Title of Thesis:

Reproductive Competition in the Context of a Superorganism

A thesis

Submitted in partial fulfilment

of the requirements for the degree

of

Master of Science (Research) in Ecology and Biodiversity

at

The University of Waikato

by

Epernay Lynne Sarda Carta

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2025

Abstract

Population density is a fundamental ecological factor that shapes the intensity of competition within a species. Increased density often intensifies competition for resources and mates, driving the evolution of diverse adaptive responses in behaviour, morphology and physiology. A key area where density-mediated competition manifests is in sperm competition. This occurs when sperm from multiple males compete to fertilise the ova of a female. Species facing intense competition exhibit a range of adaptations, including adjustments in sperm production, ejaculate characteristics, and mating strategies. Elevated population density is a major factor that can exacerbate sperm competition, further amplifying these evolutionary pressures. Within this context, this thesis investigates the effects of apiary density on reproductive strategies and drone physiology in the honey bee (*Apis mellifera*), a highly social insect that exhibits a complex interplay between individual and colony-level responses. Apiary density, characterised by the number of colonies within a defined area, was manipulated across three treatments: low (8 colonies), medium (60-68 colonies), and high (120 colonies). Drone brood production and comb allocation (both drone and worker) were measured monthly to assess colony-level investment in reproduction versus worker production. Total sperm count and the proportion of viable sperm were assessed in drones collected from each density treatment to evaluate sperm competition dynamics. Heat Shock Protein 70 (HSP70) levels, a biomarker of cellular stress, were quantified in drones from each treatment using an enzyme-linked immunosorbent assay. Colonies in high-density apiaries exhibited significantly increased drone brood production and higher total sperm counts compared to those in low-density apiaries. However, worker comb production was significantly reduced in high-density apiaries, suggesting a trade-off between

resource allocation towards reproduction and colony maintenance. Sperm viability varied across densities and seasons, with low-density treatments having a higher proportion of viable sperm in early summer and medium-density treatments showing higher viability in late summer. This suggests that while high-density may favour increased sperm quantity, lower densities might facilitate greater investment in sperm quality. Apiary density significantly predicted HSP70 concentration in drones, with drones from high-density apiaries displaying significantly elevated levels, indicating increased physiological stress. These findings suggest that colonies in high-density environments experience intensified sperm competition, responding by increasing drone and sperm production. However, the observed variation in sperm viability and reduced worker comb production implies that this augmented reproductive investment may compromise overall colony function. The elevated HSP70 levels further corroborate that drones in high-density apiaries are subjected to greater physiological stress. Crucially, this study provides a unique perspective by demonstrating that apiary density, a factor often controlled in beekeeping, acts as a significant driver of reproductive strategies and physiological stress at both the colony and individual drone level within a superorganism. This highlights the profound impact of a managed environmental parameter on the evolutionary dynamics of a highly social species, offering critical insights for sustainable apicultural practices.

Acknowledgements

First and foremost, I would like to express my deepest gratitude to my incredible supervisors, Ashley and Chrissie. Your guidance, understanding, and expertise have been invaluable throughout this journey. Thank you for your patience and encouragement through all my challenges and setbacks, and for always pushing for my best.

I am also immensely grateful to the Bee and Biology Team at Plant & Food Research, especially James, Felicia, Charles, Rafael, Nicolas, and Gracie. Your expertise, generosity, and assistance (particularly with fieldwork when I was unable to) were instrumental to the success of this project. A special thank you to Charles, my lab partner, whose dedication, camaraderie, and willingness to endure countless late nights in the lab with me made an immeasurable difference. I am deeply grateful for your friendship.

My sincere thanks go to Reva for her invaluable assistance with the HSP analyses and to Sam for creating the automated computer model that saved me from manually counting an endless number of sperm photos.

To my partner, Isabel, your unwavering love, support, and encouragement have been invaluable. Thank you for believing in me, for your patience through the long hours and stressful moments, and for celebrating every milestone with me.

To my family, Mum, Dad, Esperance, and all my extended family, thank you for being my constant source of support. I could not have done this without you.

Table of Contents

List of Figures Page

List of Abbreviations

AIC	Akaike Information Criterion
BIC	Bayesian Information Criterion
BSA	Bovine Serum Albumin
DCAs	Drone Congregation Areas
ELISA	Enzyme-Linked Immunosorbent Assay
FOB	Frames of Bees
HSP	Heat Shock Protein
JH	Juvenile Hormone
MDA	Malondialdehyde
PLC	Phospholipase C
ROS	Reactive Oxygen Species
SFPs	Seminal Fluid Proteins
SOD	Superoxide Dismutase
TMB	3,3',5,5'-Tetramethylbenzidine

CHAPTER 1

Literature Review

Reproductive Competition

Sexual reproduction inherently generates competition among males, who often strive to maximise their reproductive success by siring as many offspring as possible (Andersson & Iwasa, 1996). However, their opportunities are fundamentally limited by the presence and competitiveness of other males (Kokko et al., 2014). This dynamic, known as male-male competition, is central to the evolution of reproductive traits and strategies (Boomsma et al., 2005; Kokko et al., 2014). It drives the process of sexual selection, where traits that enhance a male's ability to outcompete rivals in securing mates and fertilisations become more prevalent within a population. These traits can range from physical weaponry used in direct confrontations to elaborate displays that attract females, and even to subtle physiological adaptations at the level of sperm (Boomsma et al., 2005; Kokko et al., 2014). Ultimately, the intensity of male-male competition, and the nature of the traits it favours, depend on a range of ecological and social factors.

Population Density and the Intensification of Sexual Selection

Population density is a key demographic factor that significantly influences the intensity of sexual selection, particularly through its effects on male-male competition for mates (Bretman et al., 2013). As the number of males within a given area increases, so too does the competition for limited resources, mating, and fertilisation opportunities. This intensified competition subsequently amplifies the selective pressures associated with sexual selection, driving the evolution of more elaborate or effective traits that improve mating and fertilisation success (Evans & Magurran, 2000; Grant & Kramer, 1992; Vanpé et al., 2008). Crucially, variation in population

density across different populations can result in differences in trait expression and behaviour, even to the point of loss of a sexually selected trait in areas with low-density (Kokko & Rankin, 2006).

Population density affects the selection landscape in several fundamental ways, such as indirectly influencing sexual selection by altering access to resources (Winkler et al., 2023). In denser populations, competition for resources, such as food or nesting sites, can increase, which then ultimately affects the strength and direction of sexual selection (Cattelan et al., 2020; Gwynne, 1990). Therefore, in high-density populations, males may face intense selection for traits that allow them to effectively compete for limited resources, in addition to direct competition for mates. Moreover, population density also influences the frequency and nature of interactions between individuals of the same species (Winkler et al., 2023; Kokko & Rankin, 2006). As density increases, so does the probability of encounters with other members of the same species. The increased frequency of interactions can intensify competition for mates but also alter mate searching and mate choice dynamics (Kokko & Rankin, 2006). Higher densities can lead to increased mate sampling by females, as well as decreased monopolisation of mating opportunities by males due to a greater frequency of competitive interactions (Watts et al., 2022; Aronsen et al., 2013).

The effect of density on male-male competition is evident from observations across a range of species, including insects, fish, and mammals (Evans & Magurran, 2000; Grant & Kramer, 1992; Vanpé et al., 2008). Consequently, males face greater selective pressures to develop superior traits that improve their chances of mating and passing on their genes. Traits can manifest as elaborate mating displays, such as the exaggerated plumage in birds-of-paradise or the complex courtship rituals of bowerbirds (Ligon et al., 2018; Uy & Borgia, 2000). Additionally, more aggressive

behaviours can emerge, where direct contests or dominance hierarchies are more prevalent, as seen in red deer (Vanpé et al., 2008). Males may also invest more resources into sperm production, adjusting ejaculate quality, quantity, and composition in response to increased competition (Evans & Magurran, 2000; Gage, 1998).

Furthermore, denser populations amplify sperm competition. In these higher-density settings, females are more likely to mate with multiple males (Kokko & Rankin, 2006). Multiple mating leads to greater competition for fertilisation opportunities, which drives the selection of traits that enhance a male's ability to outcompete rival sperm (Gage, 1998). The link between population density and sperm competition illustrates the interconnectivity between demography and sexual selection, where the environment shapes the dynamics of mating and fertilisation**.** While the theoretical underpinning of these processes is strong, there are relatively few studies directly exploring the effect of population density on sexual selection. Despite the strong predictions for the influence of population density on sexual selection, this effect is often overlooked in experimental studies. Population density strongly influences sexual selection, accelerating the evolution of traits linked to reproductive competition.

Reproductive Competition: A Powerful Evolutionary Force

A central theme in reproductive biology is the evolutionary process of sperm competition, where sperm from multiple males compete to fertilise a single female’s ovum. (Parker, 1970). This phenomenon is prominent in polyandrous species, where females mate with several males during one or multiple reproductive cycles (Birkhead & Møller, 1998). Sperm competition acts as a strong selective pressure,

shaping various male reproductive traits and strategies aimed at increasing fertilisation success (Simmons, 2001; Snook, 2005). These adaptations are diverse, reflecting the intricate nature of this competition, and include variations in sperm quantity, morphology, ejaculate composition, and mating behaviour (Simmons, 2001; Snook, 2005).

Behavioural and Physiological Strategies

Males have evolved a range of behavioural and physiological strategies to maximise their fertilisation success in the face of reproductive competition. The specific strategies favoured often depend on the nature of this competitive pressure, particularly the patterns of sperm precedence, which dictate the relative fertilisation success of sperm from different males (Simmons, 2001). Last-male sperm precedence, where the last male to mate fathers a greater proportion of offspring, is a common pattern in many species (Birkhead & Møller, 1998). This pattern selects for strategies that ensure a male's sperm is the last to enter the female's reproductive tract. A prevalent strategy under last-male precedence is mate guarding, where males actively prevent rival males from accessing the female after copulation (Moller & Birkhead, 1993). This behaviour is particularly common in birds and insects, where males may remain with the female for extended periods, physically blocking other suitors. Males also engage in frequent copulation to maximise the likelihood that their sperm is the most recently deposited (Simmons, 2001). This can be seen as a form of "defensive" mate guarding, ensuring that any subsequent matings by rivals are less likely to displace the male's sperm (Simmons, 2001). In species of damselflies, males have evolved specialised structures to actively remove or displace the sperm of previous males from the female’s

reproductive tract before transferring their own (Siva-Jothy & Tsubaki, 1989). This behaviour can be highly effective in ensuring paternity under last-male precedence.

In contrast to last-male precedence, first-male sperm precedence favours males who are the first to mate with a female. This pattern is often found in species where females store sperm for extended periods or where the first sperm to reach the eggs has a significant advantage in fertilisation (Simmons, 2001). Under first-male precedence, selection strongly favours males who are adept at locating and inseminating virgin females quickly. This can involve the evolution of enhanced sensory capabilities to detect female pheromones or other cues (Herberstein et al., 2017). Males may exhibit protandry, emerging earlier in the breeding season than females to gain a temporal advantage in the race to find receptive mates (Herberstein et al., 2017). Once a female is located, rapid copulation is crucial, and sperm may be adapted to resist displacement by subsequent males, helping to preserve the first-male advantage. This can be achieved through specialised structures that anchor sperm within the female's reproductive tract or chemical components in the seminal fluid that inhibit the movement or viability of rival sperm (Simmons, 2001). Adaptations that allow sperm to quickly reach and fertilise eggs, such as increased motility or enhanced ability to penetrate the egg's outer layers, would be highly advantageous under first-male precedence.

Where sperm mixing or incomplete precedence occurs, males may focus investment into sperm quality, and quantity or even employ chemical strategies through seminal fluid to incapacitate rival sperm or manipulate female behaviour (Firman, 2018). This often occurs in species where females mate with multiple males and the sperm of various males' mixes within the reproductive tract (Poiani, 2006; Rhodes et al., 2011; Tofilski et al., 2012).

Morphological Adaptations to Sperm Competition

The risk and intensity of sperm competition can drive the evolution of specific morphological adaptations (Pitnick, 1996). Males in populations experiencing intense sperm competition tend to develop larger testes, allowing the production of greater numbers of sperm. This suggests that the capacity to adjust testes size in response to competition is an evolved trait. Additionally, it is often correlated with the production of larger ejaculates, as observed in guppies (*Poecilia reticulata*) (Evans & Magurran, 2000; Parker, 1982). Beyond simply increasing sperm numbers, sperm competition can also drive changes in sperm morphology. For example, some *Drosophila* species produce elongated sperm under competitive conditions (Pitnick, 1996). Longer sperm may have an advantage in reaching and fertilising the egg first, or in displacing previously stored sperm. Other morphological adaptations include changes to sperm structure, such as the development of more streamlined sperm in particular bird species, that have higher mitochondrial content to increase swimming speed and efficiency (Humphries et al., 2008). Although these examples highlight general patterns of morphological adaptation in response to sperm competition, the precise mechanisms that underpin these trends remain an area of active research.

The diverse strategies employed by males highlight the variable nature of sperm competition. Some strategies, such as producing larger ejaculates or increasing mating frequency, represent a “numbers game” by simply outnumbering the sperm of competitors. Contrastingly, other strategies prioritise sperm quality over quantity. Males may invest instead in sperm that are more mobile, longer lived, or more capable of penetrating the egg (Fitzpatrick et al., 2009; Gage, 1998).

Sperm Competition in the Context of a Superorganism

Species utilise different strategies to maximise reproductive success. Sperm competition is a multifaceted process where diverse adaptations are influenced by sexual selection. The intensity of this competition is influenced by various factors, including mating frequency, the number of competing males, and female mating behaviour (Shackelford et al., 2005). While the influence of demographic factors on male-male competition and its associated evolutionary adaptations has been explored in various species, this relationship remains largely unstudied in the context of superorganisms.

Superorganisms, such as ants, termites, and some bees, exhibit a unique social structure where individuals function as interconnected parts of a larger, cohesive unit, with the reproductive division of labour (Canciani et al., 2019; Moritz & Southwick, 1992; Seeley, 1989b). This model suggests that superorganism colonies act as a unified unit of selection, analogous to a multicellular organism where individual cells function together for the organism's overall success, and the colony's overall success takes priority over the reproductive success of individual members (Page & Metcalf, 1984).

Within a superorganism, we can consider the reproductive individuals as representing the germline, while the non-reproductive individuals represent the soma (Boomsma et al., 2014). This framework allows us to explore the concept of sperm competition at two levels: the level of the individual male's sperm and the level of the colony's "sperm" (i.e., the drones it produces). Furthermore, this leads to an important question: if the colony functions as a single organism, can drones be

viewed as its flying gametes, and therefore would we expect to see sperm competition dynamics reflected in the production and traits of drones?

To address this gap, we can use the western honey bee (*Apis mellifera*) as a model superorganism. The honey bee provides a valuable system for investigating these complex social dynamics due to several practical advantages. First, First, the long-standing history of apiculture and scientific research on honey bees provides a strong foundation for studying their biology and behaviour (Canciani et al., 2019; Gates, 1914; Phillips & Demuth, 1914; Tarpy, 2024). Second, the reproductive biology of honey bees, particularly the mating dynamics at drone congregation areas, is relatively well-characterised (Baudry et al., 1998; Brutscher et al., 2019; Jaffé et al., 2009; Moškrič et al., 2020). Third, the ability to manipulate honey bee colony population densities through the transport and management of hives provides a unique opportunity to experimentally investigate the effects of density on reproductive competition. Finally, understanding how high-density management scenarios, such as those encountered during commercial pollination and honey production, affect colony physiology and reproductive success has practical relevance for beekeeping and optimising pollination services.

Within this framework, honey bee colonies are characterised by a hierarchical structure. Non-reproductive female workers undertake tasks related to foraging, nest maintenance, and brood rearing (Moritz & Southwick, 1992; Seeley, 1989b). Male drones are solely dedicated to reproduction, while the queen's principal role is to lay both fertilised (diploid) eggs, which develop into queens or workers, and unfertilised (haploid) eggs, which develop into drones (Slater et al., 2021). Approximately five days after emerging, queens will embark on mating flights, typically undertaking one to five such flights over a week (Koeniger et al., 2014; Seeley, 1989).

Characteristically polyandrous, a single queen will mate with numerous drones. Recent research suggests that the number of drone matings may be significantly higher than previously thought, with estimates averaging 54.5 mates and ranging up to over 90, based on the discovery of cryptic royal subfamilies that are not represented in worker offspring (Withrow & Tarpy, 2018). While earlier estimates based on worker paternity analyses suggested an average of around 12-20 matings, these likely underestimated the true extent of polyandry (Winston, 1991). The sperm obtained from these matings is then stored in the queen's spermatheca and must suffice for the duration of her life (Rhodes et al., 2011; Shafir et al., 2009; Tofilski et al., 2012).

This complex social organisation greatly influences the dynamics of sperm competition. Drones from neighbouring colonies compete for opportunities to mate with queens (Jasper et al., 2020; Shafir et al., 2009; Tofilski et al., 2012). Drones achieve this by gathering in drone congregation areas (DCAs), specific aerial locations where drones from many colonies congregate, sometimes in the thousands, waiting for the arrival of virgin queens. While the general location of DCAs are known to remain consistent, the stability of a DCA on any given day, in terms of drone numbers, can vary, and the precise mechanisms that regulate daily DCA formation and drone attendance are not fully understood (Ruttner, 1966). Drones, however, do need to return to their colonies periodically, presumably to be fed, approximately every 20-30 minutes (Koeniger et al., 2005). This need to refuel likely imposes a selective pressure to attend DCAs that are relatively close to their home colony, minimising travel time and maximising the time spent at the DCA, increasing their odds of encountering a queen (Koeniger et al., 2005).

Within the DCA, drones are generally dispersed until a queen arrives. The presence of a queen is initially detected through her pheromones, which trigger a rapid and dramatic change in drone behaviour (Page, 1986; Tofilski et al., 2012). Drones then use visual cues to orient towards the queen, forming a characteristic "comet" of hundreds of drones chasing her from below and behind (Bastin et al., 2017). Competition at this stage is primarily a numbers game, with drones relying on their flight speed and agility to increase their chances of reaching the queen (Loper et al., 1992). Interestingly, there is a remarkable lack of direct physical contact or fighting between drones as they compete to mate, suggesting an evolutionary strategy that prioritises speed and positioning over aggression (Rangel & Fisher, 2019). Furthermore, the presence of the "mating sign" (the endophallus of the previously mated drone) on the queen appears to enhance her attractiveness to other drones, potentially increasing the intensity of competition (Baer, 2005; Zhao et al., 2021).

While queens do have the ability to reject further mating attempts, the specific circumstances and reasons why a queen might be present at a DCA but not receptive to mating are not fully understood. Research using tethered queens flown at DCAs has shown that queens are not always receptive to mating, but the factors influencing their receptivity in natural settings require further investigation (Jacobson, 2012). Once a drone successfully mates with a queen, they do so in flight. The drone will transfer its sperm via its endophallus (Figure 1-1) (Slater et al., 2021). Eversion of the endophallus during mating is fatal for the drone, and the endophallus itself detaches and remains in the queen, rendering the male obligately monogynous (i.e., single male mating) (Slater et al., 2021). The sperm is then subject to mixing in the queen’s oviducts as they contract (Page, 1986; Tofilski et al., 2012). However, only a small portion of the total sperm in the queen’s spermatozoa is stored for ova

fertilisation, the rest is expelled through the sting chamber (Liberti et al., 2019; Tofilski et al., 2012).

By framing drones as the colony's 'sperm', we can investigate how the colony might adapt its reproductive strategy in response to varying levels of competition (Baer, 2005; Boomsma et al., 2005). This includes examining both the abundance of drones produced by the colony and the individual traits of those drones, such as sperm quality and quantity (Rangel & Fisher, 2019). For example, in populations with high competition (i.e., high population density or male-biased sex ratio), the colony may invest in producing more drones or in enhancing drone sperm quality or quantity to increase its chances of successfully mating with queens. By addressing the colony's response to male-male competition at both the population and individual drone levels, we can gain a deeper understanding of how the colony's reproductive strategy, as a superorganism, interacts with individual drone biology. This perspective highlights the link between demography and reproduction in honey bees, suggesting new avenues of research into how colonies adapt to varying levels of competition.

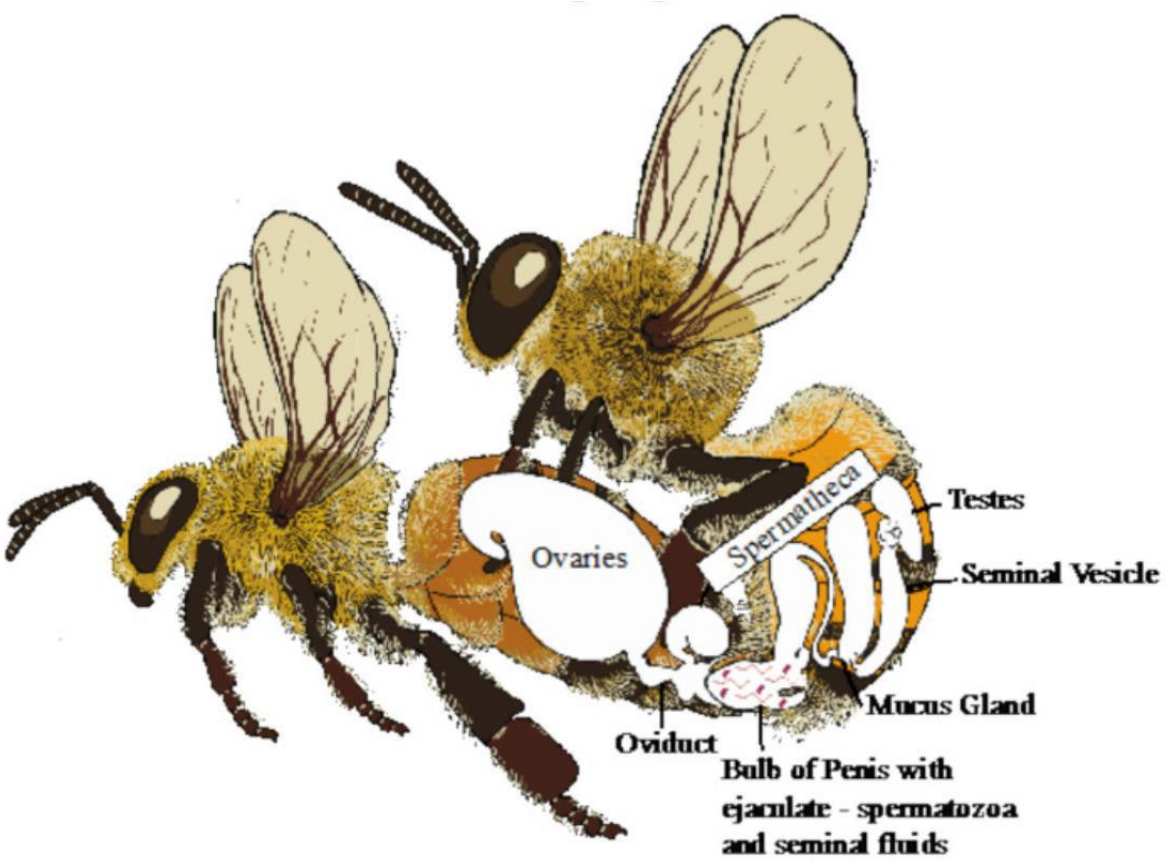

Figure 1-1. During mating, the male drone deposits spermatozoa and seminal fluids into the queen's oviduct, as illustrated in this depiction of the mating process (Slater et al., 2021).

