien Giang	10.390647,106.428341
TG3.1	*A.mellifera*	Tien Giang	10.3967,106.431593
TG3.2	*A.mellifera*	Tien Giang	10.3967,106.431593
TG3.3	*A.mellifera*	Tien Giang	10.3967,106.431593
TG4.1	*A.mellifera*	Tien Giang	10.342799,106.352951
TG4.2	*A.mellifera*	Tien Giang	10.342799,106.352951
TG4.3	*A.mellifera*	Tien Giang	10.342799,106.352951
TG6.1	*A.mellifera*	Tien Giang	10.330761,106.317722
TG6.2	*A.mellifera*	Tien Giang	10.330761,106.317722
TG6.3	*A.mellifera*	Tien Giang	10.330761,106.317722
TG7.1	*A. cerana*	Tien Giang	10.3435,106.314
TG7.2	*A. cerana*	Tien Giang	10.3435,106.314
TG7.3	*A. cerana*	Tien Giang	10.3435,106.314