Physiological markers of stress in Western honey bees

Apis mellifera

Apis mellifera collecting pollen from a Cosmos flower

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Abstract

Honey bees likely undergo stress in densely populated apiaries as a result of inter-colony competition. Furthermore, overcrowding of honey bee colonies in an area may negatively affect the regional ecosystem. We aim to define markers for early identification of inter-colony competition stress to allow beekeepers to take timely action and maximize the health and productivity of their colonies. Thisstudy investigates the male reproductive output of drones, energetic costs of competition such as rate of respiration and glycogen storage in foragers, and the expression of heat shock proteins in larvae to detect the physiological markers of competitive stress in honey bees. An experimental competition trial was set up where honey bee colonies were transported and distributed amongst three sites with high (120 colonies), medium (30 colonies), and low (8 colonies) density for four weeks. The samples collected at four time points showed that the respiratory rate of honey bee foragers might be rigid, but the glycogen reserves were depleted in foragers at high density site. Heat shock protein expression was highest after transportation and not due to inter-colony competition. The glycogen stores of honey bees can be used as a potential marker of competitive stress. Analyzing more such physiological indicators, can help identify markers of stress at an early stage to prevent the detrimental effects of stress on honey bees, which will benefit the beekeeping industry and the ecosystem

Acknowledgment

I would like to express my sincere gratitude to my supervisors, Dr. David Pattemore, Dr. Ashley Mortensen and Dr. Tony Hickey for guiding me throughout this process and a pandemic. I am forever in debt for the knowledge and wisdom I have imbibed from your teachings.

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I would like to thank the species without which this research wouldn't be possible, the Western honey bees (*Apis mellifera*). Thank you for teaching me all that you have. You'll be the closest to my heart …(after dogs of course!).

Chapter 1: Introduction

1.1 Background

The sustenance of biodiversity and crop production is highly dependent on pollinators such as Western honey bees (*Apis mellifera*); there arises the need to investigate the physiological processes involved in the stress response of honey bees (Even et al., 2012). Although the honey bee population in New Zealand has increased year by year (MPI, 2021), concerns have been raised over the overstocking of honey bee hives at apiary sites (Newstrom-Lloyd, 2015). Overcrowding of honey bee population often leads to competition for resources, mates and survival (Seeley & Smith, 2015) and can reduce honey collection.

This study focuses on inter-colony competition as a stressor and aims to identify markers of this competitive stress through physiological studies. These markers can help beekeepers identify stress at an early stage to develop a better management strategy, reducing negative impacts on the apiary and surrounding native flora and fauna.

1.2 Stress: What is stress?

The term "stress," being derived from physics, was used by W.Cannon and H.Selye to describe the processes that take place within a body when the state of homeostasis is challenged (Chrousos & Gold, 1992; Selye, 1950). Stress brings about changes in physiology and behaviour that lead to a decline in the productivity of bodily mechanisms. Organisms have evolved physiological processes to mitigate or cope with various environmental stressors (Bordier et al., 2017; Stratakis & Chrousos, 1995).

The general adaptation system (GAS) (Selye, 1950) is one of the most preferred models to assess stress responses in a wide range of organisms (Johnson & White, 2009). It simply states that physiological and cognitive responses can be seen as the alarm, adaptation, and exhaustion phase and various stress responses (Figure 1.1). The organism firstdetects the stress and responds to it as a "fight or flight" response. If the stressor still cannot be avoided,the stress leads to exhaustion and, finally, death (Selye, 1956).