Honey Bee Sperm Morphology

Sperm Polymorphism

Honey bee sperm competition involves a range of mechanisms that operate at the molecular and cellular levels. First, the phenomenon of sperm polymorphism, where two distinct subpopulations exist within the drone ejaculate, presents a crucial starting point (Tofilski et al., 2012). Tofilski et al. (2012) identified two distinct populations of live spermatozoa in honey bee drone semen. They also noted that proteins present in the seminal fluid of the non-viable sperm tended to coagulate when exposed to the spermatozoa of another drone. This suggests a potential

offensive mechanism for sperm competition, where one subpopulation of sperm, or its associated seminal fluid, may be allocated to incapacitating or eliminating the sperm of rival males. It is plausible that different sperm morphs are selected for different roles. These roles could include directly competing for fertilisation or having alternative functions like blocking or eliminating rival sperm through their interactions with seminal fluid. Such a mechanism would align with observations in other species, where certain sperm morphs are specialised for competitive functions rather than fertilisation. For example, in the sawfly parasitic wasp (*Dahlbominus fuscipennis*), sperm morphs have been shown to exhibit differential fertilisation capabilities. These morphs may also influence the sex ratio of the offspring by differentially fertilising or blocking access to the egg (Lee & Wilkes, 1965; Swallow & Wilkinson, 2002). The dextral and sinistral sperm coils observed in the sawfly parasitic wasp serve as evidence that different sperm morphs may have evolved specific functions (Figure 1-2). Furthermore, this mechanism is similar to that seen in *Drosophila melanogaster*, where seminal fluid proteins can incapacitate rival sperm, thus increasing the chance of fertilisation by a particular male (Harshman & Prout, 1994). Further research should focus on confirming the role of the two subpopulations, but also on the genetics of these differences and what trade-offs exist between them. This could involve using advanced imaging techniques to visualise their interactions within the reproductive tract of the queen. Alongside these methods, molecular techniques can be utilised to identify genetic and proteomic differences that are responsible for the observed distinct morphological forms.

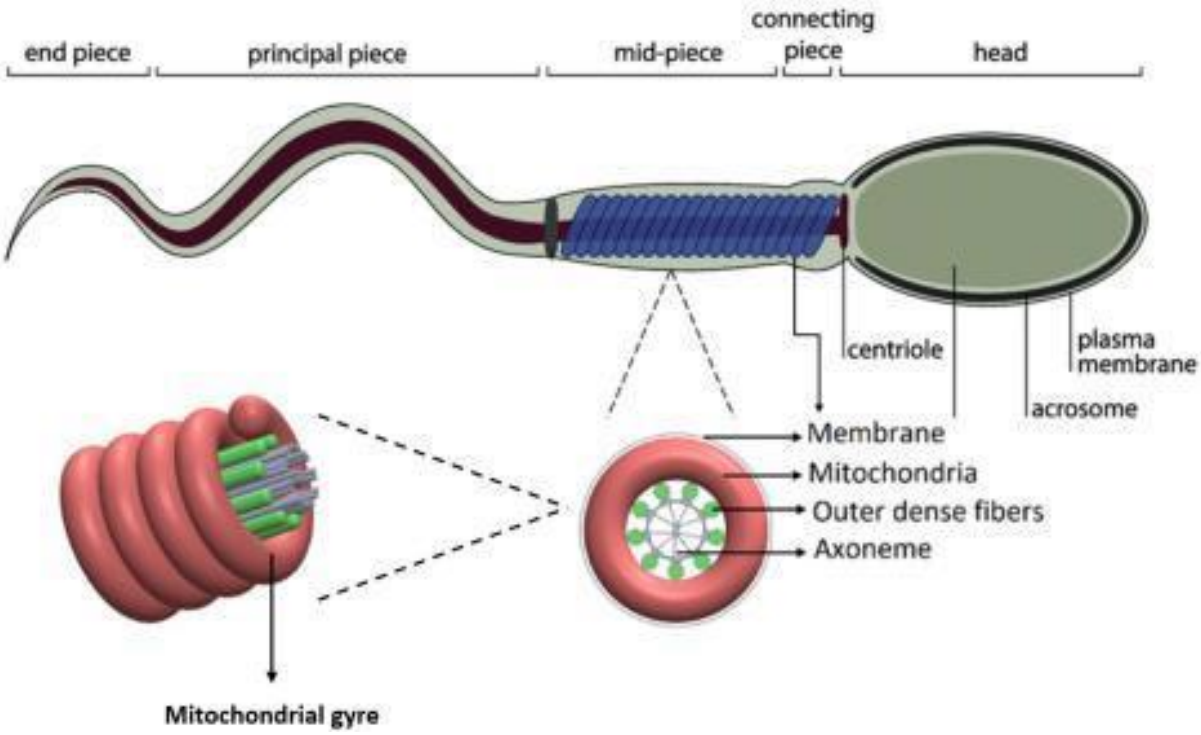

Figure 1-2. A detailed view of a sperm cell, including a cross-section of the mid-piece. The mid-piece encompasses a mitochondrial sheath, a helical formation that encloses the outer dense fibres and axoneme. A three-dimensional representation of the mid-piece is shown to demonstrate the mitochondrial gyres—the spiralling structures formed by the mitochondrial sheath (Bracke et al., 2017; Nassir et al., 2022).

Quality Over Quantity?

Beyond the quantity of sperm produced, both sperm quality and ejaculate volume are important determinants of fertilisation success (Baer, 2005; Bratu et al., 2022; Gençer & Kahya, 2020; Metz & Tarpy, 2022). Drones with a higher proportion of viable sperm and fewer morphological abnormalities, such as defects to the head, acrosome, or tail, are better equipped to navigate the female reproductive tract and successfully fertilise the queen's eggs (Bratu et al., 2022; Rhodes et al., 2011). These structural components are critical for the overall functionality of the sperm. The head, which contains the genetic material, must be properly formed to enable

penetration of the egg; the acrosome must have the necessary enzymes for fertilisation; and the tail's integrity directly impacts motility. Furthermore, the structural integrity of the mitochondrial gyres, which surround the midpiece, is also essential for energy production and proper sperm motility (Bracke et al., 2017; Nassir et al., 2022). Any structural or biochemical abnormalities in these areas are likely to compromise a sperm’s ability to reach the egg and be successful at fertilisation. This delicate balance is further emphasised by the high concentration of sperm within ejaculates produced by honey bee drones. Evolutionary pressures have resulted in a strategy of producing abundant and highly concentrated sperm, which may compensate for any sperm loss within the queen’s reproductive tract (Bratu et al., 2022; Gençer & Kahya, 2020; Metz & Tarpy, 2022). Furthermore, because drones only mate once in their lifetime, there is likely a strong selection to maximise the transfer of sperm during that single mating opportunity. This “all-in” reproductive strategy further reinforces the importance of ejaculate size and sperm concentration. Unlike in other species, where sperm dilution can be a potential issue, honey bees seem to have developed a strategy to ensure a large quantity of sperm is available for fertilisation. As Calhim et al. (2007) highlight, it is not just the number of sperm, but also their quality that is important. Therefore, drones must strike a balance between the number of sperm they produce and the quality of those sperm to maximise their chances of reproductive success. This complex interaction emphasises the delicate evolutionary balance between sperm quality and quantity and underscores the need for drones to optimise sperm output and maximise their fitness while maintaining sufficient levels of sperm viability.

Population Density: A Driver of Competition and Reproductive Strategies

In the context of honey bees, population density refers to the number of colonies within a given area, encompassing both managed apiaries and feral colonies. This density, regardless of whether it's concentrated in a few large apiaries or dispersed across many smaller ones, exerts a significant influence on colony dynamics and reproductive strategies (Lee & Winston, 1987). It acts as an environmental pressure, dictating the intensity of competition for resources, space, and mating opportunities. From the perspective of the honey bee colony, viewed as a superorganism, the primary goal is to maximise reproductive fitness. Therefore, colonies could be predicted to respond to the level of competition imposed by surrounding colonies. In high-density settings, this response could manifest through colonies producing more drones, thereby increasing the number of individuals available to compete in mating events (Rangel & Fisher, 2019; Seeley & Mikheyev, 2003). However, this increased drone production could also lead to heightened intra-colony competition for resources, particularly food, potentially influencing their development and raising stress levels. The need to reallocate colony resources to support this increased drone production poses a significant challenge, particularly as high-density settings can also intensify foraging pressure and lead to more rapid resource depletion (Boes, 2010; Seeley & Mikheyev, 2003; Smith et al., 2015). The balance between increased drone output and energetic costs remains a critical question. It remains unclear what additional strategies colonies may employ under high-density conditions. The way that colonies allocate their resources (especially in relation to worker bees and honey production) in the face of intense competition is also important and requires further investigation. The specific trade-offs and

consequences of high-density environments require further research to fully grasp how colonies optimise their reproductive strategies under density-driven competition.

As previously discussed, drones, acting as the 'sperm' of the superorganism, play a crucial role in the colony's reproductive success (Baer, 2005; Boomsma et al., 2005). The parallel between drones and sperm becomes clear when we consider their roles in competition: just as sperm from individual males compete to fertilise an egg, drones from individual colonies compete to mate with a queen. This dynamic leads to a different manifestation of sperm competition at the individual drone level compared to the colony-level adaptations. As competition intensifies, particularly in high-density apiaries, individual drones are under greater pressure to invest in sperm quality and quantity to outcompete rivals (Gage, 1998). This mirrors patterns observed in many non-eusocial animals, where increased density results in intensified competition for mates and resources (Bretman et al., 2013; Evans & Magurran, 2000; Grant & Kramer, 1992; Vanpé et al., 2008). Therefore, it is reasonable to expect a similar response in honey bees, with high apiary density leading to intensified resource competition within the colony and a heightened intensity of sperm competition among drones. In fact, apiary density can be seen as a critical demographic factor that directly shapes the competitive landscape and evolutionary pressures experienced by both individual drones and the colony as a whole. This highlights the need for research exploring how drones perceive levels of competition and how their behavioural and physiological responses are directly impacted by environmental factors like apiary density. The question that remains is how will honey bees balance the trade-offs that they are facing when they are surrounded by a high number of competing colonies? Furthermore, how might this affect the overall health, productivity, and long-term viability of the colony?

A Molecular Marker of Stress in Competitive Environments

The physiological consequences of intensified competition and abiotic stressors on individual drones, specifically in the context of varying apiary densities remains poorly understood. Especially when considering the potential for long-term stress to impair cellular mechanisms, including the essential stress response pathways (Morimoto, 1998).

For organisms to maintain cellular homeostasis under duress they must rely on several stress response mechanisms. One of the most important mechanisms involves the upregulation of heat shock genes, which encode heat shock proteins (HSPs) (Morimoto, 1993). Heat shock proteins (HSPs), particularly HSP70, are central to cellular homeostasis. They act as molecular chaperones that respond to stressful conditions (King & MacRae, 2015; Mayer & Bukau, 2005). These assist in protein folding, preventing aggregation, and facilitating the degradation of damaged proteins (King & MacRae, 2015). HSP70 is a highly conserved protein and is a critical part of the cellular stress response pathway across all living organisms. Additionally, HSP70 plays a key role in repairing damaged proteins, enabling cells to withstand environmental stressors relevant to apiary conditions, including thermal stress, starvation, pesticide exposure, and pathogen load (Mayer & Bukau, 2005). Therefore, it is a crucial factor in maintaining cellular integrity under duress.

Initially discovered in response to heat stress, it is now well-established that HSPs, including the extensively studied heat shock protein 70 (HSP70), are upregulated in response to a wide array of stressors such as starvation, toxins, oxygen deprivation, and crucially, competition for resources (Feder & Hofmann, 1999; King & MacRae, 2015). HSP70 expression responds rapidly to stressors, with concentrations increasing within minutes of exposure, making it a more responsive indicator than

other cellular markers like oxidative regulation or DNA damage (Beckham et al., 2008; Dhama et al., 2019; Farcy et al., 2009; Moreira-de-Sousa et al., 2018; Mosser et al., 2000). Studies on cell lines have also demonstrated that HSP70 expression is proportional to the toxicity level of stressors (Gibney et al., 2001). Furthermore, the signalling pathways that regulate HSP70 expression during stress responses are highly conserved across diverse taxa, including vertebrates, invertebrates, and single-celled organisms. This highlights the fundamental importance of this protein in maintaining cellular integrity (Morimoto, 1998).

In honey bees, the significance of HSP70 is increasingly recognised. Studies have shown its involvement in thermotolerance, immune response, pesticide exposure, parasites and bacterial infections (Abou-Shaara, 2024; Al-Ghzawi et al., 2022; Dubovskiy et al., 2013; King & MacRae, 2015; Y. Li et al., 2017; McMenamin et al., 2020; Scharlaken et al., 2008; Shi et al., 2024; Wojda et al., 2009). Furthermore, research on other insect species indicates that food limitation, a direct consequence of resource competition, can induce physiological stress and influence HSP expression (Chen et al., 2018; Wang et al., 2007)**.** The carob moth (*Ectomyelois ceratoniae*), for example, during caloric restriction has been shown to modulate the expression of various HSPs, including HSP70, suggesting a link between resource availability and the cellular stress response (Farahani et al., 2020). While direct evidence specifically linking competition for resources to HSP70 upregulation in honey bee drones is still emerging, these findings in other insects, combined with the known sensitivity of HSP70 to a wide range of stressors, suggest that resource competition in high-density apiaries could indeed be a significant factor influencing drone physiology and HSP70 levels. However, most research has centred on worker bees, leaving a significant gap in our understanding of HSP70's role in drones

(Figure 1-3) (McAfee et al., 2022). Given the drone's importance in passing on their queen's genes, understanding how apiary density and colony population density affect their physiological condition is important (Slater et al., 2021).

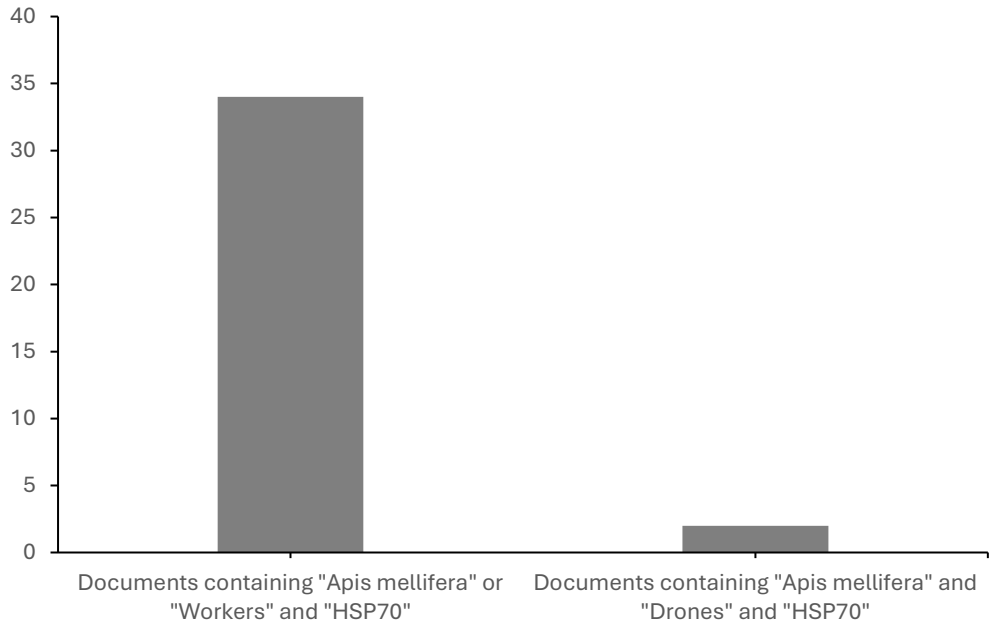

Figure 1-3. A bar graph comparing the number of publications identified in Scopus between 1999 and 2025 for two distinct search terms. The bar containing the terms "*Apis mellifera*" and/or "worker" and "HSP70," represents 34 publications (Scopus, n.d.-b). This indicates a focus either generally on honey bees or honey bee workers. The bar representing only two publications contains the terms "*Apis mellifera*," "drone," and "HSP70". This highlights a significant gap in research on drones and HSP70 (Scopus, n.d.-a).

Thesis Aims

In this thesis, I aim to investigate the effects of apiary density on honey bee (*Apis mellifera*) reproductive strategies and drone physiology, viewed through the lens of the superorganism concept. To do this, I examine how colonies and individual drones respond to varying levels of competition, as facilitated by apiary density. This research contributes to a deeper understanding of the complex interplay between

demography, reproductive investment, and physiological trade-offs in honey bees. This research explores the practical application of these findings for apiary management and honey bee conservation.

In **Chapter Two** I present an empirical study investigating the effects of apiary density on reproductive investment. Using a field study design, I compare colonies across low, medium, and high-density apiaries. At the colony level, I test the hypothesis that high-density settings will result in increased drone brood production, measured through drone comb and brood area. At the individual level, I analyse sperm quantity and quality in drones from each density treatment. I test the hypothesis that drones from high-density apiaries will have higher total sperm counts but also explore the potential trade-off between sperm quantity and quality by examining the proportion of viable sperm.

In **Chapter Three**, I investigate the impact of apiary density on drone stress responses by quantifying HSP70 expression. Drones are sampled from each density treatment and analysed using an ELISA kit. I test the hypothesis that drones from high-density apiaries will exhibit higher HSP70 levels, indicating elevated physiological stress. The results are interpreted in the context of insect stress responses and their implications for drone health and reproductive potential.

In **Chapter Four**, I synthesise my research findings, discussing the implications for our understanding of honey bee reproductive biology in the context of the superorganism concept and reproductive competition theory. I evaluate the support for the initial hypotheses, address the study's limitations, and propose avenues for future research. Finally, I consider the real-world implications of this research for

apiary management and honey bee conservation, emphasising how these findings can contribute to more sustainable beekeeping practices.

CHAPTER 2

High-Density Apiaries Drive Drone Production and Sperm Counts, but Reduce Worker Comb

Abstract

Honey bee (*Apis mellifera*) colonies function as superorganisms where reproductive competition increases is predicted to increase with higher apiary densities. This increased competition may drive adaptive reproductive strategies, including changes in sperm characteristics within individual drones, and adjustments in drone production at the colony level. Our study investigates the reproductive strategies employed by honey bees in high-, medium-, and low-density apiaries, particularly focusing on drone production and their sperm characteristics. We hypothesised that honey bee colonies respond to increased reproductive competition, driven by higher apiary density, through both colony-level and individual-level adaptations. At the colony level, we predicted increased drone production. At the individual level, we predicted both higher total sperm counts and a greater proportion of viable sperm in drones from high-density apiaries compared to those from low-density apiaries. We set up three levels of apiary density (low, medium, and high). To assess colony-level resource allocation we measured drone comb and drone brood production across three months, whereas worker comb production was measured across two months. We also collected sexually mature drones from colonies in each density treatment to determine sperm quantity and viability. Colonies in high-density apiaries exhibited increased drone brood production and significantly higher total sperm counts compared to those in low-density apiaries. However, worker comb production was reduced in high-density apiaries, suggesting a trade-off between reproduction and colony maintenance. Furthermore, sperm viability varied across densities and seasons, with low-density treatments having a higher proportion of viable sperm in early summer and medium-density treatments showing higher viability in late summer. Our findings suggest that colonies in high-density environments face

intensified sperm competition, which they respond to with increased drone and sperm production. However, it is important to note that these observations are based on unreplicated apiary densities and a truncated data collection period that missed the early stages of drone production. Moreover, the observed variability in sperm viability, coupled with reduced worker comb production, suggests that this increased reproductive effort could come at the expense of overall colony function. This highlights the intricate relationship between environmental pressures, particularly apiary density, and resource allocation within honey bee superorganisms, impacting both reproductive output and colony maintenance.

Introduction

Mating systems, which describe the patterns of mating between males and females within a population, play a fundamental role in shaping reproductive strategies and the evolution of sexual traits (Boomsma et al., 2005; Kokko et al., 2014). Monogamous and polygamous mating systems impose different selective pressures on reproductive traits and strategies. Polygamy can be further divided into polygyny (one male mating with multiple females) and polyandry (one female mating with multiple males). The prevalence of each mating system within a species is influenced by various ecological and social factors, including resource distribution, parental care requirements, and the operational sex ratio (the ratio of sexually receptive males to females) (Boomsma et al., 2005; Safonkin, 2011).

A direct consequence of polyandry is the post-mating mechanism of sperm competition. This occurs when the sperm from two or more males compete to fertilise the ovum of a single female (Parker, 1970). Sperm competition, therefore, is the

result of the female mating with multiple males and can occur within a single reproductive cycle or across multiple cycles. Subsequently, polyandry creates a scenario where sperm from multiple males coexist within the female reproductive tract, driving the evolution of diverse sperm adaptations to enhance the fertilisation success of any given male (Birkhead & Møller, 1998; Shackelford et al., 2005). These include variations in sperm number, sperm morphology, ejaculate composition, and mating behaviours (Simmons, 2001; Snook, 2005). For example, in species with high levels of sperm competition, males often produce larger ejaculates with more sperm (Parker, 1982). Alternatively, males may invest in producing higher quality sperm that are more mobile, longer lived, or better able to penetrate the egg (Fitzpatrick et al., 2009; Gage, 1998).

The intricate social structure of honey bee (*Apis mellifera*) colonies, often described as a "superorganism," relies on the coordinated efforts of individual bees to achieve the colony's collective reproductive success (Moritz & Southwick, 1992; Seeley, 1989b) Within this system, drones (male bees whose sole purpose is to mate with queens) play an essential role in colony-level reproduction (Gençer & Kahya, 2020; Tofilski et al., 2012). Honey bee queens are highly polyandrous, mating with multiple drones (54.5 on average) during their nuptial flights (Withrow & Tarpy, 2018). Meanwhile, drones, are monogynous and die after mating (Schlüns et al., 2005; Withrow & Tarpy, 2018). This mating system creates intense competition among drones, both for securing a mating opportunity with a queen and to have their sperm successfully fertilise her eggs (Baer, 2005).

Producing drones is resource intensive, requiring substantial investments of energy and food by the colony. These investments are influenced by environmental factors such as seasonality, resource availability, and colony size (Boes, 2010; Seeley &

Mikheyev, 2003; Smith et al., 2015). As a superorganism, a honey bee colony's reproductive success depends on its ability to strategically allocate these resources (Baer, 2005; Boomsma et al., 2005). At the individual level, honey bee drones compete through sperm competition to fertilise a queen's eggs. Unlike non-social insects where individual males compete directly, in honey bees, drones can be considered as the "sperm" of the superorganism, collectively representing the colony's reproductive output (Baer, 2005; Boomsma et al., 2005). This parallel is reinforced by the fact that drones are haploid, developing from unfertilised eggs. This means that drones within a colony are exceptionally closely related, much like sperm cells produced by a single diploid individual. Therefore, unlike many other systems, competition occurs not only at the level of individual drones, whose sperm compete directly within the queen's reproductive tract but alsThio at the colony level, where colonies effectively compete through the production of their drones.

Sperm quality is another critical component of reproductive success. In many species, males respond to sperm competition not only by increasing sperm numbers but also by modifying sperm quality (Gage, 1998). Baer et al. (2005) emphasised that sperm viability significantly impacts a drone's chances of fertilising eggs. Higher proportions of viable sperm confer a competitive advantage. Environmental factors, including nutrition and genetics, are known to influence sperm (Stürup et al., 2013; Zhao et al., 2021). However, the role of apiary density in shaping these dynamics remains unclear.

Our study used population density to refer to the number of honey bee colonies within a given area. High population density, therefore, implies a greater number of colonies coexisting and potentially competing for resources and mating opportunities within a defined spatial range. Comparable patterns have been observed in other

animals, where increased population density intensifies competition. In many animals, increased density often leads to intensified male-male competition for access to mates and resources (Bretman et al., 2013). For instance, in some fish species, high-density populations facilitate increased aggressive encounters between males and the establishment of dominance hierarchies and can also increase the intensity of sperm competition (Evans & Magurran, 2000). Across various taxa, from insects to mammals, studies have demonstrated that males adjust their ejaculate investment in response to the perceived density of male rivals. For example, male guppies (*Poecilia reticulata*) will alter their sperm allocation based on the number of surrounding males (Evans & Magurran, 2000). In the red deer (*Cervus elaphus*), increased population density is correlated with an increase in male reproductive skew, demonstrating the significant impact density can have on male mating success (Vanpé et al., 2008). These findings underscore the general principle that population density can be a potent driver of both behavioural and physiological responses related to competition.

Areas with high densities of honey bee colonies result in intensified reproductive competition due to a larger pool of drones competing for limited queen mating opportunities at nearby DCAs. Thereby, driving colonies to adjust their reproductive strategies by increasing their production of drones and/or optimising sperm quality in their drones to improve fertilisation success under competitive conditions.

Our study investigates the effects of apiary density on reproductive strategies at both the level of the colony (drone quantity) and the level of the drone (sperm quantity and quantity). Specifically, we examine whether colonies in high-density apiaries respond to reproductive competition intensity by producing more drones and whether drones from these environments exhibit higher sperm quantity and quality. At the

colony level, we predicted that colonies in high-density apiaries would produce a greater number of drones compared to those in lower-density apiaries**.** We predicted that drones from high-density apiaries would have a greater proportion of viable sperm relative to non-viable sperm, compared to lower density apiaries. Additionally, we expected that drones from high-density apiaries would exhibit higher total sperm counts compared to those from low-density apiaries. This dual focus applies the superorganism concept by treating drones as the colony's functional sperm.

Methods

Study Sites and Experimental Design

The experiment was conducted across three apiary sites located in Tauwhare (37°45'04"S 175°28'27"E), Matangi (37°48'26"S 175°23'45"E), and Ruakura (37°46'32"S 175°18'46"E), Aotearoa, New Zealand (Figure 2-1). These sites represented three different colony density conditions: low (Tauwhare, housed 8 colonies), medium (Matangi, housed 60–68 colonies), and high (Ruakura housed 120 colonies; Figure 2-2). All three apiaries were situated in rural or semirural areas characterised by a similar landscape dominated by pastures used for livestock grazing. Each site was approximately 10.6 km apart, with a minimum distance of 8.1 km between them. Due to the geographic proximity and similar landscape attributes of each apiary, it is unlikely that there were notable differences in the types of foraging resources available to colonies in each treatment. However, it is important to acknowledge that resource abundance may have varied to some degree between sites.

Eight ’focal colonies’ were monitored within each of the experimental apiaries. The focal colonies were comprised of European-derived honey bees (*Apis mellifera spp.*) and were housed in standard, 10-frame, Langstroth hive equipment consisting of a solid bottom board, a full-depth brood box, a queen excluder, and one medium-depth super above the queen excluder containing black plastic foundation. The focal colonies were chosen from forty colonies at Plant and Food Research, Ruakura. We assessed colony health and strength using the ‘number of frames’ (FOB) method (Nasr et al., 1990). This is a standard technique that estimates the adult bee population by counting the number of frames fully covered with bees. We selected twenty-four of the strongest colonies with FOB values between 7.5 and 8 were selected as the focal colonies. The 24 focal colonies were relocated to a neutral site without nearby managed colonies for 28 days (from 21st September to 17th October 2023) to allow a new generation of bees to develop in the absence of outside competition. Eight focal colonies were assigned to each of the three density treatment groups based on the initial assessment of drone and worker brood to ensure no noticeable differences between the groups.

Figure 2-1. Aerial map of the Waikato Region with the locations of the three apiary sites (Google Earth, n.d)

A

B

C

Figure 2-2. Visualisations of **(A)** the low-density apiary site (Tauwhare) with 8 colonies, **(B)** the medium-density apiary site (Matangi) with 60-68 colonies, **(C)** The high-density apiary site (Ruakura) with 120 colonies.

Colony Management and Monitoring

On 17th October 2023, cluster assessments were conducted on each focal colony to confirm that each colony was queen-right, all three brood stages (eggs, larvae, and pupae) were present, and we found no visible signs of disease. We applied Ecrotek Bayvarol© strips to all colonies to manage varroa mite (*Varroa destructor*) infections, following New Zealand's beekeeping guidelines.

On the morning of the 19th of October 2023, the three treatment apiaries were established and then we transported the focal colonies into their respective density treatment apiaries sites (Figure 2-1). Once in place at the treatment apiaries, the frame in position '8' of the bottom deep hive box was replaced with one blank wired frame and the frame in position '4' of the bottom deep hive box was replaced with one drone cell frame containing 1120 cm^2 of pre-established drone comb (Figure 2-3, 2-4). These frames were pre-drawn by non-focal colonies, after which the amount of existing drone comb was assessed and equalised across all frames. The standardised frames were then distributed to the focal colonies, allowing for consistent baseline conditions before further comb production was measured. The drone frames were made of plastic and had a cell size of 7.1 mm, notably larger than the standard worker cell size of 5.4 mm.

Focal colonies underwent weekly swarm checks from October 19th to 15th December 2023 then 15th January to 9th February 2024. We attended to colonies that required requeening and reestablished any colonies that had swarmed with nucleus colonies.

A

B

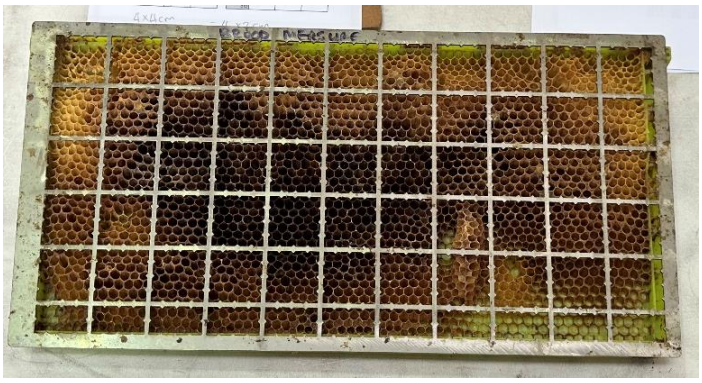

Figure 2-3. Visualisations of **(A)** the blank wired frames added to the focal colonies and **(B)** a drone cell frame containing 170cm^2 of pre-established drone comb.

Figure 2-4. The layout of the 10 frames in the hive box for each focal colony. Each frame is labelled from 1-10. The drone cell frame (with pre-established drone comb) was placed at position ‘4’. The blank frame was placed at position ‘8’.

Colony Data Collection

Colony assessments of drone production were conducted on the 10th of November 2023, 1st December 2023, and 15th January 2024. During each assessment, we recorded the following measurements: (1) capped drone brood (the total area of capped drone cells across all ten frames in the hive box), (2) drone comb (the area of comb dedicated to drone production on both the blank frame and the drone cell frame), and (3) worker comb (the area of comb used for worker brood and resource storage on the blank frame only). Each assessment was conducted in the field by removing frames from the hive and overlaying them with a brood grid during inspections. To calculate area, we used a brood grid the size of a deep frame. The grid was divided into 11 rows and 5 columns of 5 cm x 5 cm squares and one row of 5 cm x 2.5 cm rectangles. We overlaid the grid over a frame to measure comb and brood areas (Figure 2-5a). We then counted the number of full squares containing the relevant feature (e.g., capped drone brood). To convert the number of squares counted to cm^2, we multiplied the number of 5 cm x 5 cm squares by 25 (as each square represents 25 cm^2) and the number of 2.5 cm x 5 cm rectangles by 12.5 (as each rectangle represents 12.5 cm^2) for further analysis (Figure 2-5b). By measuring both drone and worker comb, we assessed how colonies proportionately allocated resources between reproduction and maintenance under different density conditions.

Drone Collection

To assess viable, non-viable, and total sperm counts, we collected drones from five randomly selected colonies at each apiary at two time points (4th to 12th December 2023 and February 5th to 7th, 2024). To ensure a representative sample, we collected a surplus of sexually mature drones from the front entrance of the hive while they

were departing for or returning from mating flights. Drones were then transported live to the laboratory in ventilated containers. From these, a minimum of five drones were randomly selected and analysed per colony at each sampling point.

Sperm Analyses

For each drone, we gently pressed the abdomen to extrude the endophallus, and the semen was removed using fine forceps (Collins & Donoghue, 1999). The semen was immediately transferred into a 1.5 mL microcentrifuge tube containing 20 µL of Beadle saline solution (128.3 mM NaCl, 4.7 mM KCl, and 23 mM $CaCl_2$) to preserve sperm viability (Thomas & Simmons, 2007). We then prepared the samples using 2 µL of reagents from the LIVE/DEAD® Sperm Viability Kit (Molecular Probes, Eugene, OR, USA), including SYBR 14 and propidium iodide. The sperm solution was mixed with an equal volume of 1:50 diluted SYBR-14 (1 mM) and incubated in the dark for 10 minutes. After incubation, 4 µL of 2.4 mM propidium iodide was added. We left the samples for an additional 10 minutes before removing 5 µL aliquot of each sample placing it on a clean microscope slide and covering it with a coverslip (Thomas & Simmons, 2007). This staining process differentiated live (green) and dead (red) sperm. Samples were examined under an Olympus BX53F2 microscope using Olympus cellSens Standard software. Sperm were viewed at 40x magnification with a blue excitation filter (λ = 510 nm). Six randomly selected fields per slide were then photographed using an Olympus D22 microscope camera.

A

B

Figure 2-5. Visualisation of **(A)** the brood grid used to calculate drone brood, drone comb and worker comb and **(B)** an example of researchers collecting data.

Automated Sperm Analysis

Live, dead and total sperm were manually counted on 200 images using a clicker counter. Those 200 manually annotated images were used to train an automated computer model to improve efficiency and consistency in the scoring of the total data set of over 900 images. The model used for sperm detection is a Faster R-CNN model using the ResNeXt-101 backbone and a feature pyramid network called Faster R-CNN X101-FPN. A generic pre-trained model was fine-tuned on our sperm images using our custom annotations, to adapt it to the task of sperm detection. Annotations were created using the Supervisely software platform, (Deep Systems, USA). Model training was performed using custom in-house software based on the Detectron2 framework. The model identified and counted viable, non-viable, and total sperm cells using a machine-learning algorithm to differentiate between the fluorescent signals of viable (green) and non-viable (red) sperm. Validation against the 200 images that we manually counted showed the model achieved 91.3% accuracy for non-viable sperm counts and 88.0% accuracy for viable sperm counts.

Statistical Analysis

Linear mixed-effects models were used to analyse the effects of apiary density and sampling date on drone brood, drone comb and worker comb production. Data were analysed separately for the drone cell frame, the blank frame, and their combined total. Models were fitted using the *lme4* package in R Version 4.0.0 (R Core Team 2024), with *Treatment* (apiary density) and *Date* as fixed effects, and *Colony* as a random effect to account for repeated measures within hives. Statistical significance was assessed at $p < 0.05$. Three outliers in the January data were identified, which represented three colonies – one from the medium-density treatment and two from the high-density treatment- that were found to be dead during health assessments. These colonies had no visible stages of brood present, suggesting no queen was present and were subsequently removed from any further analysis.

The initial model for each response variable included an interaction term between *Treatment* and *Date*. Model comparison was conducted using likelihood ratio tests to evaluate the necessity of the interaction term. Likelihood ratio tests (ANOVA) were performed to compare the full model with a reduced model excluding the interaction. Additional comparisons with simpler models (including models with only *Date* or *Treatment* as fixed effects) were conducted to identify the best-fitting model for each variable based on Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC).

Post hoc pairwise comparisons between treatment groups were performed using Tukey's method to adjust for multiple comparisons. The *emmeans* package was used to calculate estimated marginal means for the treatment groups, allowing further investigation of differences across apiary densities.

Non-parametric tests were used for sperm count data due to non-normal distributions and unequal variances. Data was analysed separately for the December 2023 and February 2024 sampling points. Kruskal-Wallis rank-sum tests were conducted to compare total sperm counts and the proportion of viable sperm across density treatments. Post hoc pairwise comparisons were performed using Dunn's test with Bonferroni corrections to adjust for multiple comparisons.

Results

Colony Management and Maintenance

During the initial setup of the experiment, some colonies required intervention to ensure their health and stability. Specifically, on October 17th, 2023, one colony from the medium-density apiary and one colony from the high-density apiary were found to be queenless. These colonies were immediately requeened, and the new queens were caged until October 19th, 2023, to allow for acceptance by the colony. Additionally, on October 20th, 2023, one colony from the low-density apiary was discovered to have swarmed. This colony was promptly combined with a nucleus colony to restore its population. No additional interventions were needed beyond routine weekly swarm checks for the remaining colonies. These checks were conducted from October 19th to December 15th, 2023, and then resumed from January 15th to February 9th, 2024.

Drone Brood

No significant interaction between *Treatment* and *Date* was found for drone brood production on drone cell frames ($\chi 2 = 3.445$, df = 4, $p = 0.486$; Figure 2-7a), and there was no effect of treatment ($\chi 2 = 14.10$, df = 2, $p > 0.05$) showing that drone

brood production on the drone cell frames was similar across all density levels tested. However, there was a significant effect of sampling date for drone brood production on the drone cell frames ($\chi2$= 3.94, df = 2, $p < 0.001$, Figure 2-6) with average drone brood production being highest in November (606 ± 65 cm²), and lowest in December (304 ± 75 cm²).

A model using the additive effects of *Treatment* ($\chi2 = 7.34$, df = 2, $p = 0.026$; Figure 2-7b) and *Date* ($\chi2 = 9.18$, df = 2, $p = 0.010$) had a significant impact of drone brood production on blank frames (AIC = 916.11). Post hoc tests revealed that the high-density treatment produced 381 ± 96 cm² more drone brood compared to the low-density treatment on the blank frames ($p = 0.012$). No significant differences were found between the medium and low-density treatments ($p > 0.05$) or between the high and medium-density treatments ($p > 0.05$). Regarding Date, there were only significant differences found between December 2023 and November 2023. In December less drone brood was produced on the blank frames compared to November.

The additive model indicated that *Treatment* ($\chi2 = 15.53$, df = 2, $p < 0.001$; Figure 2-7c) and *Date* ($\chi2 = 6.57$, df = 2, $p = 0.037$) had a significant effect on total drone brood production across combined frames (AIC = 1047.5). A lower AIC value indicates a more parsimonious model, balancing fit with simplicity. In November 2023, colonies produced significantly more drone brood compared to December 2023. Post hoc analysis showed a marginally significant difference between the high-density and low-density treatments. The high-density treatment produced 680 ± 232cm² more drone brood than the low-density treatment ($p = 0.006$). There were no significant differences between the medium and low treatments ($p > 0.05$) or between the high and medium treatments ($p > 0.05$).

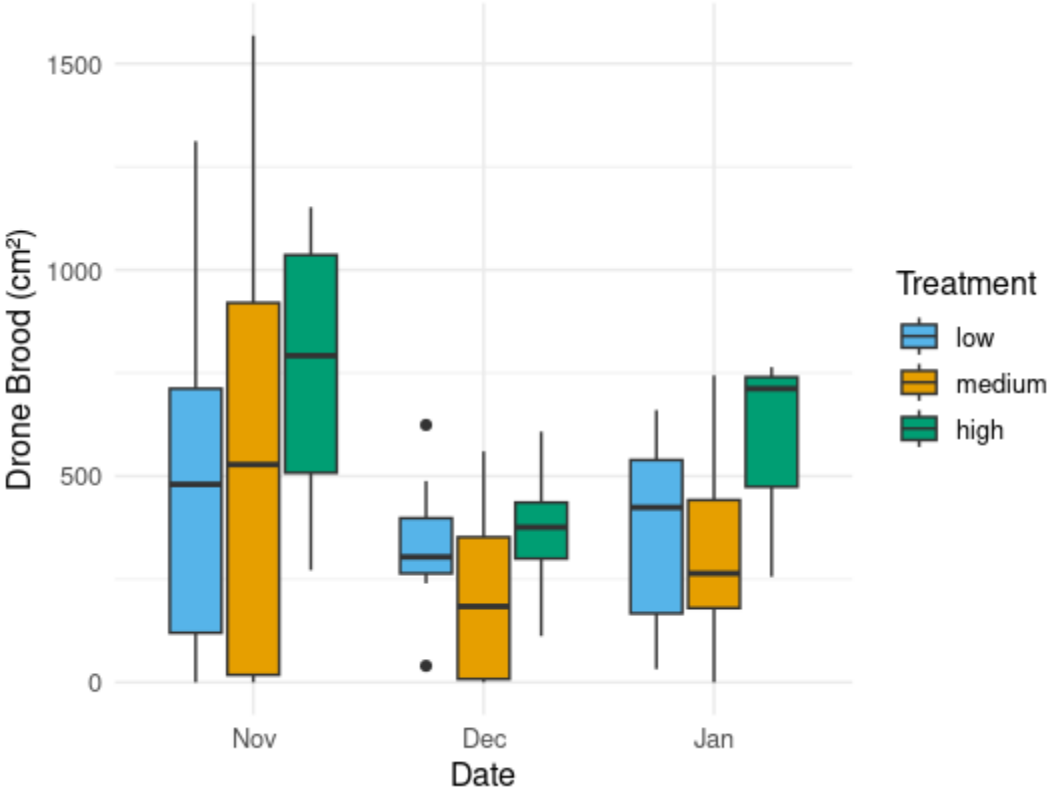

Figure 2-6: Effect of date across treatments for drone brood (cm²) produced on the drone cell frame. Box plots represent the distribution of drone brood for each treatment group (low, medium, and high) across three dates (November, December, and January). The central line within each box represents the median amount of drone comb, the edges of the box indicate the interquartile range (IQR), and the whiskers extend to 1.5 times the IQR. Outliers are displayed as individual points beyond the whiskers. The key indicates which coloured box plot represents each treatment.

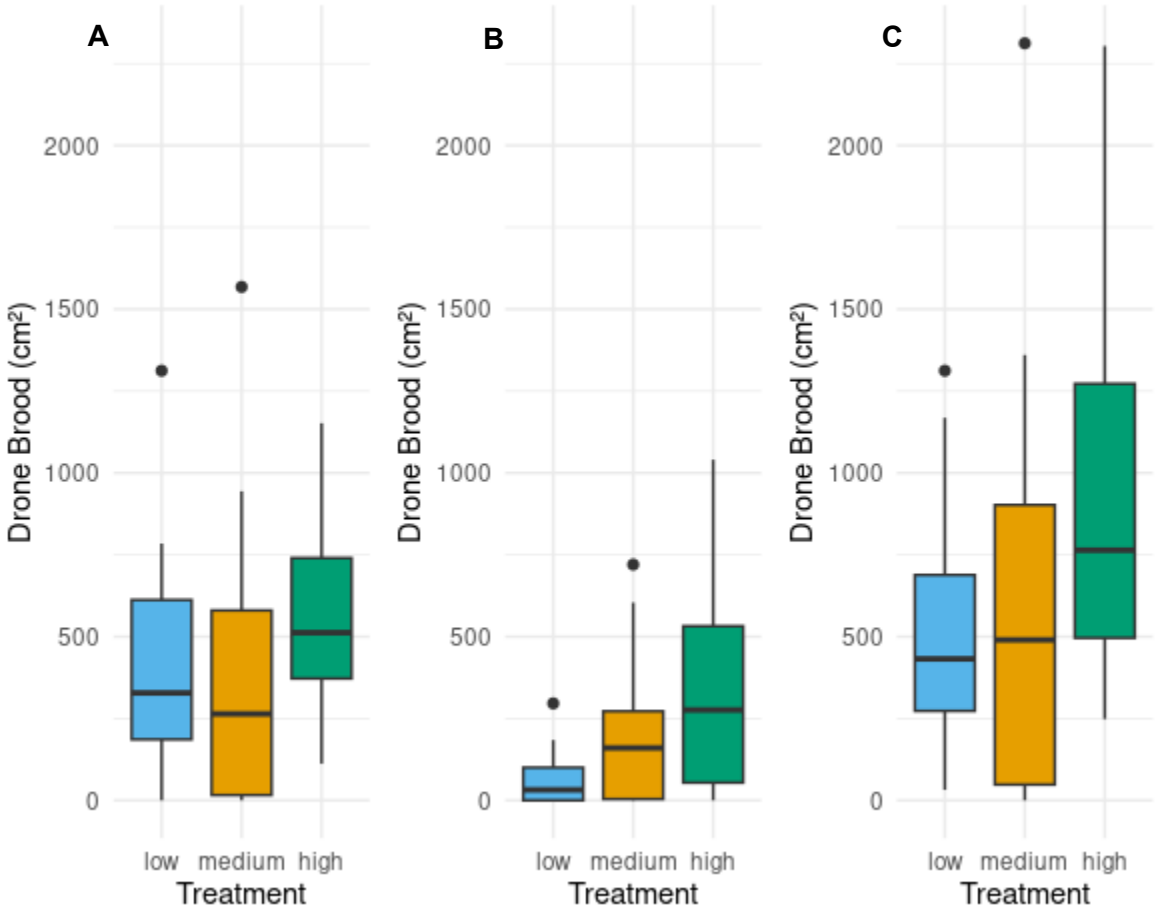

Figure 2-7. Effect of apiary density on drone brood production across different frame types. The panels show the amount of drone brood (cm^2) measured on **(A)** drone frames, **(B)** blank frames, and **(C)** combined frames across the three treatments. The central line within each box represents the median area of the drone brood, the edges of the box indicate the interquartile range (IQR), and the whiskers extend to 1.5 times the IQR. Outliers are displayed as individual points beyond the whiskers.

Drone Comb

Similar to drone brood production, no significant differences were found in drone comb production on the drone cell frames across the density treatments ($\chi 2 = 57.76$, df = 2, $p = 0.175$; Figure 2-9a). The interaction between *Treatment* and *Date* was not significant ($\chi 2 = 2.62$, df = 4, $p > 0.05$). Instead, drone comb production on the drone

cell frame varied significantly by *Date* ($\chi 2 = 3.48$, df = 2, $p < 0.001$; Figure 2-8), with colonies producing notably less drone comb in November than in either January or December.

However, *Treatment* ($\chi 2 = 12.73$, df = 2, $p = 0.0017$; Figure 2-9b) and *Date* ($\chi 2 = 2.34$, df = 2, $p < 0.001$) together as additive effects had a significant influence of drone comb production on blank frames (AIC = 1027.5). Colonies produced significantly fewer drone comb cells in January compared to November. Post hoc comparisons showed that the high-density treatment produced 802 ± 234 cm² more drone comb than the low-density treatment ($p < 0.001$). However, there were no significant differences in drone comb production between either the medium-density and the low-density treatment ($p > 0.05$) or the medium and high-density treatment ($p > 0.05$).

Additionally, the combined production of drone comb was significantly impacted by the additive effects of *Treatment* ($\chi 2 = 29.6$, df = 2, $p < 0.001$; Figure 2-9c) and *Date* ($\chi 2 = 12.95$, df = 2, $p = 0.0015$) (AIC = 1070.2). Colonies produced significantly more drone comb in December compared to November across the combined frames. Post hoc analysis indicated that the high-density treatment produced on average 856 ± 276 cm² more drone comb than the low-density treatment ($p = 0.0021$). There were no significant differences between the medium-density and low-density treatments ($p = 0.0823$) or the medium-density and high-density treatments ($p = 0.215$).

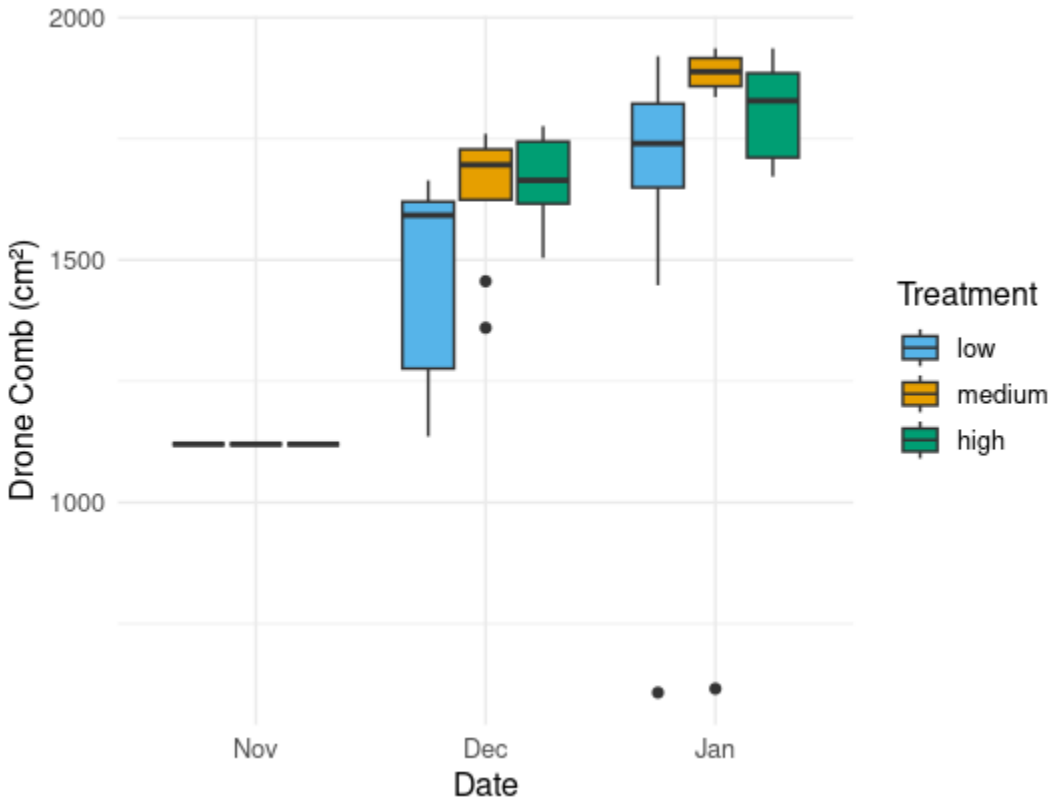

Figure 2-8. Effect of date across treatments on the amount of drone comb (cm²) on the drone cell frame. Box plots represent the distribution of drone comb for each treatment group (low, medium, and high) across three dates (November, December, and January). In November 2023 each focal colony began with 1120 cm² of pre-established drone comb on the drone frame. The central line within each box represents the median amount of drone comb, the edges of the box indicate the interquartile range (IQR), and the whiskers extend to 1.5 times the IQR. Outliers are displayed as individual points beyond the whiskers. The key indicates which coloured box plot represents each treatment.

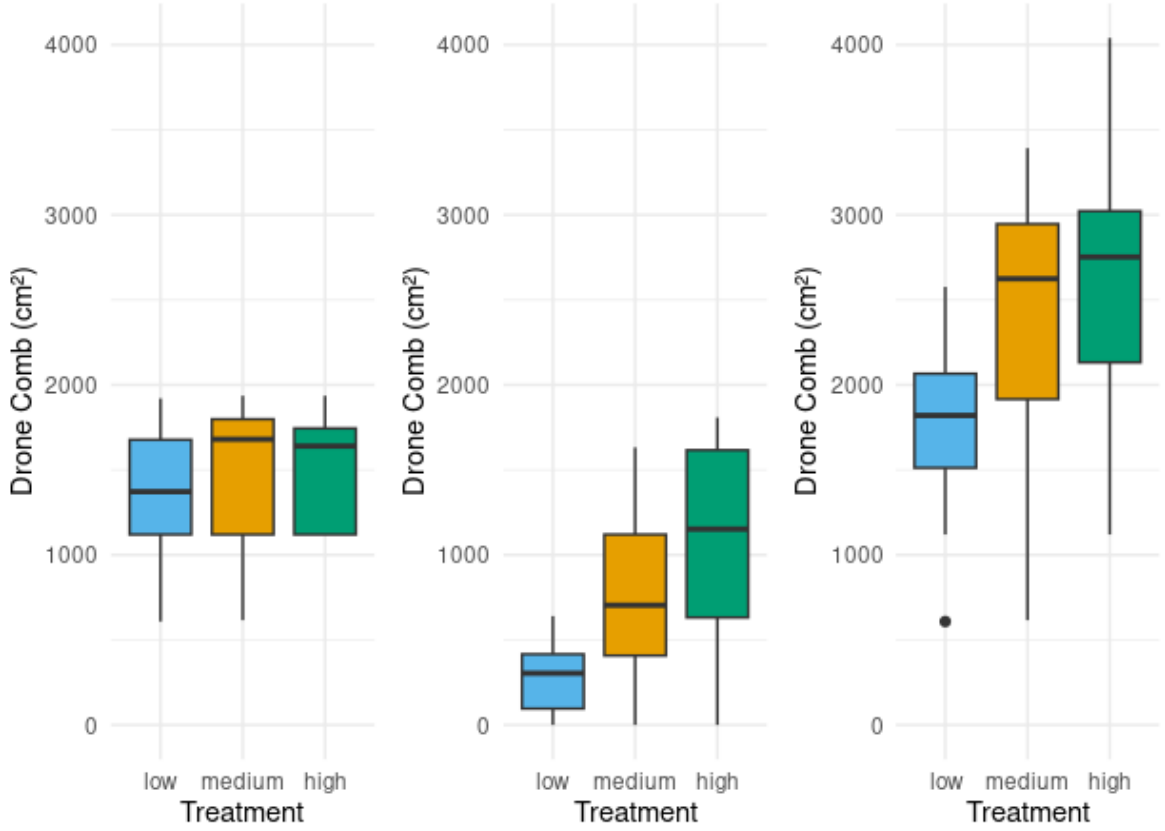

Figure 2-9. Effect of apiary density on drone comb production across different frame types. The panels show the amount of drone brood (cm^2) measured on **(A)** drone frames, **(B)** blank frames, and **(C)** combined frames across the three treatments. The central line within each box represents the median amount of drone comb, the edges of the box indicate the interquartile range (IQR), and the whiskers extend to 1.5 times the IQR. Outliers are displayed as individual points beyond the whiskers.

Worker Comb on Blank Frames

Worker comb was not recorded during the initial November assessment, as this variable was only added to the protocol beginning in December. The additive model, which included *Treatment* ($\chi 2 = 6.35$, df = 1, $p = 0.012$; Figure 2-10), *Date* ($\chi 2 = 8.57$, df = 2, $p = 0.014$) and the random effect of *Colony* demonstrated a significant influence on worker comb production. The low-density treatment produced 176.08 ± 68.1 cm² more worker comb compared to the high-density treatment ($p = 0.007$).

However, there were no significant differences between the high and medium-density treatments ($p > 0.05$) or the low and medium-density treatments ($p > 0.05$). Worker comb production was significantly higher in January compared to December (89.96 ± 33.38 cm², $t = 2.695$, $p = 0.011$; Figure 2-11).

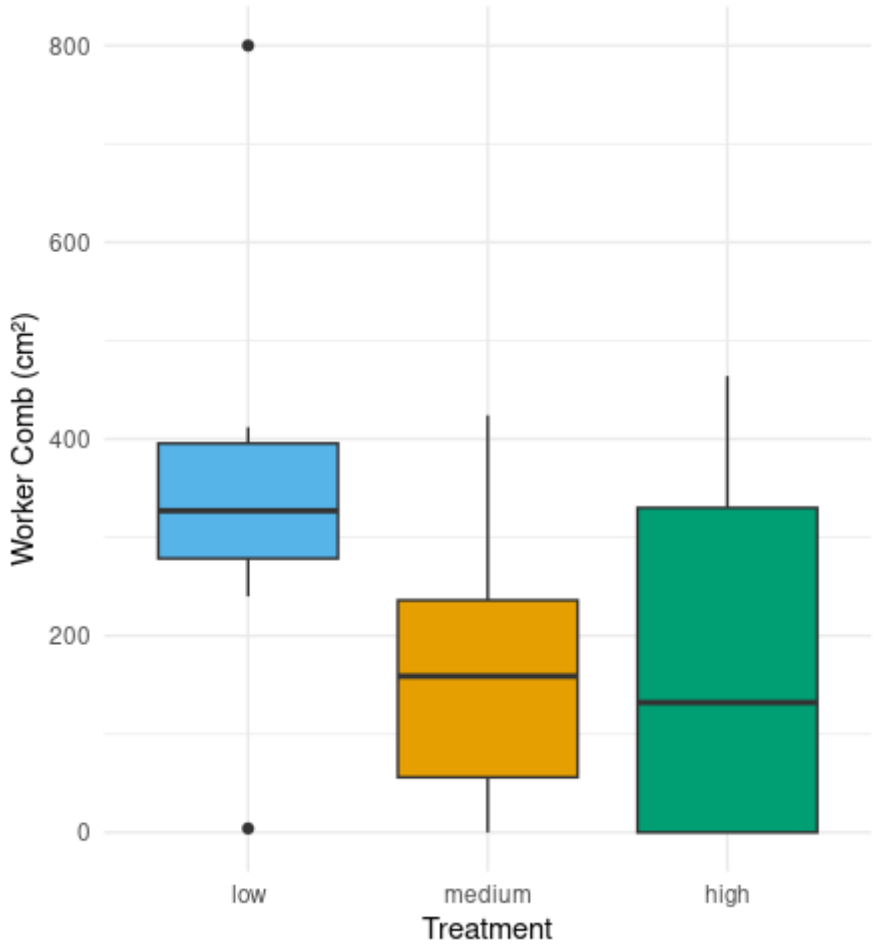

Figure 2-10. Effect of apiary density on worker comb production (cm^2) on blank frames across the three treatments. The central line within each box represents the median worker comb area, the edges of the box indicate the interquartile range (IQR), and the whiskers extend to 1.5 times the IQR. Outliers are displayed as individual points beyond the whiskers.

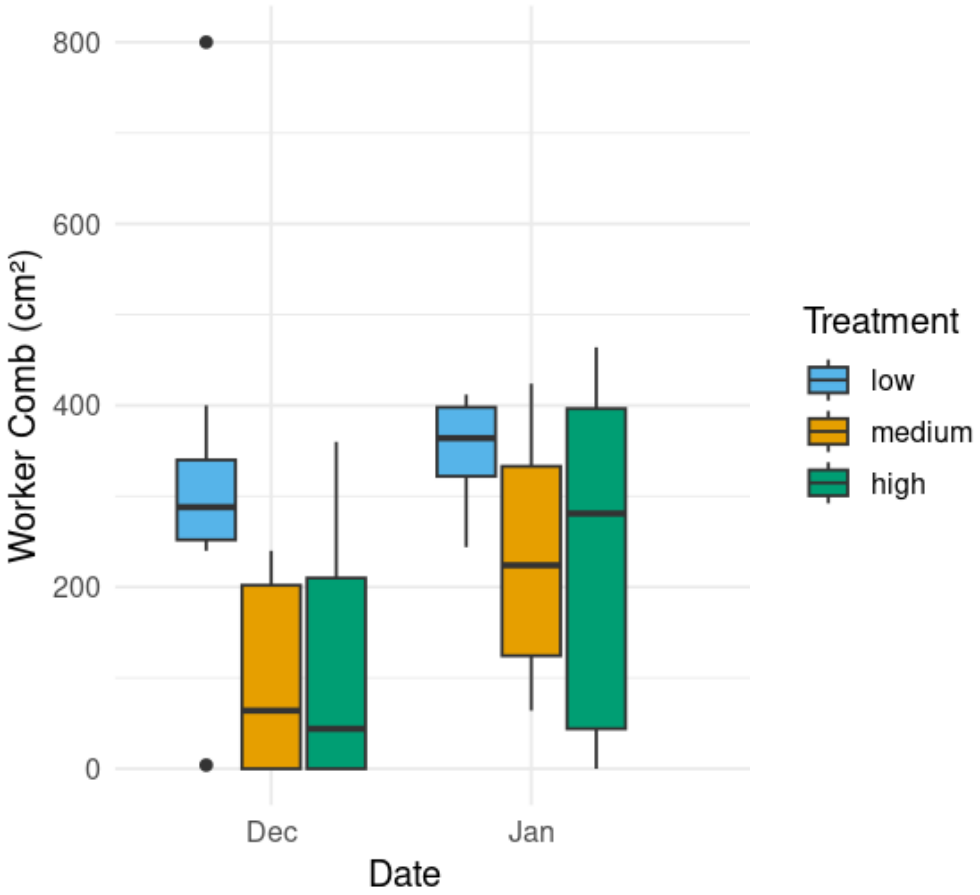

Figure 2-11. Effect of date across treatments on the amount of worker comb (cm²) on the blank frame. Box plots represent the distribution of drone comb for each treatment group (low, medium, and high) across two dates (December, and January). The central line within each box represents the median amount of drone comb, the edges of the box indicate the interquartile range (IQR), and the whiskers extend to 1.5 times the IQR. Outliers are displayed as individual points beyond the whiskers. The key indicates which coloured box plot represents each treatment. Note: Worker comb data collection began in December; no measurements were taken in November.

Effect of Apiary Density on Total Sperm Count

Total sperm counts during December 2023 sampling differed significantly across treatments ($\chi^2 = 114.67$, df = 2, $p < 0.001$; Figure 2-12a). A Dunn's post-hoc test with Bonferroni correction indicated that total sperm count in the low-density treatment was significantly lower than in both the medium ($Z = -8.63$, $p < 0.001$) and high-

density treatments (Z = -9.37, p < 0.001). However, no significant difference was detected between the medium and high treatments (Z = 1.06, p = 0.87).

Likewise, the total sperm counts differed significantly across treatments during the February 2024 sampling ($\chi^2 = 59.50$, $p < 0.001$; Figure 2-12b). Dunn's test revealed that total sperm count was significantly lower in the low-density treatment compared to both the medium (Z = -7.58, p < 0.001) and high-density treatments (Z = -3.68, p < 0.001). Additionally, the high-density treatment exhibited a significantly higher sperm count than the medium-density treatment (Z = 4.60, p < 0.001).

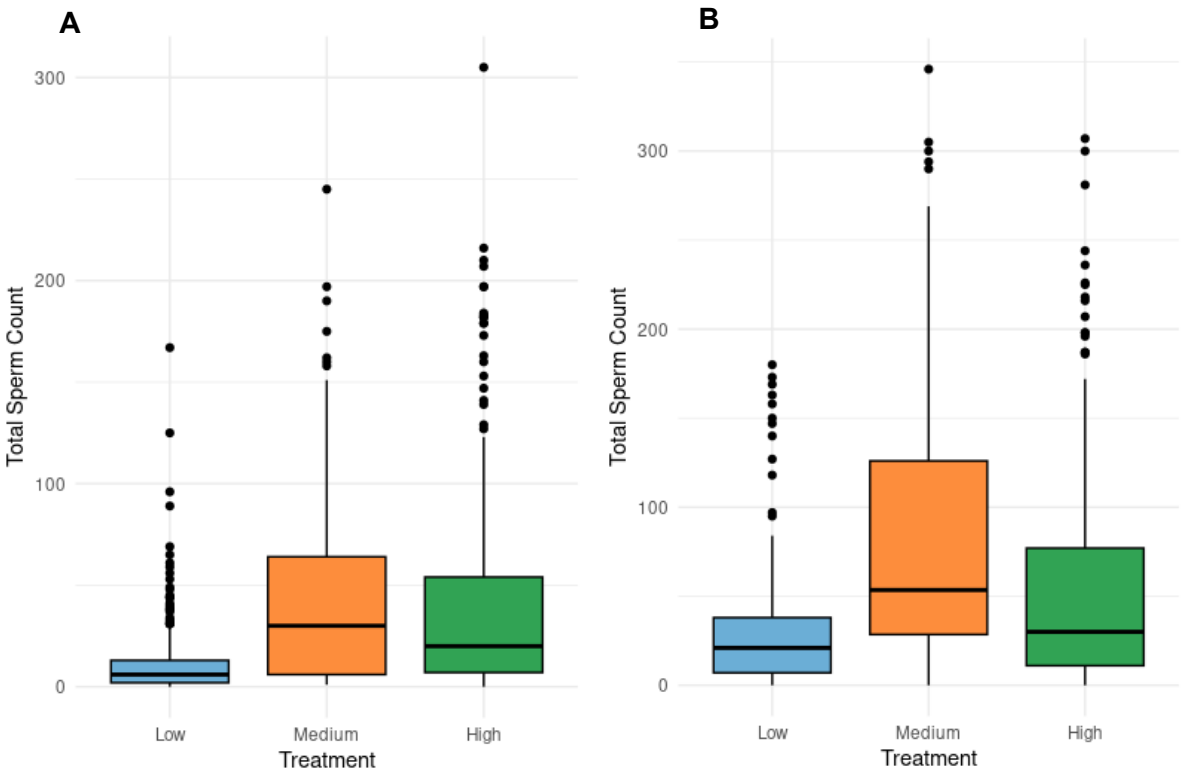

Figure 2-12. Total sperm counts of drones collected in **(A)** December 2023 and **(B)** February 2024 across different apiary densities (low, medium, high). The central line within each box represents the median total sperm count in, the edges of the box indicate the interquartile range (IQR), and the whiskers extend to 1.5 times the IQR. Outliers are displayed as individual points beyond the whiskers

Effect of Apiary Density on the Proportion of Viable Sperm

Across the treatments, the Kruskal-Wallis tests showed significant differences in the proportion of viable sperm in December 2023 ($\chi 2 = 48.925$, df = 2, $p < 0.001$; Figure 2-13) and February 2024 ($\chi 2 = 6.5259$, df = 2, $p = 0.038$; Figure 2-14). For December, post-hoc Dunn's tests with Bonferroni adjustments showed that the low-density treatment had a significantly higher proportion of viable sperm than the high-density treatment ($p < 0.001$). The low-density treatment also had a significantly higher proportion of viable sperm than the medium-density treatment ($p = 0.045$). The high and medium-density treatments were significantly different ($p = 0.003$). Dunn's post hoc tests for the February 2024 data showed no significant difference between the high-density and low-density treatments ($p > 0.05$). The high-density treatment had a significantly lower proportion of viable sperm than the medium-density treatment ($p = 0.036$). The low and medium-density treatments were not significantly different ($p > 0.05$).

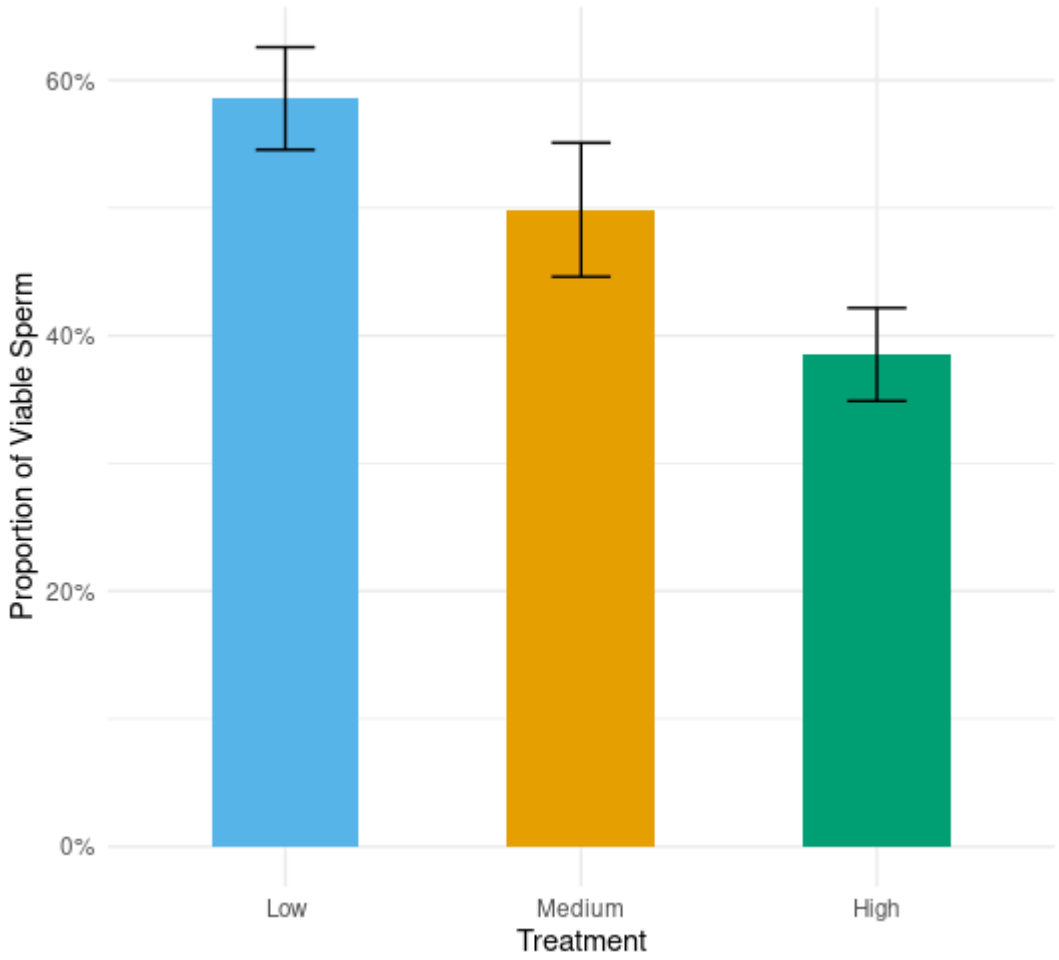

Figure 2-13. Proportion of viable sperm from drones collected in December 2023 across different apiary density treatments (low, medium, high). Bars represent the median proportion of viable sperm, and error bars represent the standard error of the median.

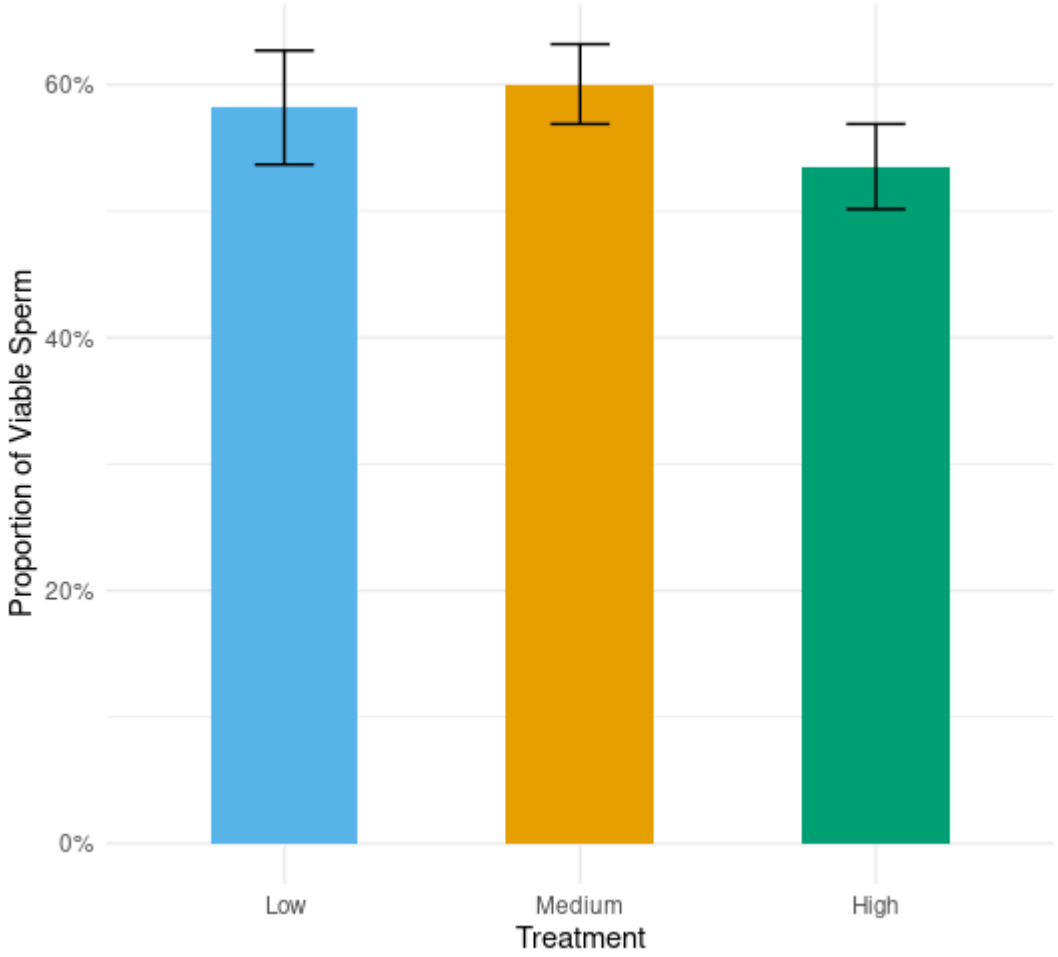

Figure 2-14. Proportion of viable sperm from drones collected in February 2024 across different apiary density treatments (low, medium, high). Bars represent the median proportion of viable sperm, and error bars represent the standard error of the median.

Discussion

Our study demonstrates that apiary density significantly influences both drone production and sperm characteristics in honey bees. Colonies in the higher-density apiary produced significantly more drone brood and had higher total sperm counts compared to those in the low-density apiary. Despite this, the increased reproductive output appears to come at a cost, as evidenced by the reduced worker comb production in those same colonies in the high-density apiary. This suggests a

potential trade-off between worker allocation and drone production. However, when considering the viability of sperm we found variable results. Interestingly, in early summer (December 2023), drones from the low-density treatment exhibited a significantly higher proportion of viable sperm compared to medium and high-density apiaries. In contrast, drones sampled in late summer (February 2024) from the medium-density treatment had a significantly higher proportion of viable sperm compared to those from high-density apiaries. Notably, in both months, the lowest proportion of viable sperm was consistently observed in the highest-density treatment, suggesting another potential cost associated with increased apiary density.

These findings partially support our hypothesis that reproductive competition in high-density environments prompts colonies to increase reproductive investment. While the increased drone production and total sperm counts align with this hypothesis, the observed variations in sperm viability across density treatments and sampling times indicate a more complex relationship between apiary density and sperm quality. Specifically, the higher proportion of viable sperm in the low-density treatment in early summer and the medium-density treatment in late summer, coupled with the consistently lower viability in the high-density treatment, suggest that colonies might be experiencing trade-offs between the quantity and quality of their sperm output. Colonies in high-density environments may invest more heavily in overall drone production at the expense of individual drone sperm quality, potentially reducing the proportion of viable sperm per drone. This could be a strategy to maximize the chances of some drones succeeding in a highly competitive mating environment, even if the average sperm viability per drone is lower.

Previous studies provide valuable context for this investigation and suggest that our findings when considered alongside those of other researchers, help address a critical gap in understanding how environmental factors shape honey bee reproductive strategies. For example, Jaffé et al. (2009) explored how inter-colony competition influences mating dynamics, highlighting the importance of external pressures on reproductive strategies. Our results align with the predictions from the reproductive competition theory, which suggests that organisms increase reproductive investment in response to heightened competition for mates and fertilisation opportunities (Wedell et al., 2002). This is further supported by studies in other taxa, such as the increased sperm production observed in humans under competitive conditions (Baker, 1995). However, the specific mechanisms and trade-offs may differ across species. In honey bees, the superorganism concept adds another layer of complexity, where a single colony potentially adjust their reproductive strategy collectively (Baer, 2005).

The findings on sperm viability, however, present a more nuanced picture. While the increased drone production and total sperm counts align with the general predictions of reproductive competition theory, the consistently lower proportion of viable sperm in the high-density treatment across both sampling periods suggests that higher density may favour greater investment in sperm quantity at the expense of quality. This divergence in the allocation of resources (between sperm quality and quantity) is a pattern that has been observed in other species, where males under increased competition may adjust their ejaculate characteristics. Thomas and Simmons (2007), for example, found that male field crickets (*Teleogryllus oceanicus*) adjusted the proportion of viable sperm in their ejaculates based on a female's mating history. They observed that males ejaculated sperm of lower viability when mating with virgin

females, increased sperm viability when mating with singly mated females, and reduced sperm viability when mating with multiply mated females. This strategic adjustment is likely an adaptation to maximise paternity. Males allocate the highest proportion of viable sperm when there is a moderate risk of sperm competition, suggesting an optimal level of investment. However, when sperm competition becomes too intense, males reduce the proportion of viable sperm, likely because the probability of successfully outcompeting numerous rivals is low, making it more advantageous to conserve resources and potentially seek out females with fewer prior mates.

However, individual male sperm strategies are unlikely to be the primary drivers of these patterns in honeybees. Unlike crickets or many other species where males can mate multiple times, honeybee drones are monogamous and die upon mating (Betti & Lee, 2020; Thomas & Simmons, 2007). This single, fatal mating makes individual ejaculate adjustment at the drone level a less likely explanation. Instead, the observed variations in sperm quality are more likely a reflection of colony-level resource allocation and reproductive strategies influenced by the environmental conditions they experience. The observed patterns, particularly the lower proportion of viable sperm in high-density apiaries, contrast with findings from a review study by Boomsma (2013), which examined ejaculate investment strategies across a broad range of social and non-social insects. The review indicated that, at the individual level, males in many species tend to allocate a higher proportion of viable sperm when facing increased sperm competition. This pattern holds for both non-social insects and some social insects, such as certain ant and termite species where males may compete for access to a single, long-lived queen. However, this difference is likely due to the unique mating dynamics of honey bees, particularly the

lack of opportunity for post-mating, individual-level ejaculate adjustment in drones. Honey bee queens are highly polyandrous, creating intense sperm competition within the queen's reproductive tract. Yet, individual drones, having only one opportunity to mate before they die, cannot adjust their ejaculate in response to the competitive environment. Instead, our findings suggest that in honey bees, colony-level responses to environmental conditions, such as apiary density, are more influential in determining sperm viability. While other eusocial insects with different mating systems (e.g., monogamous species) may exhibit individual-level adjustments in sperm viability, the constraints on honey bee drones make this less likely (Boomsma, 2013).

It's plausible that colonies in low-density apiaries may experience less stress and better resource availability, enabling them to produce a higher proportion of high-quality sperm. Conversely, while high-density apiaries might prompt colonies to increase total sperm output by producing more drones, these resources may be spread more thinly, potentially decreasing the proportion of viable sperm per drone. High-density stress itself may also alter resource allocation within a colony, further reducing the resources available to produce higher-quality sperm (Tihelka, 2018). This is consistent with findings by Fisher and Rangel (2018) who demonstrated that exposure to pesticides during development negatively impacts drone sperm viability, underscoring the role of environmental factors in reproductive health.

Resource availability is likely a critical factor for drone production and sperm quality. Colonies in high-density environments probably face increased resource competition, as evidenced by the reduced worker comb production on blank frames. However, the results suggest that reproductive priorities may override resource constraints, at least to some extent. Similarly, Perry et al. (2015) and Schneider &

McNally (1992), noted that honey bee colonies can maintain reproductive investment under competitive pressures, even when resources are limited. However, Danner et al. (2017) and Pasquale et al. (2013) emphasise that resource availability, particularly pollen and nectar, is a key determinant of colony health and reproduction. The lack of resource quantification in this study limits our ability to fully understand how colonies balanced resource competition with not only reproductive investment (i.e. drone production) but also the production of female workers. This is particularly relevant given our observation that colonies in the high-density apiary produced more drones but exhibited reduced worker comb production. This suggests a potential trade-off where increased competitive pressures may force colonies to prioritise reproductive output at the expense of workers or resource storage. Therefore, future research should include direct measures of resource availability and foraging behaviour to better understand how colonies manage resource competition in high-density environments.

Our findings of increased drone production in high-density apiaries may come at the expense of other functions, such as colony maintenance, worker production or immunity (DeGrandi-Hoffman & Chen, 2015). This increased investment in reproduction under competitive pressure could make colonies more susceptible to stressors, including varroa mites, which are known to impair drone production and sperm quality (Rangel & Fisher, 2019). Although varroa mites were managed in this study, their prevalence was not directly monitored and we did not measure the prevalence of any other honey bee parasites or pathogens The potential for increased stress due to resource allocation towards reproduction in high-density environments underscores the complex interplay between environmental factors, colony health, and reproductive success. Ultimately, the ability of colonies to

successfully navigate these trade-offs between reproduction and other vital functions will determine their long-term survival and contribution to the surrounding ecosystem.

By expanding on previous studies to include the effects of apiary density at both the colony level and individual level, our research addresses a critical gap in understanding how environmental factors shape honey bee reproductive strategies. Our findings also offer practical insights for beekeeping. Higher apiary densities could pose risks if colonies are unable to sustain reproductive investment without compromising health or reducing investment in worker production. A decrease in the number of workers could, in turn, lead to lower honey production. Strategies such as optimising apiary density to balance colony health and productivity are essential for sustainable practices and the long-term success of honey bee populations (Nganso et al., 2024). For instance, Rangel & Fisher (2019) emphasised the need to maintain optimal environmental conditions to support colony function under stress. Therefore, beekeepers must carefully balance the potential benefits of increased drone production with the need to maintain overall colony health and resilience.

In addition to the effects of apiary density, we also observed variations in drone comb and drone brood production over time during our experiment. We found that colonies produced the greatest and most variable amounts of drone brood in November and the least in December. In contrast, colonies produced notably less drone comb in November than in either January or December, regardless of apiary density. This suggests a strong seasonal influence on drone comb and brood production. This aligns with the typical pattern of drone production, which peaks in spring and early summer, coinciding with the swarming season and the availability of virgin queens (Smith et al., 2015). It is plausible that our sampling was conducted towards the end

of the typical drone comb building period, and we may have missed earlier seasonal variability since our data collection did not begin until November.

Our study has several limitations that need to be addressed in future studies. Most importantly, the lack of replication of the three apiary size treatments (low, medium, and high) and the limited range of apiary sizes tested prevent us from drawing definitive conclusions about the causal relationship between apiary density and the observed patterns in drone production and sperm characteristics. While our results are consistent with the hypothesis that higher apiary density leads to increased drone production but potentially lower sperm quality, we cannot rule out the possibility that these patterns are due to chance or other unmeasured factors that varied between our experimental apiaries. Future studies should prioritise replicating these findings across a greater number of apiaries and a wider range of colony densities to establish a more robust link between apiary size and reproductive strategies. The lack of direct measures of resource availability and foraging behaviour limits our understanding of how colonies managed resource competition. Danner et al. (2017) demonstrated that foraging success is closely tied to reproductive outcomes, particularly in resource-limited environments. Including metrics such as forager activity, pollen loads, and nectar collection would provide a clearer picture of how colonies cope with competitive pressures.

The potential influence of genetic factors on drone reproductive success also warrants further investigation. Variations in genes involved in spermatogenesis, sperm motility, or the synthesis of seminal fluid components could lead to differences in sperm competitiveness. For example, certain alleles might confer advantages in sperm longevity, swimming speed, or the ability to penetrate the egg, ultimately affecting a drone's fertilisation success (Jaffé et al., 2010). Exploring these genetic

influences could be further enhanced by investigating potential heritable variations in reproductive traits across different subspecies of *A. mellifera*, such as the Italian honey bee (Jaffé et al., 2010). These studies would help explain the complex interaction between genetics and environmental pressures on honey bee reproductive strategies.

Finally, investigating the physiological mechanisms underlying the observed trade-offs between sperm quantity and quality would provide valuable insights into the reproductive biology of honey bees. Our study indicates that reproductive competition (due to increased population density) drives a response to increase sperm competition through the production of more drones and sperm production. Here, we contribute to understanding how honey bee colonies allocate resources under competitive pressures and highlight the complex trade-offs between quantity and quality in reproductive investment. By addressing limitations such as resource quantification, varroa monitoring, and foraging activity in future research, a more comprehensive understanding of these dynamics can be achieved. The results also offer practical insights for apiary management, emphasising the need to balance density to optimise both reproductive output and colony health.

CHAPTER 3

Elevated HSP70 in Honey Bee Drones Indicates Increased Stress in High-Density Apiaries

Abstract

Population density is a critical ecological factor that significantly influences selection pressures and reproductive strategies, primarily through its effects on resource competition. In many species, increased density intensifies competition, leading to trade-offs between resource allocation towards reproduction versus survival and maintenance. This is particularly evident in social insects, where colony-level responses are crucial. Our study investigates this dynamic in honey bees (*Apis mellifera*), focusing on how apiary density, a manageable environmental factor, impacts drone physiology. Specifically, we measured Heat Shock Protein 70 (HSP70), a biomarker of cellular stress, to assess the physiological consequences of density-mediated competition. We hypothesised that drones reared in colonies within higher-density apiaries would exhibit higher HSP70 levels than those in lower-density apiaries, reflecting increased stress due to resource competition. Drones were collected from colonies managed in high (120 colonies), medium (60-68 colonies), and low (8 colonies) density apiaries, and their HSP70 levels were quantified using an ELISA kit. Drones from high-density apiaries exhibited significantly higher HSP70 levels than those from low- and medium-density apiaries. No significant difference in HSP70 levels was found between drones from low- and medium-density apiaries. Our findings suggest a link between apiary density and increased physiological stress in honey bee drones, as indicated by elevated HSP70 levels. The results align with previous research on stress responses in insects and highlight the potential impact of environmental factors on colony health. While further research is needed to explore the underlying mechanisms and long-term consequences, our study provides valuable insights into the physiological responses

of honey bees to varying population densities, emphasizing the trade-offs associated with increased competition.

Introduction

Honey bees (*Apis mellifera*) are eusocial insects that live in colonies with a complex social structure and a highly specialised division of labour (Slater et al., 2021). Within this superorganism, the queen's reproductive success is essential, but the quality and quantity of male drones are also crucial; these factors directly influence the colony's reproductive success and, consequently, its fitness within the population (Page & Metcalf, 1984). We previously found that apiary density (the concentration of bee colonies within a given area) significantly impacts drone production at the colony level, likely in response to sperm competition **(Chapter 2)**. Specifically, colonies in higher-density apiaries produced a greater number of drones, potentially intensifying male-male competition for mating opportunities. However, while a colony may be able to increase reproductive success in response to increased density, the effects of competition and environmental stress on drone physiology remain poorly understood.

Long-term exposure to stress can impair the cellular mechanisms that manage an organism’s response to stress (Morimoto, 1998). Disruptions can lead to protein misfolding, unfolding, or aggregation, triggering a cascade of biochemical reactions that may result in cell apoptosis. Such disruptions are mitigated by the transcription of highly conserved heat shock genes (Morimoto, 1993). These genes encode heat shock proteins (HSPs), a diverse group of proteins produced in response to various stressors. HSPs function as molecular chaperones, assisting in the proper folding of

newly synthesised or denatured proteins, thus maintaining cellular homeostasis under duress (Feder & Hofmann, 1999; Georgopoulos & Welch, 1993). While the term "heat shock proteins" implies a specific response to heat, it's important to note that these proteins are also upregulated by a variety of other stressors, including starvation, exposure to toxins, and oxygen deprivation (King & MacRae, 2015).

Among the HSPs, Heat Shock Protein 70 (HSP70) has been extensively studied and recognised as a sensitive biomarker for detecting stress in animals and cell culture lines. Compared to other cellular markers, such as oxidative regulation, DNA damage, and cell cycle regulation, HSP70 expression responds rapidly to environmental, physical, or chemical stressors (Dhama et al., 2019; Farcy et al., 2009; Moreira-de-Sousa et al., 2018). Stress protein concentrations in cells can increase within minutes of exposure, making HSP70 an efficient indicator of cellular stress response (Beckham et al., 2008; Mosser et al., 2000). For example, mouse cell lines exposed to heavy metals expressed HSP70 in proportion to the toxicity level and corresponding lethal dosage (LC50) of the metals (Gibney et al., 2001). In *Drosophila melanogaster*, HSP70 is upregulated under anoxia-like conditions, with increased expression linked to impaired nervous system function and weakened wing muscles in adult flies (Feder & Hofmann, 1999). Even at critically low temperatures, rodents exhibit HSP70 expression as part of their stress response.

The signalling pathways that regulate HSP70 expression during stress responses are conserved across vertebrates, invertebrates, and single-celled organisms such as yeast (Beere, 2004; Morimoto, 1998). Conservation of these pathways extends to honey bees, where increased HSP70 expression has been observed in response to heat, toxic substances, bacterial infections, and parasites (Al-Ghzawi et al., 2022; Feder & Hofmann, 1999; G. Li et al., 2018; Scharlaken et al., 2008; Shi et al., 2024).

Recent studies have explored the role of HSP70 in honey bees, highlighting its involvement in thermotolerance and immune responses (Abou-Shaara, 2024; Al-Ghzawi et al., 2022; McMenamin et al., 2020). Al-Ghzawi et al. (2022) found that increased HSP70 expression in honey bees was associated with enhanced survival rates under heat stress conditions. Similarly, Abou-Shaara (2024) demonstrated that HSP70 plays a role in modulating the immune response of bees to bacterial pathogens, showing that its upregulation is an important factor in pathogen defence. However, most research has focused on worker bees, with limited attention given to drones, particularly regarding the role of HSP70 in their stress response and physiology. Previous studies on drones have primarily focussed on the role of drones in mating behaviour and sperm transfer, while the relationship between competition, resource availability, and physiology in drones remains underexplored (McAfee et al., 2022). Addressing this gap is critical for understanding honey bee reproductive biology within the superorganism context.

Drones represent the colony's genetic investment in future generations, making it crucial to understand how environmental factors, such as apiary density, affect their physiological condition. Dense apiary settings could exacerbate competition for resources (e.g. access to high-quality food provided by nurse bees within the hive), possibly increasing stress levels for drones. This potential stress can be reflected in HSP70 levels, providing a critical indicator of cellular health and stress response. By investigating how drone physiology, specifically stress responses, is modulated by environmental factors, we can gain valuable insights into the adaptive strategies of honey bee colonies and the factors influencing their reproductive success.

Here, we investigate the physiological stress response of drone honey bees to population density by quantifying HSP70 levels in drones collected from colonies

managed in apiaries of different densities. We hypothesise that drones in colonies experiencing higher competition will exhibit upregulated HSP70 expression. This study is restricted in scope to the individual-level effects of apiary density on drone physiology, as opposed to broader effects of apiary density on the physiology of the colony level, such as overall colony productivity.

Methods

Sample Collection and Preparation

To investigate the effects of apiary density on drone stress physiology, we collected honey bee drones from three apiary sites in the Waikato region of Aotearoa, New Zealand, each representing a different density treatment: high (120 colonies), medium (60-68 colonies), and low (8 colonies). These apiary sites were established on October 19th, 2023, as part of a larger experimental trial outlined in **Chapter 2**, and were located in Tauwhare (low-density), Matangi (medium-density), and Ruakura (high-density). The focal colonies within each apiary were monitored until the final drone sampling on February 5th - 7th, 2024.

For this specific analysis, we initially collected a surplus of drones from five randomly selected colonies within each apiary between 2:00 pm and 4:00 pm on February 5th-7th, 2024, coinciding with peak mating flight hours to ensure the capture of sexually mature drones. Drones were collected by hand at the entrance of each colony as they were returning from or departing on mating flights (as described in **Chapter 2**). From this larger pool of collected drones, a subset was immediately frozen at -20°C for subsequent HSP70 analysis. This prevented desiccation and protein degradation. However, limited drone availability during this late-season sampling period, coupled

with the loss of some specimens from the high-density apiary due to a freezer malfunction, resulted in a reduced and unequal final sample size between treatments. Ultimately, 31 drones were analysed from the high-density treatment, 56 from the medium-density treatment, and 39 from the low-density treatment for the protein assays.

Once ready for the assay to commence we placed the drones on ice. Within one hour of collection, we carefully dissected each drone's abdomen using a sterile scalpel. We chose the abdomen as the sample tissue because it contains a significant portion of the drone's haemolymph and fat body, key tissues for stress response analysis (McAfee et al., 2022). We transferred each abdomen into a labelled 2ml Eppendorf tube, followed by 600µl of PRO-PREP™ protein extraction solution (Intron Biotechnology, Cat. No. 17081) to facilitate protein extraction. A sterile micro-pestle was used to homogenise the tissue within each tube thoroughly. The tubes were then stored at -20°C for 30 minutes to further aid cell lysis.

After freezing, we centrifuged the tubes in an Eppendorf™ 5415R centrifuge at 13,000 RCF (4°C) for 5 minutes. This separated cellular debris from the protein-containing supernatant. We carefully aspirated the supernatant using a pipette, transferred it to new, labelled tubes and stored it at -20°C for further analysis.

Protein assay- PRO-MEASURE™ procedure

Total protein concentration was determined using the PRO-MEASURE™ Protein Measurement Solution (Intron Biotechnology, Cat. No. 21072). We followed the manufacturer's protocol, where we first generated a standard curve using serial dilutions of Bovine Serum Albumin (BSA, New Zealand origin, standard grade). A stock BSA solution was prepared according to the manufacturer's instructions. Serial

dilutions were then performed as outlined in the PRO-MEASURE™ manual. Distilled water was used as the diluent. A blank was prepared using 100µL of distilled water. For each assay, 1000µL of PRO-MEASURE™ solution was added to each tube containing a standard or blank. Then, 200µL of each standard, blank, and sample was pipetted in triplicate into a 96-well microplate. The absorbance was measured at 595 nm using a FLUOstar® Omega plate reader (BMG Labtech) within one hour.

Due to a limited amount of BSA available due to budget constraints, only one standard curve was used for all the plates. The experiments were run under the same conditions. Several standards were run and then the standard with the highest R^2 value was chosen to be the blank for all the rounds. The standards were prepared in 5 tubes. 100uL of distilled H2O was pipetted into each tube and a serial dilution followed with modifications as described in the PRO-MEASURETM solution manual. Bovine Serum albumin (New Zealand Bovine Serum Albumin - Standard grade - BSA NZ origin) was used to determine a standard curve and distilled H2O as diluent. A blank was prepared by adding 100µL of distilled H2O. 1000µL of PRO-MEASURETM solution was added to each tube. 200µL of blanks, standards, and samples were added to the microplate in triplicates. Absorbance was measured at OD 595 nm using a FLUOstar® Omega plate reader (FLUOstar® Omega, BMG Labtech) within the hour.

Measurement of HSP70 Concentration Using ELISA kit

To quantify HSP70 levels, we used a commercially available Enzyme-Linked Immunosorbent Assay (ELISA) kit, following the manufacturer's protocol with minor modifications. All reagents were prepared as instructed in the kit manual. Wash buffer and assay buffer were prepared fresh for each assay, based on the number of samples being analysed. Biotin-conjugate and Streptavidin-HRP were diluted 1:100

with assay buffer immediately before use. The human HSP70 standard was reconstituted with 260 µL of distilled water and allowed to stand for 30 minutes before use. The required number of microplate strips were placed in the provided holder. Wells were washed twice with 400 µL of wash buffer. After the final wash, the plate was inverted and tapped on a paper towel to remove excess liquid. Reagents were added to the wells within 15 minutes of washing.

Serial dilutions of the HSP70 standard were prepared according to the "external standard dilution" protocol in the kit manual. For blanks, 100 µL of sample diluent was added to designated wells. For samples, 50 µL of sample diluent was added to each well, followed by 50 µL of the protein sample. As the number of kits was limited and costly, biological replicates were prioritised over technical replicates. Each sample was analysed in a single well rather than in duplicate or triplicate. The plate was covered with an adhesive film and incubated on a microplate shaker for 2 hours at room temperature.

After incubation, the plate contents were discarded, and the wells were washed six times with wash buffer. Freshly prepared biotin-conjugate (100 µL) was added to each well. The plate was covered and incubated on a shaker for 1 hour at room temperature. Streptavidin-HRP was prepared 15 minutes before the end of the incubation period. After this incubation, the plate was washed six times, and 100 µL of Streptavidin-HRP was added to each well. The plate was covered and incubated for 30 minutes at room temperature on a shaker. Following this, the plate was washed six times.

Subsequently, 100 µL of TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution was added to each well. The plate was covered and incubated in the dark for 30 minutes

at room temperature. The absorbance was monitored at 450 nm at 15-minute intervals using the FLUOstar® Omega plate reader. Once a sufficient colour change was observed, 100 µL of stop solution was added to each well to terminate the reaction (Figure 3-1). The final absorbance was measured at 450 nm within one hour of adding the stop solution. For 11 absorbance values, the model could not estimate their concentration as the values were outside the range of the standard curve. Therefore, the samples were removed from the dataset as their absorbance values could not be interpolated reliably.

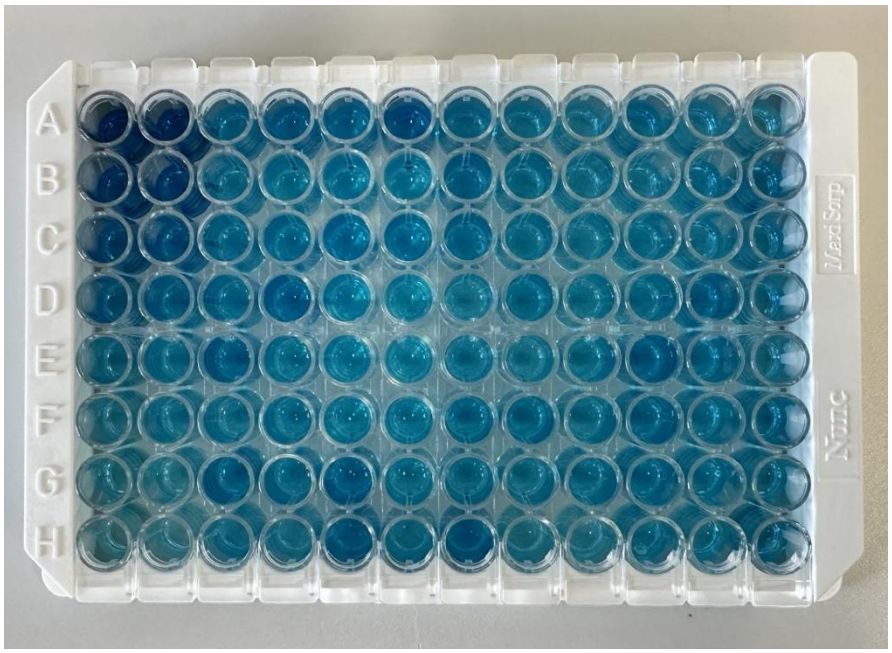

Figure 3-1. ELISA plate showing the detection of HSP70 in samples, with colour development observed following the addition of TMB substrate and termination of the reaction with stop solution.

Statistical Analysis

We determined the concentration of HSP70 in each drone (ng/mg of total protein) by first determining the concentration of total protein in the assay. From the standard

curve, the protein concentration was calculated and multiplied by the dilution factor (1:2). To standardise the HSP70 concentration to 1 mg of total protein, a scaling factor was determined by dividing 1000 µg (equivalent to 1 mg) by the protein concentration used resulting in a scaling factor. The HSP70 concentration obtained from the standard curve was then multiplied by the scaling factor to yield a concentration.

Linear mixed-effects models (LMMs) were used to analyse the effects of treatment (apiary density: high, medium, and low) on HSP70 concentrations in drones. The concentration of HSP70 was measured in ng/mg of total protein. The models were fitted with the *lme4* package in R (version 4.0.0). *Treatment* was included as a fixed effect, while *Colony* was included as a random effect within *Treatment*. This accounted for the hierarchical sampling design and the non-independence of drones within colonies. Model assumptions were assessed using diagnostic plots and the *DHARMa* package.

To address the issues of non-normality and outliers identified in the initial analysis, log transformation and square root transformation of the response variable (HSP70 concentration) were explored. The best-fitting model, based on Akaike Information Criterion (AIC), was the log-transformed model (HSP70_log). This model exhibited no significant deviations from homogeneity of variance or normality. These conclusions were supported by the results of *DHARMa* diagnostic tests for dispersion ($p = 0.992$), uniformity ($p = 0.1463$), and outliers ($p = 0.5983$).

A likelihood-ratio test was conducted to determine if *Treatment* significantly explained variation in HSP70 levels. The full model, which included *Treatment* as a fixed effect, was compared to a reduced model excluding *Treatment*.

The statistical significance of fixed effects was assessed using Type III Analysis of Variance with Satterthwaite's method. Post hoc pairwise comparisons between treatment groups were performed using estimated marginal means (EMMs) with Tukey's adjustment for multiple comparisons, calculated via the *emmeans* package.

Results

The likelihood-ratio test indicated that *Treatment* significantly improved model fit (χ^2= 16.541, df = 2, $p < 0.001$). The full model with *Treatment* also had lower AIC (355.10), and BIC (368.78) values compared to the null model (AIC = 367.64, BIC = 375.85), further supporting the inclusion of *Treatment*. The linear mixed model (LMM) with a log transformation demonstrated that apiary density was a significant predictor of HSP70 concentration in drones ($F_{(2, 9.96)} = 8.40$, $p = 0.007$).

Post hoc tests indicated that drones from high-density treatments had significantly higher HSP70 concentrations than those from low-density (estimate = 1.315, SE = 0.349, $p = 0.0047$; Figure 3-2) and medium-density treatments (estimate = 1.437, SE = 0.325, $p = 0.0022$; Figure 3-2). No significant difference was observed between low- and medium-density treatments (estimate = 0.122, SE = 0.319, $p = 0.923$; Figure 3-2).

Estimated marginal means (EMMs) were back-transformed to the original scale for interpretability. Mean HSP70 concentrations (ng/mg of total protein) were highest in high-density treatments (2.72 ng/mg, 95% CI: 2.19–3.25), followed by low-density (1.40 ng/mg, 95% CI: 0.88–1.92) and medium-density treatments (1.28 ng/mg, 95% CI: 0.80–1.76).

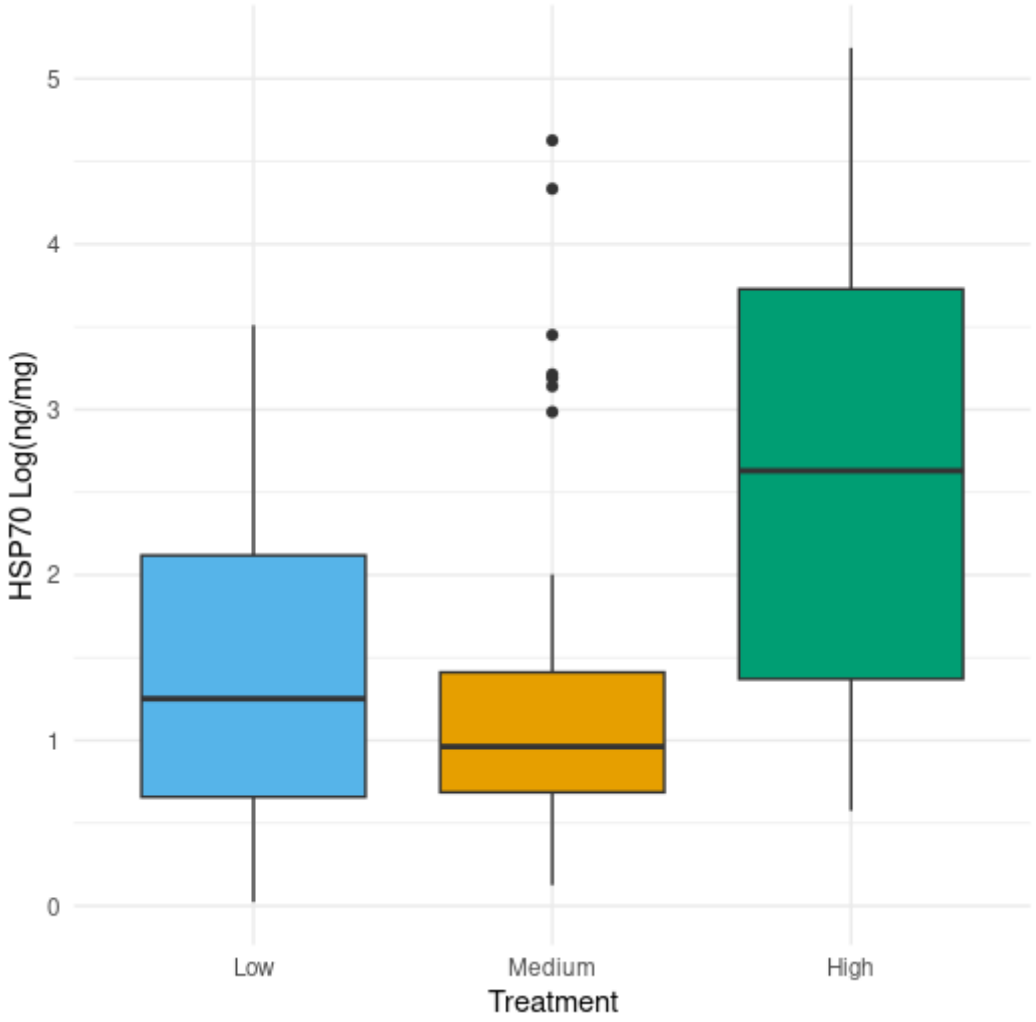

Figure 3-2. Box plot of log-transformed HSP70 concentrations (ng/mg) in honey bee drones sampled from apiaries with different apiary densities. Drones from high-density apiaries exhibited significantly higher HSP70 levels compared to those from both low-density ($p = 0.000047$) and medium-density ($p = 0.0022$) apiaries. No significant difference was found between the low- and medium-density treatments ($p = 0.9225$). The central line within each box represents the median HSP70 concentration in Log(ng/mg), the edges of the box indicate the interquartile range (IQR), and the whiskers extend to 1.5 times the IQR. Outliers are displayed as individual points beyond the whiskers.

Discussion

This study investigated the relationship between apiary density and physiological stress in honey bee drones, using HSP70 expression as a biomarker. Our findings

provide evidence of a direct link between apiary density and quantifiable stress response in drones. We found that drones from high-density apiaries exhibited significantly higher HSP70 concentrations compared to those in medium- and low-density settings. Furthermore, it offers a novel perspective on a key member of the honey bee superorganism: the drone.

The observed pattern of increasing HSP70 levels with increasing apiary density aligns with our initial hypothesis and suggests a dose-response relationship between environmental stress and physiological response in drones. This finding is consistent with a broad body of literature demonstrating the upregulation of HSP70 in response to various stressors across insect taxa (Bodlah et al., 2023; Farahani et al., 2020; Martínez-Paz et al., 2014; Rix & Cutler, 2022). However, our study extends these findings by demonstrating a direct link between apiary density, which serves as a proxy for resource competition and environmental stress, and HSP70 expression specifically in honey bee drones. This is important as drones have a distinct physiological and behavioural profile compared to workers.

The observed trend linking higher apiary density to increased stress levels in honey bees aligns with previous research on colony health and productivity (Smith et al., 2015). Nevertheless, the lack of a significant difference in HSP70 levels between medium- and low-density apiaries in our study warrants further consideration. One possible explanation is that the medium-density treatment did not create sufficient resource competition or environmental stress to elicit a significant HSP70 increase relative to the low-density treatment. Alternatively, drones in low-density apiaries may experience other unidentified stressors that contribute to slightly higher basal HSP70 levels. It's also possible that there is a threshold effect, where HSP70 expression is only significantly upregulated when apiary density exceeds a certain critical level.

The lack of a significant difference in HSP70 levels between medium- and low-density apiaries, however, suggests a potential threshold effect. HSP70 expression may be only significantly upregulated when apiary density exceeds a certain critical level. This is supported by research on other social insects, where stress responses can vary non-linearly with colony size or density (Easton-Calabria et al., 2023; Khaliq et al., 2014; Perez & Aron, 2020). Similar results were observed in a related study by Vispute (2022), who investigated HSP70 responses in worker honey bees across three apiary densities (high, medium, and low). While Vispute (2022) found that worker bees generally exhibited elevated HSP70 levels in response to transportation stress, similar to what was observed in our study with drones, there was no significant difference in HSP70 expression between workers from the medium- and low-density apiaries. Although their medium-density treatment (30 hives) was smaller than our medium-density treatment (60-68 hives), both studies suggest that a threshold density may be required to elicit a significant HSP70 response. It is plausible that the medium-density treatments in both studies did not create sufficient resource competition or environmental stress to induce a measurable increase in HSP70 levels compared to the low-density treatments. Alternatively, other unidentified stressors could be contributing to slightly higher basal HSP70 levels in low-density apiaries. It is also important to acknowledge that both our study and Vispute's (2022) work faced limitations regarding the replication of apiary densities. Nonetheless, the congruence in findings between these studies strengthens the hypothesis of a potential threshold effect for density-dependent HSP70 responses in honey bees.

Another factor to consider is the time of year; drone production drastically reduces towards the end of the mating season. Our sampling for this experiment was

conducted in February, towards the end of the known mating season for this region. This natural decline in drone production might have influenced resource allocation within the colonies, potentially affecting drone physiology regardless of treatment. As shown in **Chapter 2**, drone brood production was notably lower in December 2023 and January 2024 compared to November 2023 across all density treatments (Figure 2-6). This decline aligns with the expected seasonal pattern of drone production, which typically peaks in spring and early summer. Therefore, the drones sampled in February for HSP70 analysis may have developed under conditions of reduced drone rearing and potentially altered resource allocation within the colony. It is plausible that the stress experienced by drones towards the end of the mating season, when resources might be more limited and drones are no longer a priority for the colony, could be exacerbated in higher-density scenarios (Betti & Lee, 2020). This could potentially contribute to the significantly higher HSP70 levels observed in drones from the high-density apiary compared to the medium- and low-density apiaries. In other words, while we did observe a clear treatment effect, the late-season sampling might have amplified the differences in stress responses between the density treatments. This is because drones in high-density apiaries may have experienced a combination of increased competition-related stress throughout their development *and* the heightened stress associated with the natural decline in drone production at the end of the season (McNally & Schneider, 1994; Rangel & Fisher, 2019; Seeley & Mikheyev, 2003).

Our study focused solely on HSP70 as a biomarker of stress. Future research should incorporate a wider range of physiological and biochemical indicators, such as heat shock proteins HSP27 and HSP90, and hormone levels related to stress or reproductive status (e.g. juvenile hormone, vitellogenin) (Xu et al., 2009).

Conversely, markers of oxidative stress could be examined to provide a more comprehensive assessment of drone health and stress responses (Lalouette et al., 2011). Investigating the direct link between HSP70 levels and drone reproductive fitness, potentially through metrics such as sperm viability, mating success, or offspring quality, would also be a valuable avenue for future research. Research should also prioritise investigating a wider range of apiary densities, beyond the limited scope of low, medium, and high densities tested in this study. This is a crucial limitation in the present study as it restricts our ability to draw definitive conclusions about the specific relationship between apiary density and drone stress responses. Incorporating a more continuous range of densities, along with more refined measures of resource availability and competition, would provide a more comprehensive understanding of how these factors interact to affect drone physiology. It would be beneficial to examine HSP70 expression across different seasons or different stages of drone development to provide a more nuanced understanding of how stress responses vary throughout the drone lifecycle. By addressing these questions, we can gain a deeper understanding of what drone physiology reveals about the overall "stress" experienced by a colony. This could increase appreciation for the value of drone health as an indicator of colony health and productivity, particularly in the context of increasing apiary density. Furthermore, this research will help us understand how colonies respond to "overstocking" in productive scenarios and how we might assess if colonies are stocked at a density that induces competition and stress that could negatively impact productivity. Ultimately, this will improve our understanding of how environmental changes, particularly those related to management practices, impact the intricate social dynamics and physiological responses within honey bee colonies.

CHAPTER 4

General Discussion

In this thesis, I aimed to investigate the effects of apiary density on reproductive strategies in honey bees (*Apis mellifera*), with a particular focus on how these strategies are influenced by sperm competition and mediated through the lens of the superorganism concept **(Chapter 2).** Additionally, I explored the physiological responses of drones to varying apiary densities, using HSP70 expression as a biomarker for stress **(Chapter 3)**. My findings indicate that colonies in higher-density apiaries produced significantly more drone brood and had higher total sperm counts compared to those in low-density apiaries. However, early in the breeding season, drones from the low-density treatment exhibited a significantly higher proportion of viable sperm compared to medium and high-density apiaries. Later in the breeding season, drones from the medium-density treatment had a significantly higher proportion of viable sperm compared to those from high-density apiaries. Drones from high-density apiaries consistently exhibited significantly higher levels of HSP70, indicating increased physiological stress. Here, I discuss the significance of these results for our understanding of honey bee reproductive biology, the trade-offs involved in reproductive investment, and the potential implications for beekeeping management. I also highlight promising avenues for future research, particularly concerning the role of seminal fluid proteins in mediating competitive interactions.

Apiary Density Drives Reproductive Investment but with Trade-offs

My results provide strong support for the hypothesis that increased reproductive competition in high-density apiaries prompts colonies to increase their reproductive investment. This is evident by the significantly greater drone brood production and higher total sperm counts observed in drones from high-density treatments. My findings align with predictions from reproductive competition theory, which suggests

that organisms will enhance reproductive output in response to intensified competition for mates and fertilisation opportunities (Wedell et al., 2002). This is further supported by studies across various taxa, including observations of increased sperm production in humans under competitive conditions (Baker, 1995).

However, the data on sperm viability present a more nuanced picture. The significantly higher proportion of viable sperm found in the low-density treatment in December 2023 and in the medium-density treatment in February 2024 suggests that lower apiary density may favour greater investment in sperm quality over quantity. This contrasts with findings by Boomsma (2013), who, in a review of the literature, found evidence that a higher proportion of viable sperm under competitive conditions is a consistent pattern across a wide range of both social and non-social organisms. Several factors could contribute to this discrepancy. Colonies in low-density apiaries may experience less competition for resources, allowing them to invest more heavily in the quality of each drone produced. Alternatively, high-density environments may introduce stressors that negatively impact sperm quality, as suggested by Tihelka (2018). Furthermore, the observed pattern could reflect a colony-level strategic investment, where colonies in high-density environments prioritise the production of a larger number of drones, potentially at the cost of lower average sperm quality within individual drones. This could be a bet-hedging strategy, increasing the chances of at least some drones succeeding in a highly competitive mating environment, even if the average sperm viability per drone is lower.

Physiological Stress Increases with Apiary Density

The significantly higher HSP70 concentrations observed in drones from high-density apiaries provide compelling evidence of a direct link between apiary density and physiological stress. This finding is consistent with the broader literature demonstrating the upregulation of HSP70 in response to various stressors across insect taxa (Bodlah et al., 2023; Farahani et al., 2020; Martínez-Paz et al., 2014; Rix & Cutler, 2022). However, my study extends these findings by demonstrating a direct link between apiary density, which serves as a proxy for resource competition and environmental stress, and HSP70 expression specifically in honey bee drones.

The observed trend linking higher apiary density to increased stress levels in honey bees aligns with previous research on colony health and productivity (Smith et al., 2015). The lack of a significant difference in HSP70 levels between medium- and low-density apiaries, however, suggests a potential threshold effect. HSP70 expression may only be significantly upregulated when apiary density exceeds a certain critical level. This is supported by research on other social insects, where stress responses can vary non-linearly with colony size or density (Easton-Calabria et al., 2023; Khaliq et al., 2014; Perez & Aron, 2020). Similarly, Vispute (2022) found no difference in HSP70 expression in worker honey bees between low and medium-density apiaries but observed significantly higher levels in high-density apiaries. These findings, combined with our results, suggest that a similar threshold effect may be operating in honey bee responses to apiary density.

Future Research Directions

While my thesis provides valuable insights into the effects of apiary density on honey bee reproductive strategies and drone physiology, several limitations should be addressed in future studies. My findings, particularly the differences in sperm viability between density treatments, raise intriguing questions about the potential role of seminal fluid proteins (SNPs) in mediating competitive interactions. It is plausible that variations in apiary density could influence the composition or activity of SNPs in drones, potentially contributing to the observed differences in sperm viability and overall reproductive success. Seminal fluid proteins are specialised molecules found in the seminal fluid of male insects, including honey bee drones (Dosselli et al., 2019). These proteins can play crucial roles in modulating female reproductive physiology and behaviour after mating. They are known to influence processes such as sperm storage, immune responses and hormonal regulation in females. Extensive studies in *Drosophila* and an increasing amount of evidence in other insects including ants and crickets have illustrated the effects of SNPs on female fertility and behaviour (Avila et al., 2011; den Boer et al., 2010; Jasper et al., 2020; Wagner et al., 2001; Wolfner, 2002). In *Drosophila*, SNPs alone can trigger post-mating changes, including increased egg-laying and decreased receptiveness to further mating (Ram & Wolfner, 2007). These findings highlight the potential for a similar mechanism to operate in honey bees, although much remains unknown about the specific roles of SNPs in honey bee queens.

Emerging evidence suggests that SNPs influence honey bee queen physiology in several ways. Research by Jasper et al. (2020) and Liberti et al. (2019) has established a connection between SNPs and decreased sexual receptivity, as well as changes in queen vision. For instance, queens inseminated with seminal fluid

exhibited upregulation of vision-related genes, such as those coding for phospholipase C (PLC), a critical enzyme for light perception in retinal cells (Liberti et al., 2019). This genetic alteration correlates with phenotypic changes, including reduced visual responsiveness to light stimulation in both compound eyes and ocelli cells (Liberti et al., 2019). Such impairments in visual performance may reduce a queen's ability to navigate during mating flights and prematurely return to their hives during mating flights, potentially incurring survival costs cells (Liberti et al., 2019). However, further research is needed to understand whether these effects are solely due to disrupted visual perception or whether other physiological mechanisms induced by seminal fluid are also involved.

Building on the observed effects on vision and receptivity, additional insights into SNPs' roles have come from studies examining the upregulation of specific proteins in queens following insemination. For example, the proteases serine protease snake (a Drosophila-derived protein involved in proteolytic cascades), and Antitrypsin/Serpin 4 have been identified as upregulated in the fat bodies of inseminated queens (Jasper et al., 2020). Serine protease snake, observed in male ant sperm, is implicated in sperm competition and rival sperm degradation, with subsequent neutralisation by queen spermathecal secretions (Dosselli et al., 2019). Similarly, Antitrypsin/Serpin 4, involved in immunity, may also play a role in sperm competition (Brutscher et al., 2015). Both proteases are upregulated in *Drosophila* females after mating and are enriched in the spermathecal proteome, suggesting potential roles in regulating proteolytic pathways within the honey bee reproductive system (LaFlamme & Wolfner, 2013). These findings underscore the need for further investigation into the molecular targets and receptors within queens that interact with SFPs to drive post-mating changes.

Collectively, this body of evidence supports the hypothesis that seminal fluid may mediate sexual conflict between queens and drones. By impairing queen vision, seminal fluid might reduce the likelihood of additional mating flights, thereby aligning with the reproductive interests of male bees by minimising sperm competition. This highlights the role of seminal fluid proteins in manipulating the female to favour the male's own sperm by strategically limiting further mating opportunities and is a testament to the ongoing evolutionary arms race between males and females.

Another step forward would be to incorporate direct measures of resource availability. Quantifying parameters such as pollen and nectar stores within the hive, as well as assessing foraging activity and success rates, would significantly enhance our understanding of how colonies manage the trade-offs between resource competition and reproductive investment. This would allow for a more comprehensive picture of colony resource dynamics and how they influence reproductive decisions under different density conditions. For example, studies have shown that resource availability can significantly impact both colony growth and reproductive output in honey bees (Seeley, 1989a). Limited resources can lead to reduced brood rearing, decreased drone production, and even colony failure (Seeley, 2016). Furthermore, increased foraging effort in resource-poor environments can come at a cost to individual forager lifespan and colony productivity (Seeley, 2016). Therefore, directly measuring resource availability in future studies would provide crucial context for interpreting the observed physiological and reproductive responses to varying apiary densities.

Future studies should also include regular monitoring of varroa mite infestation levels. Specifically, the quantity of varroa mite loads should be measured in both adults and brood throughout the study period. This would involve established

methods such as alcohol washes or sugar shakes to determine mite prevalence in adult honey bees and detailed brood inspections to assess infestation rates within brood cells (Dietemann et al., 2013; Pietropaoli et al., 2021). Given that varroa mites are known to impact drone production, sperm quality, and stress responses, assessing mite loads alongside measurements of drone production (e.g., area of drone brood), sperm parameters (e.g., total sperm count, proportion of viable sperm), and HSP70 expression would help to separate the effects of competition from those of parasite load on these observed patterns (Rangel & Fisher, 2019). Additionally, Tlak Gajger & Mutinelli (2024) found a positive correlation between colony density and varroa mite infestation levels. This is likely attributed to several factors, including increased drifting of bees between colonies, which facilitates mite transfer, and potentially reduced hygienic behaviour in densely populated apiaries where detection and removal of infested brood may be less efficient. Therefore, measuring mite loads concurrently with the other parameters would help to separate the effects of competition, driven by resource availability and influenced by apiary density, from those of parasite-induced stress on the observed patterns of drone production, sperm quality, and HSP70 expression. This is essential for accurately interpreting the relationship between apiary density and reproductive outcomes and determining the specific mechanisms through which apiary density exerts its effects, whether it be primarily through resource limitation, increased parasite load, or a combination of both.

Incorporating genetic analyses is a crucial next step for future research in this area. Investigating potential genetic differences between colonies, particularly in traits related to drone reproductive success, could elucidate the complex interplay between genetic factors and the environmental pressures imposed by varying

population densities. For instance, variations in genes involved in spermatogenesis, sperm motility, or the synthesis of seminal fluid components could lead to differences in sperm competitiveness between drones from different colonies. Certain alleles might confer advantages in sperm longevity, swimming speed, or the ability to penetrate the egg, ultimately affecting a drone's fertilisation success and, consequently, the colony's reproductive output. Such analyses could involve examining the genetic relatedness and diversity of drones and queens within and across apiaries, potentially using microsatellite markers or whole-genome sequencing. Furthermore, exploring potential heritable variations in these reproductive traits across different subspecies of *A. mellifera*, known to exhibit diverse mating strategies, could provide valuable insights into the evolutionary forces shaping honey bee reproductive biology (Jaffé et al., 2010). This line of research will be essential to determine the extent to which genetic factors, in concert with environmental pressures like population density, influence both colony-level reproductive strategies and individual drone reproductive success.

Expanding the range of physiological and biochemical indicators examined would also be beneficial. While this study focused on HSP70 as a biomarker of stress, relying on a single indicator might provide an incomplete picture of the complex stress response. Including a suite of other heat shock proteins (HSPs) such as HSP27 and HSP90 would offer a more comprehensive understanding of the specific stressors experienced by drones. Different HSPs have distinct roles and respond to different types and intensities of stress; HSP27 is involved in cytoskeletal stabilisation and apoptosis prevention, whilst HSP90 is associated with hormone receptor signalling and protein degradation (Feder & Hofmann, 1999; Mizrahi et al., 2010; Tanguay & Hightower, 2015). Studies have shown that different stressors can

elicit distinct HSP profiles. For example, increased resource competition in high-density apiaries may induce a strong HSP70 response, while increased pathogen load might alternatively upregulate HSP27 or HSP90 (Chen et al., 2018; King & MacRae, 2015; Sørensen, 2010; Sørensen et al., 2003). Therefore, measuring a range of HSPs could provide a more detailed understanding of the specific stressors experienced by drones in high-density apiaries.

Furthermore, examining hormone levels, such as juvenile hormone (JH) and vitellogenin, would offer valuable insights into the drones' physiological and reproductive status. JH is a key regulator of insect development and reproduction, and its levels are known to be affected by stress (Bloch & Grozinger, 2011). Vitellogenin, a yolk protein precursor, is involved in immunity and oxidative stress resistance in addition to its reproductive function (Seehuus et al., 2006). In honey bees, vitellogenin has been shown to be involved in the regulation of worker behaviour and longevity, and it may also have similar roles in drones (Page Jr. & Amdam, 2007; Seehuus et al., 2006). Fluctuations in JH and vitellogenin levels could indicate hormonal imbalances and altered reproductive investment in response to apiary density. Additionally, incorporating markers of oxidative stress, such as malondialdehyde (MDA) or superoxide dismutase (SOD) activity, is important for assessing cellular damage and the overall physiological toll of environmental stressors on drones. Oxidative stress occurs when there's an imbalance between the production of reactive oxygen species (ROS) and the ability of the organism to detoxify them (Zhang et al., 2022). It's often associated with various stressors, including pathogen infection, resource competition, and increased activity levels, all of which could be exacerbated in high-density apiaries (Della Noce et al., 2019; Diaz-Albiter et al., 2011; Kunat-Budzyńska et al., 2025). This imbalance can lead to

cellular damage, lipid peroxidation, and impaired physiological function (Della Noce et al., 2019; Diaz-Albiter et al., 2011; Kunat-Budzyńska et al., 2025; Moreira & Hermes-Lima, 2024; Sun et al., 2023). Turnell et al. (2021) demonstrated that in *Drosophila melanogaster*, manipulating ROS production and scavenging through genetic modification affected sperm ageing and male reproductive fitness. Specifically, males with reduced ROS production had sperm that maintained their fertilising ability longer, supporting the idea that oxidative stress contributes to sperm senescence. Therefore, measuring oxidative stress markers alongside HSPs and hormone levels would provide a more comprehensive assessment of drone health and help determine whether the observed increase in HSP70 in high-density apiaries is associated with oxidative damage, potentially contributing to reduced sperm viability.

Future research should also aim to establish a direct link between HSP70 levels, sperm characteristics, and drone reproductive fitness. Measuring metrics such as sperm viability, mating success, or offspring quality would help to determine the functional consequences of the observed physiological responses to apiary density. This would provide a more complete picture of how environmental pressures ultimately impact reproductive success.

A wider range of apiary densities should be tested and incorporated with more refined measures of resource availability and competition to further enhance our understanding of density-dependent effects. This could involve establishing experimental apiaries with a wider range of colony densities and manipulating resource availability to examine how these factors interact to shape reproductive strategies. Importantly, more levels of density manipulation would reveal where a threshold may lie more precisely. It should also be noted that there was only one

replicate for each density treatment in this study. Therefore, we can't say for sure if the differences we saw were due to density or some other environmental factors due to where the hives were. Although efforts were made to minimise confounding factors by selecting apiaries in relatively similar environments and relocating focal colonies to a neutral site before the experiment to allow a new cohort of bees to develop under controlled conditions. Additional research should aim for multiple, independent replicates of each density treatment to ensure the robustness of the findings. This is a crucial step in establishing a clear cause-and-effect relationship between apiary density and the various reproductive and physiological parameters investigated. Addressing this limitation would require significant logistical effort and resources, highlighting the challenges inherent in conducting large-scale ecological experiments with honey bees.

Implications for Beekeeping Management

The findings of this thesis carry significant implications for beekeeping management practices, particularly in the context of optimising reproductive output and maintaining colony health. The observation that adjusting apiary density can influence drone production suggests a potential tool for beekeepers, especially those involved in breeding programs where increasing the availability of drones is a key objective. By strategically manipulating density, it may be possible to enhance drone production to meet specific breeding goals.

However, the double-edged nature of high apiary density cannot be ignored, particularly concerning the trade-off with worker production. Our study found that high-density colonies produced significantly less worker comb on blank frames

(Chapter 2). This suggests that colonies in densely populated apiaries may be diverting resources towards drone production at the expense of worker production and potentially honey production. This is a crucial consideration for beekeepers who are not primarily focused on drone rearing but rather on maintaining strong, productive colonies for pollination services or honey yield. The increased physiological stress observed in drones from high-density apiaries, as evidenced by elevated HSP70 levels, further raises concerns about the potential long-term consequences for overall colony health. If colonies are pushed to sustain increased reproductive investment without adequate resources or in the face of excessive competition and increased parasite loads, their overall health, productivity, and ability to perform essential tasks like foraging and brood rearing could be compromised. This underscores the critical importance of striking a delicate balance: optimising density to achieve desired reproductive outcomes while simultaneously safeguarding colony well-being and maintaining a robust worker population. Beekeepers must be mindful of the potential trade-offs involved and consider factors such as resource availability, disease prevalence, desired balance between drone and worker production, and overall colony health when determining optimal apiary densities. Regular monitoring of both drone and worker populations, as well as indicators of colony health like brood patterns and honey stores, is essential for managing apiaries effectively under different density conditions. Further research into the long-term effects of varying apiary densities on colony productivity and resilience is warranted to inform best management practices.

Final Remarks

In conclusion, I found evidence that apiary density significantly influences reproductive strategies and physiological stress responses in honey bees. The findings support the application of the superorganism concept and reproductive competition theory to understanding honey bee biology, while also highlighting the complex trade-offs involved in reproductive investment. The observed patterns of drone production, sperm characteristics, and HSP70 expression underscore the intricate interplay between environmental pressures, colony-level responses, and individual drone physiology. By addressing the limitations identified and pursuing the suggested avenues for future research, particularly the investigation of seminal fluid proteins, we can further refine our understanding of these dynamics and develop more effective strategies for managing and conserving honey bee populations. The insights gained from this research contribute to a deeper understanding of social insect biology and provide a foundation for sustainable beekeeping practices that support both ecological and practical objectives.

References

Abou-Shaara, H. F. (2024). The response of heat shock proteins in honey bees to abiotic and biotic stressors. *Journal of Thermal Biology*, *119*, 103784. https://doi.org/10.1016/j.jtherbio.2024.103784

Al-Ghzawi, A. A.-M. A., Al-Zghoul, M. B., Zaitoun, S., Al-Omary, I. M., & Alahmad, N. A. (2022). Dynamics of heat shock proteins and heat shock factor expression during heat stress in daughter workers in pre-heat-treated (rapid heat hardening) *Apis mellifera* mother queens. *Journal of Thermal Biology*, *104*, 103194. https://doi.org/10.1016/j.jtherbio.2022.103194

Andersson, M., & Iwasa, Y. (1996). Sexual selection. *Trends in Ecology & Evolution*, *11*(2), 53–58. https://doi.org/10.1016/0169-5347(96)81042-1

Avila, F. W., Sirot, L. K., LaFlamme, B. A., Rubinstein, C. D., & Wolfner, M. F. (2011). Insect Seminal Fluid Proteins: Identification and Function. *Annual Review of Entomology*, *56*, 21–40. https://doi.org/10.1146/annurev-ento-120709-144823

Baer, B. (2005). Sexual selection in Apis bees. *Apidologie*, *36*(2), 187–200. https://doi.org/10.1051/apido:2005013

Baker, R. R. (with Bellis, M. A.). (1995). *Human sperm competition: Copulation, masturbation, and infidelity*. Chapman & Hall.

Bastin, F., Savarit, F., Lafon, G., & Sandoz, J.-C. (2017). Age-specific olfactory attraction between Western honey bee drones (Apis mellifera) and its chemical basis. *PLOS ONE*, *12*(10), e0185949. https://doi.org/10.1371/journal.pone.0185949

Baudry, E., Solignac, M., Garnery, L., Gries, M., Cornuet, J., & Koeniger, N. (1998). Relatedness among honeybees (Apis mellifera) of a drone congregation. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *265*(1409), 2009–2014. https://doi.org/10.1098/rspb.1998.0533

Beckham, J. T., Wilmink, G. J., Mackanos, M. A., Takahashi, K., Contag, C. H., Takahashi, T., & Jansen, E. D. (2008). Role of HSP70 in cellular thermotolerance. *Lasers in Surgery and Medicine*, *40*(10), 704–715. https://doi.org/10.1002/lsm.20713

Beere, H. M. (2004). “The stress of dying” — the role of heat shock proteins in the regulation of apoptosis. *Journal of Cell Science, 117*(13), 2641–2651. https://doi.org/10.1242/jcs.01284

Betti, M. I., & Lee, I. (2020). The effects of diploid male production on honey bee colony evolution and survival. *Theoretical Population Biology*, *135*, 49–55. https://doi.org/10.1016/j.tpb.2020.08.001

Birkhead, T. R., & Møller, A. P. (1998). *Sperm competition and sexual selection* (1st ed.). Academic Press.

Bloch, G., & Grozinger, C. M. (2011). Social molecular pathways and the evolution of bee societies. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *366*(1574), 2155–2170. https://doi.org/10.1098/rstb.2010.0346

Bodlah, M. A., Iqbal, J., Ashiq, A., Bodlah, I., Jiang, S., Mudassir, M. A., Rasheed, M. T., & Fareen, A. G. E. (2023). Insect behavioral restraint and adaptation strategies under heat stress: An inclusive review. *Journal of the Saudi Society of Agricultural Sciences*, *22*(6), 327–350. https://doi.org/10.1016/j.jssas.2023.02.004

Boes, K. E. (2010). Honeybee colony drone production and maintenance in accordance with environmental factors: An interplay of queen and worker decisions. *Insectes Sociaux*, *57*(1), 1–9. https://doi.org/10.1007/s00040-009-0046-9

Boomsma, J. J. (2013). Beyond promiscuity: Mate-choice commitments in social breeding. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1613), 20120050. https://doi.org/10.1098/rstb.2012.0050

Boomsma, J. J., Baer, B., & Heinze, J. (2005). The Evolution of Male Traits in Social Insects. *Annual Review of Entomology*, *50*, 395–420.

Boomsma, J. J., Huszár, D. B., & Pedersen, J. S. (2014). The evolution of multiqueen breeding in eusocial lineages with permanent physically differentiated castes. *Animal Behaviour*, *92*, 241–252. https://doi.org/10.1016/j.anbehav.2014.03.005

Bracke, A., Peeters, K., Punjabi, U., Hoogewijs, D., & Dewilde, S. (2017). A search for molecular mechanisms underlying male idiopathic infertility. *Reproductive BioMedicine Online*, *36*. https://doi.org/10.1016/j.rbmo.2017.12.005

Bratu, I. C., Igna, V., Simiz, E., Dunea, I. B., & Pătruică, S. (2022). The Influence of Body Weight on Semen Parameters in Apis mellifera Drones. *Insects*, *13*(12), 1141. https://doi.org/10.3390/insects13121141

Bretman, A., Westmancoat, J. D., & Chapman, T. (2013). Male control of mating duration following exposure to rivals in fruitflies. *Journal of Insect Physiology*, *59*(8), 824–827. https://doi.org/10.1016/j.jinsphys.2013.05.011

Brutscher, L. M., Baer, B., & Niño, E. L. (2019). Putative Drone Copulation Factors Regulating Honey Bee (Apis mellifera) Queen Reproduction and Health: A Review. *Insects*, *10*(1), Article 1. https://doi.org/10.3390/insects10010008

Brutscher, L. M., Daughenbaugh, K. F., & Flenniken, M. L. (2015). Antiviral defense mechanisms in honey bees. *Current Opinion in Insect Science*, *10*, 71–82. https://doi.org/10.1016/j.cois.2015.04.016

Calhim, S., Immler, S., & Birkhead, T. R. (2007). Postcopulatory Sexual Selection Is Associated with Reduced Variation in Sperm Morphology. *PLOS ONE*, *2*(5), e413. https://doi.org/10.1371/journal.pone.0000413

Canciani, M., Arnellos, A., & Moreno, A. (2019). Revising the Superorganism: An Organizational Approach to Complex Eusociality. *Frontiers in Psychology*, *10*. https://doi.org/10.3389/fpsyg.2019.02653

Cattelan, S., Evans, J. P., Garcia-Gonzalez, F., Morbiato, E., Pilastro, A., & Ghalambor, C. (2020). Dietary stress increases the total opportunity for sexual

selection and modifies selection on condition-dependent traits. *Ecology Letters*, *23*(3), 447–456. https://doi.org/10.1111/ele.13443

Chen, B., Feder, M. E., & Kang, L. (2018). Evolution of heat-shock protein expression underlying adaptive responses to environmental stress. *Molecular Ecology*, *27*(15), 3040–3054. https://doi.org/10.1111/mec.14769

Collins, A. M., & Donoghue, A. M. (1999). Viability assessment of honey bee, Apis mellifera, sperm using dual fluorescent staining. *Theriogenology*, *51*(8), 1513–1523. https://doi.org/10.1016/S0093-691X(99)00094-1

Danner, N., Keller, A., Härtel, S., & Steffan-Dewenter, I. (2017). Honey bee foraging ecology: Season but not landscape diversity shapes the amount and diversity of collected pollen. *PLOS ONE*, *12*(8), e0183716. https://doi.org/10.1371/journal.pone.0183716

DeGrandi-Hoffman, G., & Chen, Y. (2015). Nutrition, immunity and viral infections in honey bees. *Current Opinion in Insect Science*, *10*, 170–176. https://doi.org/10.1016/j.cois.2015.05.007

Della Noce, B., Carvalho Uhl, M. V. de, Machado, J., Waltero, C. F., de Abreu, L. A., da Silva, R. M., da Fonseca, R. N., de Barros, C. M., Sabadin, G., Konnai, S., da Silva Vaz, I., Ohashi, K., & Logullo, C. (2019). Carbohydrate Metabolic Compensation Coupled to High Tolerance to Oxidative Stress in Ticks. *Scientific Reports*, *9*(1), 4753. https://doi.org/10.1038/s41598-019-41036-0

den Boer, S. P. A., Baer, B., & Boomsma, J. J. (2010). Seminal fluid mediates ejaculate competition in social insects. *Science (New York, N.Y.)*, *327*(5972), 1506–1509. https://doi.org/10.1126/science.1184709

Dhama, K., Latheef, S. K., Dadar, M., Samad, H. A., Munjal, A., Khandia, R., Karthik, K., Tiwari, R., Yatoo, M. I., Bhatt, P., Chakraborty, S., Singh, K. P., Iqbal, H. M. N., Chaicumpa, W., & Joshi, S. K. (2019). Biomarkers in Stress Related Diseases/Disorders: Diagnostic, Prognostic, and Therapeutic Values. *Frontiers in Molecular Biosciences*, *6*. https://doi.org/10.3389/fmolb.2019.00091

Diaz-Albiter, H., Mitford, R., Genta, F. A., Sant'Anna, M. R. V., & Dillon, R. J. (2011). Reactive Oxygen Species Scavenging by Catalase Is Important for Female Lutzomyia longipalpis Fecundity and Mortality. *PLOS ONE*, *6*(3), e17486. https://doi.org/10.1371/journal.pone.0017486

Dietemann, V., Nazzi, F., Martin, S. J., Anderson, D. L., Locke, B., Delaplane, K. S., Wauquiez, Q., Tannahill, C., Frey, E., Ziegelmann, B., Rosenkranz, P., & Ellis, J. D. (2013). Standard methods for varroa research. *Journal of Apicultural Research*, *52*(1), 1–54. https://doi.org/10.3896/IBRA.1.52.1.09

Dosselli, R., Grassl, J., den Boer, S. P. A., Kratz, M., Moran, J. M., Boomsma, J. J., & Baer, B. (2019). Protein-Level Interactions as Mediators of Sexual Conflict in Ants. *Molecular & Cellular Proteomics: MCP*, *18*(Suppl 1), S34–S45. https://doi.org/10.1074/mcp.RA118.000941

Dubovskiy, I. M., Whitten, M. M. A., Yaroslavtseva, O. N., Greig, C., Kryukov, V. Y., Grizanova, E. V., Mukherjee, K., Vilcinskas, A., Glupov, V. V., & Butt, T. M. (2013). Can Insects Develop Resistance to Insect Pathogenic Fungi? *PLOS ONE*, *8*(4), e60248. https://doi.org/10.1371/journal.pone.0060248

Easton-Calabria, A. C., Thuma, J. A., Cronin, K., Melone, G., Laskowski, M., Smith, M. A. Y., Pasadyn, C. L., de Bivort, B. L., & Crall, J. D. (2023). Colony size buffers interactions between neonicotinoid exposure and cold stress in bumblebees. *Proceedings of the Royal Society B: Biological Sciences*, *290*(2003), 20230555. https://doi.org/10.1098/rspb.2023.0555

Evans, J. P., & Magurran, A. E. (2000). Multiple benefits of multiple mating in guppies. *Proceedings of the National Academy of Sciences*, *97*(18), 10074–10076. https://doi.org/10.1073/pnas.180207297

Farahani, S., Bandani, A. R., Alizadeh, H., Goldansaz, S. H., & Whyard, S. (2020). Differential expression of heat shock proteins and antioxidant enzymes in response to temperature, starvation, and parasitism in the Carob moth larvae, Ectomyelois ceratoniae (Lepidoptera: Pyralidae). *PLOS ONE*, *15*(1), e0228104. https://doi.org/10.1371/journal.pone.0228104

Farcy, É., Voiseux, C., Lebel, J.-M., & Fiévet, B. (2009). Transcriptional expression levels of cell stress marker genes in the Pacific oyster *Crassostrea gigas* exposed to acute thermal stress. *Cell Stress and Chaperones*, *14*(4), 371–380. https://doi.org/10.1007/s12192-008-0091-8

Feder, M. E., & Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annual Review of Physiology*, *61*(Volume 61, 1999), 243–282. https://doi.org/10.1146/annurev.physiol.61.1.243

Firman, R. C. (2018). Postmating sexual conflict and female control over fertilization during gamete interaction. *Annals of the New York Academy of Sciences*, *1422*(1), 48–64. https://doi.org/10.1111/nyas.13635

Fisher, A., & Rangel, J. (2018). Exposure to pesticides during development negatively affects honey bee (Apis mellifera) drone sperm viability. *PloS One*, *13*(12), e0208630–e0208630. https://doi.org/10.1371/journal.pone.0208630

Fitzpatrick, J. L., Montgomerie, R., Desjardins, J. K., Stiver, K. A., Kolm, N., & Balshine, S. (2009). Female promiscuity promotes the evolution of faster sperm in cichlid fishes. *Proceedings of the National Academy of Sciences*, *106*(4), 1128–1132. https://doi.org/10.1073/pnas.0809990106

Gage, M. J. (1998). Mammalian sperm morphometry. *Proceedings. Biological Sciences*, *265*(1391), 97–103. https://doi.org/10.1098/rspb.1998.0269

Gates, B. N. (1914). *The temperature of the bee colony: Vol. no.96* (pp. 1–40). U.S. Dept. of Agriculture. https://doi.org/10.5962/bhl.title.64474

Gençer, H. V., & Kahya, Y. (2020). Sperm competition in honey bees (Apis mellifera L.): The role of body size dimorphism in drones. *Apidologie*, *51*(1), 1–17. https://doi.org/10.1007/s13592-019-00699-4

Georgopoulos, C., & Welch, W. J. (1993). *The Biology of Heat Shock Proteins and Molecular Chaperones. The Quarterly Review of Biology*, *70*(3), 336–336. https://doi.org/10.1086/419095

Gibney, E., Gault, J., & Williams, J. (2001). The use of stress proteins as a biomarker of sub-lethal toxicity: Induction of heat shock protein 70 by 2-isobutyl piperidine and transition metals at sub-lethal concentrations. *Biomarkers*, *6*(3), 204–217. https://doi.org/10.1080/13547500010009573

Google Earth. (n.d). *Map of Apiary locations in the Waikato Region, North Island, Aotearoa, New Zealand* [Map].

Grant, J. W. A., & Kramer, D. L. (1992). Temporal clumping of food arrival reduces its monopolization and defence by zebrafish, *Brachydanio rerio*. *Animal Behaviour*, *44*(1), 101–110. https://doi.org/10.1016/S0003-3472(05)80759-6

Gwynne, D. T., & Simmons, L. W. (1990). Experimental reversal of courtship roles in an insect. *Nature (London)*, *346*(6280), 172–174. https://doi.org/10.1038/346172a0

Harshman, L. G., & Prout, T. (1994). Sperm displacement without sperm transfer in Drosophila melanogaster. *Evolution*, *48*(3), 758–767.

Herberstein, M. E., Painting, C. J., & Holwell, G. I. (2017). Chapter Seven—Scramble Competition Polygyny in Terrestrial Arthropods. In M. Naguib, J. Podos, L. W. Simmons, L. Barrett, S. D. Healy, & M. Zuk (Eds.), *Advances in the Study of Behavior* (Vol. 49, pp. 237–295). Academic Press. https://doi.org/10.1016/bs.asb.2017.01.001

Humphries, S., Evans, J. P., & Simmons, L. W. (2008). Sperm competition: Linking form to function. *BMC Evolutionary Biology*, *8*(1), 319. https://doi.org/10.1186/1471-2148-8-319

Jacobson, M. (2012). *Insect Sex Pheromones*. Academic Press.

Jaffé, R., Dietemann, V., Allsopp, M. H., Costa, C., Crewe, R. M., Dall'olio, R., DE LA Rúa, P., El-Niweiri, M. A. A., Fries, I., Kezic, N., Meusel, M. S., Paxton, R. J., Shaibi, T., Stolle, E., & Moritz, R. F. A. (2010). Estimating the density of honeybee colonies across their natural range to fill the gap in pollinator decline censuses. *Conservation Biology: The Journal of the Society for Conservation Biology*, *24*(2), 583–593. https://doi.org/10.1111/j.1523-1739.2009.01331.x

Jaffé, R., Dietemann, V., Crewe, R. M., & Moritz, R. F. A. (2009). Temporal variation in the genetic structure of a drone congregation area: An insight into the population dynamics of wild African honeybees (Apis mellifera scutellata). *Molecular Ecology*, *18*(7), 1511–1522. https://doi.org/10.1111/j.1365-294X.2009.04143.x

Jasper, W. C., Brutscher, L. M., Grozinger, C. M., & Niño, E. L. (2020). Injection of seminal fluid into the hemocoel of honey bee queens (Apis mellifera) can stimulate

post-mating changes. *Scientific Reports*, *10*(1), 11990. https://doi.org/10.1038/s41598-020-68437-w

Khaliq, A., Javed, M., Sohail, M., & Sagheer, M. (2014). Environmental effects on insects and their population dynamics. *Journal of Entomology and Zoology Studies*, *2*(2), 1–7.

King, A. M., & MacRae, T. H. (2015). Insect heat shock proteins during stress and diapause. *Annual Review of Entomology*, *60*, 59–75. https://doi.org/10.1146/annurev-ento-011613-162107

Koeniger, G., Koeniger, N., Ellis, J., & Connor, L. (2014). *Mating biology of honey bees* (R. Kim & R. Muir, Eds.; 1st edition). Wicwas Press.

Koeniger, N., Koeniger, G., Gries, M., & Tingek, S. (2005). Drone competition at drone congregation areas in four Apis species. *Apidologie*, *36*(2), 211–221. https://doi.org/10.1051/apido:2005011

Kokko, H., Klug, H., & Jennions, M. D. (2014). Mating systems. In D. Shuker & L. Simmons (Eds.), *The Evolution of Insect Mating Systems* (p. 0). Oxford University Press. https://doi.org/10.1093/acprof:oso/9780199678020.003.0003

Kokko, H., & Rankin, D. J. (2006). Lonely hearts or sex in the city? Density-dependent effects in mating systems. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *361*(1466), 319–334. https://doi.org/10.1098/rstb.2005.1784

Kunat-Budzyńska, M., Łabuć, E., & Ptaszyńska, A. A. (2025). Changes in enzymatic activity and oxidative stress in honeybees kept in the apiary and laboratory conditions during the course of nosemosis. *PLOS ONE*, *20*(1), e0317384. https://doi.org/10.1371/journal.pone.0317384

LaFlamme, B. A., & Wolfner, M. F. (2013). Identification and Function of Proteolysis Regulators in Seminal Fluid. *Molecular Reproduction and Development*, *80*(2), 80–101. https://doi.org/10.1002/mrd.22130

Lalouette, L., Williams, C. M., Hervant, F., Sinclair, B. J., & Renault, D. (2011). Metabolic rate and oxidative stress in insects exposed to low temperature thermal fluctuations. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *158*(2), 229–234. https://doi.org/10.1016/j.cbpa.2010.11.007

Lee, P. E., & Wilkes, A. (1965). Polymorphic Spermatozoa in the Hymenopterous Wasp Dahlbominus. *Science*, *147*(3664), 1445–1446. https://doi.org/10.1126/science.147.3664.1445

Lee, & Winston, M. L. (1987). Effects of reproductive timing and colony size on the survival, offspring colony size and drone production in the honey bee (Apis mellifera). *Ecological Entomology*, *12*(2), 187–195. https://doi.org/10.1111/j.1365-2311.1987.tb00997.x

Li, G., Zhao, H., Liu, Z., Wang, H., Xu, B., & Guo, X. (2018). The Wisdom of Honeybee Defenses Against Environmental Stresses. *Frontiers in Microbiology*, *9*, 722–722. https://doi.org/10.3389/fmicb.2018.00722

Li, Y., Zhao, Q., Duan, X., Song, C., & Chen, M. (2017). Transcription of four *Rhopalosiphum padi* (L.) heat shock protein genes and their responses to heat stress and insecticide exposure. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *205*, 48–57. https://doi.org/10.1016/j.cbpa.2016.12.021

Liberti, J., Görner, J., Welch, M., Dosselli, R., Schiøtt, M., Ogawa, Y., Castleden, I., Hemmi, J. M., Baer-Imhoof, B., Boomsma, J. J., & Baer, B. (2019). Seminal fluid compromises visual perception in honeybee queens reducing their survival during additional mating flights. *eLife*, *8*, e45009. https://doi.org/10.7554/eLife.45009

Ligon, R. A., Diaz, C. D., Morano, J. L., Troscianko, J., Stevens, M., Moskeland, A., Laman, T. G., & Iii, E. S. (2018). Evolution of correlated complexity in the radically different courtship signals of birds-of-paradise. *PLOS Biology*, *16*(11), e2006962. https://doi.org/10.1371/journal.pbio.2006962

Loper, G. M., Wolf, W. W., & Taylor, O. R. (1992). Honey Bee Drone Flyways and Congregation Areas: Radar Observations. *Journal of the Kansas Entomological Society*, *65*(3), 223–230.

Martínez-Paz, P., Morales, M., Martín, R., Martínez-Guitarte, J. L., & Morcillo, G. (2014). Characterization of the small heat shock protein *Hsp27* gene in *Chironomus riparius* (Diptera) and its expression profile in response to temperature changes and xenobiotic exposures. *Cell Stress and Chaperones*, *19*(4), 529–540. https://doi.org/10.1007/s12192-013-0479-y

Mayer, M. P., & Bukau, B. (2005). Hsp70 chaperones: Cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences*, *62*(6), 670. https://doi.org/10.1007/s00018-004-4464-6

McAfee, A., Metz, B. N., Milone, J. P., Foster, L. J., & Tarpy, D. R. (2022). Drone honey bees are disproportionately sensitive to abiotic stressors despite expressing high levels of stress response proteins. *Communications Biology*, *5*(1). https://doi.org/10.1038/s42003-022-03092-7

McMenamin, A. J., Daughenbaugh, K. F., & Flenniken, M. L. (2020). The Heat Shock Response in the Western Honey Bee (Apis mellifera) is Antiviral. *Viruses*, *12*(2), 245. https://doi.org/10.3390/v12020245

McNally, L. C., & Schneider, S. S. (1994). Drone production and drone comb utilization in colonies of the African honey bee, Apis mellifera scutellata Lepeletier, in Africa. *Apidologie*, *25*(6), 547–556. https://doi.org/10.1051/apido:19940605

Metz, B. N., & Tarpy, D. R. (2022). Variation in the reproductive quality of honey bee males affects their age of flight attempt. *PeerJ*, *10*, e13859. https://doi.org/10.7717/peerj.13859

Mizrahi, T., Heller, J., Goldenberg, S., & Arad, Z. (2010). Heat shock proteins and resistance to desiccation in congeneric land snails. *Cell Stress and Chaperones*, *15*(4), 351–363. https://doi.org/10.1007/s12192-009-0150-9

Moller, A. P., & Birkhead, T. R. (1993). Cuckoldry and Sociality: A Comparative Study of Birds. *The American Naturalist*, *142*(1), 118–140. https://doi.org/10.1086/285531

Moreira, D. C., & Hermes-Lima, M. (2024). Dynamics of Redox Metabolism during Complete Metamorphosis of Insects: Insights from the Sunflower Caterpillar Chlosyne lacinia (Lepidoptera). *Antioxidants*, *13*(8), Article 8. https://doi.org/10.3390/antiox13080959

Moreira-de-Sousa, C., de Souza, R. B., & Fontanetti, C. S. (2018). HSP70 as a Biomarker: An Excellent Tool in Environmental Contamination Analysis—a Review. *Water, Air, & Soil Pollution*, *229*(8), 1–12. https://doi.org/10.1007/s11270-018-3920-0

Morimoto, R. I. (1993). Cells in stress: Transcriptional activation of heat shock genes. *Science (New York, N.Y.)*, *259*(5100), 1409–1410. https://doi.org/10.1126/science.8451637

Morimoto, R. I. (1998). The Heat Shock Response: Systems Biology of Proteotoxic Stress in Aging and Disease. *Cold Spring Harbor Symposia on Quantitative Biology*, *76*, 91–99. https://doi.org/10.1101/sqb.2012.76.010637

Moritz, R. F. A., & Southwick, E. E. (1992). *Bees as Superorganisms*. Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-84666-3

Moškrič, A., Formato, G., Smodiš Škerl, M. I., & Prešern, J. (2020). Reproductive technologies in the honeybee (*Apis mellifera*). In G. A. Presicce (Ed.), *Reproductive Technologies in Animals* (pp. 229–243). Academic Press. https://doi.org/10.1016/B978-0-12-817107-3.00014-X

Mosser, D. D., Caron, A. W., Bourget, L., Meriin, A. B., Sherman, M. Y., Morimoto, R. I., & Massie, B. (2000). The Chaperone Function of hsp70 Is Required for Protection against Stress-Induced Apoptosis. *Molecular and Cellular Biology*, *20*(19), 7146–7159. https://doi.org/10.1128/MCB.20.19.7146-7159.2000

Nasr, M. E., Thorp, R. W., Tyler, T. L., Briggs, D. L., Nasr, M. E., Thorp, R. W., Tyler, T. L., & Briggs, D. L. (1990). Estimating honey bee (Hymenoptera: Apidae) colony strength by a simple method: measuring cluster size. *Journal of Economic Entomology*, *83*(3), Article 3. https://doi.org/10.1093/jee/83.3.748

Nassir, M., Levi, M., Dardikman-Yoffe, G., Mirsky, S. K., & Shaked, N. T. (2022). Prediction of Sperm Progression in Three Dimensions Using Rapid Optical Imaging and Dynamic Mechanical Modeling. *Cells*, *11*(8), Article 8. https://doi.org/10.3390/cells11081319

Nganso, B. T., Soroker, V., Osabutey, A. F., Pirk, C. W. W., Johansson, T., Elie, N., … Subramanian, S. (2024). Best practices for colony management: a neglected aspect for improving honey bee colony health and productivity in Africa. *Journal of Apicultural Research*, *63*(3), 438–455. https://doi-org.ezproxy.waikato.ac.nz/10.1080/00218839.2024.2308418

Page Jr., R. E., & Amdam, G. V. (2007). The making of a social insect: Developmental architectures of social design. *BioEssays*, *29*(4), 334–343. https://doi.org/10.1002/bies.20549

Page, R. E. (1986). Sperm Utilization in Social Insects. *Annual Review of Entomology*, *31*(1), 297–320. https://doi.org/10.1146/annurev.en.31.010186.001501

Page, R. E., & Metcalf, R. A. (1984). A Population Investment Sex Ratio for the Honey Bee (Apis mellifera L.). *The American Naturalist*, *124*(5), 680–702.

Parker, G. A. (1970). Sperm Competition and Its Evolutionary Consequences in the Insects. *Biological Reviews*, *45*(4), 525–567. https://doi.org/10.1111/j.1469-185X.1970.tb01176.x

Parker, G. A. (1982). Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. *Journal of Theoretical Biology*, *96*(2), 281–294. https://doi.org/10.1016/0022-5193(82)90225-9

Pasquale, G. D., Salignon, M., Conte, Y. L., Belzunces, L. P., Decourtye, A., Kretzschmar, A., Suchail, S., Brunet, J.-L., & Alaux, C. (2013). Influence of Pollen Nutrition on Honey Bee Health: Do Pollen Quality and Diversity Matter? *PLOS ONE*, *8*(8), e72016. https://doi.org/10.1371/journal.pone.0072016

Perez, R., & Aron, S. (2020). Adaptations to thermal stress in social insects: Recent advances and future directions. *Biological Reviews of the Cambridge Philosophical Society*, *95*(6), 1535–1553. https://doi.org/10.1111/brv.12628

Perry, C. J., Søvik, E., Myerscough, M. R., & Barron, A. B. (2015). Rapid behavioral maturation accelerates failure of stressed honey bee colonies. *Proceedings of the National Academy of Sciences*, *112*(11), 3427–3432. https://doi.org/10.1073/pnas.1422089112

Phillips, E. F., & Demuth, G. S. (1914). *The Temperature of the Honeybee Cluster in Winter*. U.S. Dept. of Agriculture.

Pietropaoli, M., Gajger, I. T., Costa, C., Gerula, D., Wilde, J., Adjlane, N., Aldea-Sánchez, P., Škerl, M. I. S., Bubnič, J., & Formato, G. (2021). Evaluation of Two Commonly Used Field Tests to Assess Varroa destructor Infestation on Honey Bee (Apis mellifera) Colonies. *Applied Sciences*, *11*(10), 4458. https://doi.org/10.3390/app11104458

Pitnick, S. (1996). Investment in Testes and the Cost of Making Long Sperm in Drosophila. *The American Naturalist*, *148*(1), 57–80. https://doi.org/10.1086/285911

Poiani, A. (2006). Complexity of seminal fluid: A review. *Behavioral Ecology and Sociobiology*, *60*(3), 289–310. https://doi.org/10.1007/s00265-006-0178-0

Ram, K. R., & Wolfner, M. F. (2007). Sustained Post-Mating Response in Drosophila melanogaster Requires Multiple Seminal Fluid Proteins. *PLOS Genetics*, *3*(12), e238. https://doi.org/10.1371/journal.pgen.0030238

Rangel, J., & Fisher, A. (2019). Factors affecting the reproductive health of honey bee (Apis mellifera) drones—A review. *Apidologie*, *50*(6), 759–778. https://doi.org/10.1007/s13592-019-00684-x

Rhodes, J. W., Harden, S., Spooner-Hart, R., Anderson, D. L., & Wheen, G. (2011). Effects of age, season and genetics on semen and sperm production in Apis mellifera drones. *Apidologie*, *42*(1), 29–38. https://doi.org/10.1051/apido/2010026

Rix, R. R., & Cutler, G. C. (2022). Review of molecular and biochemical responses during stress induced stimulation and hormesis in insects. *Science of The Total Environment*, *827*, 154085. https://doi.org/10.1016/j.scitotenv.2022.154085

Ruttner, F. (1966). The Life and Flight Activity of Drones. *Bee World*, *47*(3), 93–100. https://doi.org/10.1080/0005772X.1966.11097111

Safonkin, A. F. (2011). Polygamous strategies of insects. *Biology Bulletin Reviews*, *1*(6), 536–541. https://doi.org/10.1134/S2079086411060077

Scharlaken, B., de Graaf, D. C., Goossens, K., Peelman, L. J., & Jacobs, F. J. (2008). Differential gene expression in the honeybee head after a bacterial challenge. *Developmental and Comparative Immunology*, *32*(8), 883–889. https://doi.org/10.1016/j.dci.2008.01.010

Schlüns, H., Moritz, R. F. A., Neumann, P., Kryger, P., & Koeniger, G. (2005). Multiple nuptial flights, sperm transfer and the evolution of extreme polyandry in honeybee queens. *Animal Behaviour*, *70*(1), 125–131. https://doi.org/10.1016/j.anbehav.2004.11.005

Schneider, S. S., & McNally, L. C. (1992). Colony Defense in the African Honey Bee in Africa (Hymenoptera: Apidae). *Environmental Entomology*, *21*(6), 1362–1370. https://doi.org/10.1093/ee/21.6.1362

Scopus. (n.d.-a). *Analyze search results for apis AND mellifera AND/OR drone AND HSP70*. Scopus. Retrieved January 11, 2025, from https://www-scopus-com.ezproxy.waikato.ac.nz/results/results.uri?sort=plf-f&src=s&sid=2e0397c6d8d653062ccad901c08fad4d&sot=a&sdt=b&sl=48&s=TITLE-ABS-KEY%28honey+AND+bee+AND+drone+AND+heat+AND+shock+AND+protein%29&origin=searchbasic&editSaveSearch=&txGid=cd46143ff30e6d165d2577f7a09e5ee4&sessionSearchId=2e0397c6d8d653062ccad901c08fad4d&limit=10

Scopus. (n.d.-b). *Analyze search results for apis AND mellifera AND/OR workers AND HSP70*. Scopus. Retrieved January 11, 2025, from https://www-scopus-com.ezproxy.waikato.ac.nz/results/results.uri?st1=Honey+bee+workers+HSP&st2=&s=TITLE-ABS-KEY%28Apis+mellifera+OR+worker+AND+hsp70%29&limit=10&origin=searchbasic&sort=plf-f&src=s&sot=b&sdt=b&sessionSearchId=9623ef8b6b242d823515216258e7d975

Seehuus, S.-C., Norberg, K., Gimsa, U., Krekling, T., & Amdam, G. V. (2006). Reproductive protein protects functionally sterile honey bee workers from oxidative

stress. *Proceedings of the National Academy of Sciences*, *103*(4), 962–967. https://doi.org/10.1073/pnas.0502681103

Seeley, T. D. (1989a). Social foraging in honey bees: How nectar foragers assess their colony's nutritional status. *Behavioral Ecology and Sociobiology*, *24*(3), 181–199. https://doi.org/10.1007/BF00292101

Seeley, T. D. (1989b). The Honey Bee Colony as a Superorganism. *American Scientist*, *77*(6), 546–553.

Seeley, T. D. (2016). *Honeybee Ecology: A Study of Adaptation in Social Life: 44*. Princeton University Press.

Seeley, T. D., & Mikheyev, A. S. (2003). Reproductive decisions by honey bee colonies: Tuning investment in male production in relation to success in energy acquisition. *Insectes Sociaux*, *50*(2), 134–138. https://doi.org/10.1007/s00040-003-0638-8

Shackelford, T. K., Pound, N., & Goetz, A. T. (2005). Psychological and Physiological Adaptations to Sperm Competition in Humans. *Review of General Psychology*, *9*(3), 228–248. https://doi.org/10.1037/1089-2680.9.3.228

Shafir, S., Kabanoff, L., Duncan, M., & Oldroyd, B. P. (2009). Honey bee (Apis mellifera) sperm competition in vitro – two are no less viable than one. *Apidologie*, *40*(5), 556–561. https://doi.org/10.1051/apido/2009014

Shi, Y.-T., Zhai, Z., Liu, Z.-Y., Zhou, L., Yang, H., Chen, L., Zhang, Y.-S., & Yu, H. (2024). Expressional respones of *hsp70* genes against abiotic and entomopathgenic stresses in four different noctuid larval species (Lepidoptera: Noctidae). *Pesticide Biochemistry and Physiology*, *204*, 106059. https://doi.org/10.1016/j.pestbp.2024.106059

Simmons, L. W. (2001). *Sperm Competition and Its Evolutionary Consequences in the Insects*. Princeton University Press. https://doi.org/10.2307/j.ctvs32sr2

Siva-Jothy, M. T., & Tsubaki, Y. (1989). Variation in copulation duration in Mnais pruinosa pruinosa Selys (Odonata: Calopterygidae). *Behavioral Ecology and Sociobiology*, *24*(1), 39–45. https://doi.org/10.1007/BF00300116

Slater, G. P., Smith, N. M. A., & Harpur, B. A. (2021). Prospects in Connecting Genetic Variation to Variation in Fertility in Male Bees. *Genes*, *12*(8), 1251. https://doi.org/10.3390/genes12081251

Smith, M. L., Ostwald, M. M., & Seeley, T. D. (2015). Adaptive tuning of an extended phenotype: Honeybees seasonally shift their honey storage to optimize male production. *Animal Behaviour*, *103*, 29–33. https://doi.org/10.1016/j.anbehav.2015.01.035

Snook, R. R. (2005). Sperm in competition: Not playing by the numbers. *Trends in Ecology & Evolution*, *20*(1), 46–53. https://doi.org/10.1016/j.tree.2004.10.011

Sørensen, J. G. (2010). Application of heat shock protein expression for detecting natural adaptation and exposure to stress in natural populations. *Current Zoology*, *56*(6), 703–713. https://doi.org/10.1093/czoolo/56.6.703

Sørensen, J. G., Kristensen, T. N., & Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecology Letters*, *6*(11), 1025–1037. https://doi.org/10.1046/j.1461-0248.2003.00528.x

Stürup, M., Baer-Imhoof, B., Nash, D. R., Boomsma, J. J., & Baer, B. (2013). When every sperm counts: Factors affecting male fertility in the honeybee Apis mellifera. *Behavioral Ecology*, *24*(5), 1192–1198. https://doi.org/10.1093/beheco/art049

Sun, Y., Niu, X., Huang, Y., Wang, L., Liu, Z., Guo, X., Xu, B., & Wang, C. (2023). Role of the tyrosine aminotransferase *AccTATN* gene in the response to pesticide and heavy metal stress in *Apis cerana cerana*. *Pesticide Biochemistry and Physiology*, *191*, 105372. https://doi.org/10.1016/j.pestbp.2023.105372

Swallow, J. G., & Wilkinson, G. S. (2002). The long and short of sperm polymorphisms in insects. *Biological Reviews*, *77*(2), 153–182. https://doi.org/10.1017/S1464793101005851

Tanguay, R. M., & Hightower, L. E. (2015). *The Big Book on Small Heat Shock Proteins* (1st ed. 2015.). Springer International Publishing. https://doi.org/10.1007/978-3-319-16077-1

Tarpy, D. R. (2024). Collective decision-making during reproduction in social insects: A conceptual model for queen supersedure in honey bees (*Apis mellifera*). *Current Opinion in Insect Science*, *66*, 101260. https://doi.org/10.1016/j.cois.2024.101260

Thomas, M. L., & Simmons, L. W. (2007). Male Crickets Adjust the Viability of Their Sperm in Response to Female Mating Status. *The American Naturalist*, *170*(2), 190–195. https://doi.org/10.1086/519404

Tihelka, E. (2018). Effects of synthetic and organic acaricides on honey bee health: A review. *Slovenski Veterinarski Zbornik*, *55*(3). https://doi.org/10.26873/svr-422-2017

Tlak Gajger, I., & Mutinelli, F. (2024). Impact of Environmental Factors and Management Practices on Bee Health. *Insects*, *15*(12), Article 12. https://doi.org/10.3390/insects15120996

Tofilski, A., Chuda-Mickiewicz, B., Czekońska, K., & Chorbiński, P. (2012). Flow cytometry evidence about sperm competition in honey bee (Apis mellifera). *Apidologie*, *43*(1), 63–70. https://doi.org/10.1007/s13592-011-0089-6

Turnell, B. R., Kumpitsch, L., & Reinhardt, K. (2021). Production and scavenging of reactive oxygen species both affect reproductive success in male and female Drosophila melanogaster. *Biogerontology*, *22*(4), 379–396. https://doi.org/10.1007/s10522-021-09922-1

Uy, J. A. C., & Borgia, G. (2000). Sexual Selection Drives Rapid Divergence in Bowerbird Display Traits. *Evolution*, *54*(1), 273–278. https://doi.org/10.1111/j.0014-3820.2000.tb00027.x

Vanpé, C., Kjellander, P., Galan, M., Cosson, J.-F., Aulagnier, S., Liberg, O., & Hewison, A. J. M. (2008). Mating system, sexual dimorphism, and the opportunity for sexual selection in a territorial ungulate. *Behavioral Ecology*, *19*(2), 309–316. https://doi.org/10.1093/beheco/arm132

Vispute, R. (2022). *Physiological markers of stress in western honey bees (Apis mellifera)* [ResearchSpace@Auckland]. https://hdl.handle.net/2292/60441

Wagner, W. E., Kelley, R. J., Tucker, K. R., & Harper, C. J. (2001). Females receive a life-span benefit from male ejaculates in a field cricket. *Evolution; International Journal of Organic Evolution*, *55*(5), 994–1001. https://doi.org/10.1554/0014-3820(2001)055[0994:fralsb]2.0.co;2

Wang, H.-S., Wang, X.-H., Zhou, C.-S., Huang, L.-H., Zhang, S.-F., Guo, W., & Kang, L. (2007). cDNA cloning of heat shock proteins and their expression in the two phases of the migratory locust. *Insect Molecular Biology*, *16*(2), 207–219. https://doi.org/10.1111/j.1365-2583.2006.00715.x

Wedell, N., Gage, M. J. G., & Parker, G. A. (2002). Sperm competition, male prudence and sperm-limited females. *Trends in Ecology & Evolution*, *17*(7), 313–320. https://doi.org/10.1016/S0169-5347(02)02533-8

Winkler, L., Eilhardt, R., & Janicke, T. (2023). Population density affects sexual selection in an insect model. *Functional Ecology*, *37*(10), 2734–2747. https://doi.org/10.1111/1365-2435.14410

Winston, M. L. (1991). *The Biology of the Honey Bee* (1st edition). *Harvard University Press.

Withrow, J. M., & Tarpy, D. R. (2018). Cryptic “royal” subfamilies in honey bee (Apis mellifera) colonies. *PLOS ONE*, *13*(7), e0199124. https://doi.org/10.1371/journal.pone.0199124

Wojda, I., Kowalski, P., & Jakubowicz, T. (2009). Humoral immune response of *Galleria mellonella* larvae after infection by *Beauveria bassiana* under optimal and heat-shock conditions. *Journal of Insect Physiology*, *55*(6), 525–531. https://doi.org/10.1016/j.jinsphys.2009.01.014

Wolfner, M. F. (2002). The gifts that keep on giving: Physiological functions and evolutionary dynamics of male seminal proteins in Drosophila. *Heredity*, *88*(2), 85–93. https://doi.org/10.1038/sj.hdy.6800017

Xu, J., Shu, J., Qiu, X., Wang, Z., Zhao, F., Zhang, Z., & Zhang, Q. (2009). Effects of heat shock on ovary development and hsp83 expression in Tribolium castaneum (Coleoptera: Tenebrionidae). *Archives of Insect Biochemistry and Physiology*, *70*(3), 204–216. https://doi.org/10.1002/arch.20294

Zhang, Y.-J., Pang, Y.-B., Wang, X.-Y., Jiang, Y.-H., Herrera-Balandrano, D. D., Jin, Y., Wang, S.-Y., & Laborda, P. (2022). Exogenous genistein enhances soybean resistance to Xanthomonas axonopodis pv. Glycines. *Pest Management Science*, *78*(8), 3664–3675. https://doi.org/10.1002/ps.7009

Zhao, H., Mashilingi, S. K., Liu, Y., & An, J. (2021). Factors Influencing the Reproductive Ability of Male Bees: Current Knowledge and Further Directions. *Insects*, *12*(6), 529. https://doi.org/10.3390/insects12060529