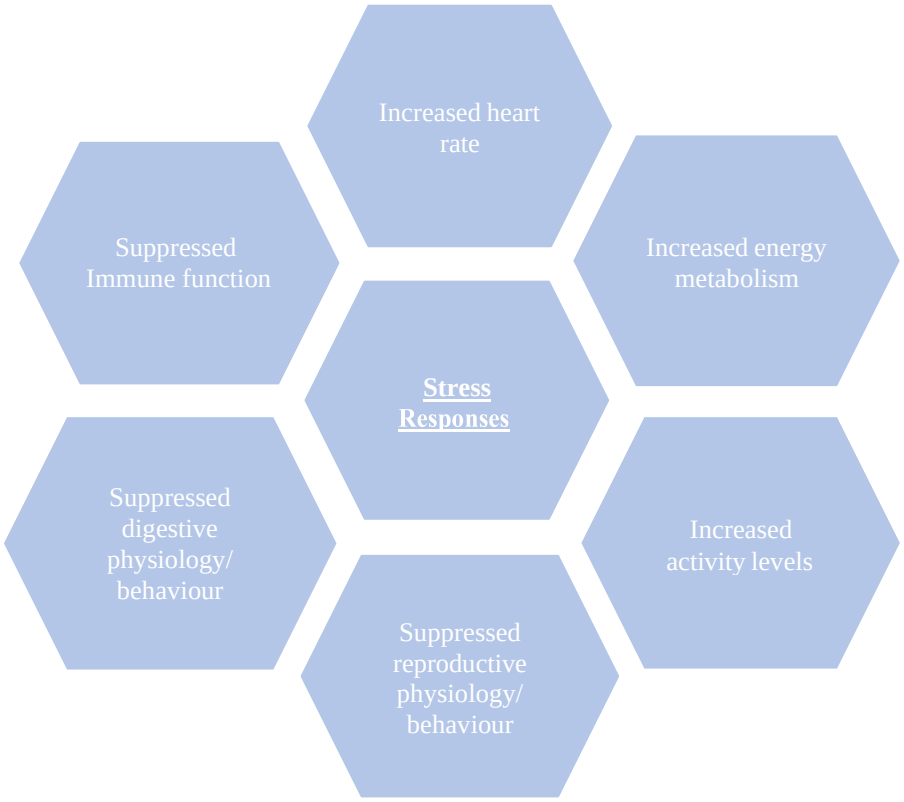

Figure 1.1 : A model of responses to environmental stressors [conceptualized from (Selye, 1950)].The model describes six primary stress responses: suppressed immune function, increased heart rate and metabolism, increased activity levels, suppressed reproductive and digesFiguretive behaviour

For example, the mammalian endocrine system responds to stress by releasing stress hormones (Riddiford, 1980). Physiologically, arthropods experiencing fight or flight situations, a hormone equivalent to noradrenaline known as octopamine, is released (Kononenko et al., 2009). Octopamine, a biogenic amine found in both vertebrates and invertebrates, modulates learning behaviours, the central motor networks, neuromuscular transmissions, and energy metabolism (Farooqui, 2012).

Stressors for honey bees can be divided into biotic stressors that include predators, bacteria, fungii; whereas abiotic stressors comprise insecticides, toxins, beekeeping practices, climate change, and habitats (Havard et al., 2020).

Physiological responses to stress in honey bees have been identified by measuring juvenile hormone (JH), stress proteins known as heat shock proteins and brain biogenic amines such as octopamine,

dopamine; peptides such as adipokinetic hormone, diuretic hormone, and insulin-like peptides neurohormones such as allatostatin-A and corazonin (Bicker & Menzel, 1989; Farooqui, 2012; Kvetnansky et al., 2009; Veenstra, 2009). While behavioural responses caused due to stress include sting extension and probosci's extension (Balderrama et al., 2002; Roussel et al., 2009).

Along with the effects of stress, the following section focuses on the effects of inter-colony competition. This study aims to look at inter-colony competition as a stressor and whether this competitive stress can be detected by investigating the physiology of honey bees.

1.3 Potential impacts of inter-colony competition on physiology

What is competition, and why do individuals compete? Individuals habitually compete over limited food resources, nesting sites, mating, and cover (Armstrong, 1991; Austad, 1983; Dowds & Elwood, 1985; Ewald & Carpenter, 1978).

In resource competition, dominant or aggressive competitors will claim the most lucrative feeding grounds and displace weak competitors spatially (Heller, 1971). For example, marine organisms such as the hermit crab residing in the intertidal zone show intra-specific competition as they compete for a highly nutritious food resource, the carrion (dead decaying animal tissues)(Wilson & Wolkovich, 2011). Due to the competitive displacement of other crabs, these dominant hermit crab species create a zonation pattern where the resources get unequally distributed.

Often when the population of a species in an area increases, it gives rise to exploitative competition for mates and food resources (Schoener, 1983). Similarly, inter-colony competition amongst honey bee species can be observed when the population increases beyond a threshold. There is a chance of inter-colony competition for various reasons such as foraging, resources, mating and territoriality that would leave the bees stressed (Newstrom-Lloyd 2015; Wojcik et al. 2018).

Eusocial hymenopteran males have exceptionally high levels of ejaculate quality, which are assumed to result from extreme selection pressures for pre- and post-copulatory male–male competition and the necessity to retain viable sperm after years of storage in the queen bee's spermathecae (Stürup et al., 2013). The spermatheca is an organ of the female reproductive tract in queen bee that stores live sperm.

Sperm viability is crucial for eusocial hymenopteran male reproductive success, as only live sperm becomes stored in the spermatheca (Collins, 2000). We assume that during inter-colony competition, there would be an increase in male reproductive output (MRO)(Koffler et al., 2016).

Moreover, honey bee foragers might compete with each other for resources in the presence of inter-colony competition. Physiologically, flying requires metabolizing energy stores that might get depleted due to increased foraging activities in the presence of competition. This makes foraging energetically costly (Schmolz et al., 1995), leaving the bees with depleted energy stores (Abou-Seif et al., 1993).

Heat shock proteins (hsp) in insects have been widely researched since the 1900's (Hranitz et al., 2010; King & MacRae, 2015; Morimoto, 1993; Neven, 2000; Pyza et al., 1997). hsp is a group of proteins that work in response to thermal stress. Heat shock protein 70 (hsp70) has become widely recognised as a sensitive biomarker for sub lethal toxicity and whole organism stress (Hranitz, Abramson, and Carter 2010). Preliminary work on hsp70 as a marker of stress in honey bees has been carried out at Plant and Food Research which acted as a baseline for my thesis chapter. We hypothesize that hsp upregulation would be seen in honey bees facing inter-colonycompetition stress.

Nevertheless, when greater number of foragers would forage for a single resource, behavioural dynamics might change and inter-colony competition might cause changes in behaviour such as aggressive foragers.

1.4 Effects of over-stocking at managed apiary sites

Amongst the managed pollinators, the Western honey bee is a cornerstone because the pollination of many crops in most parts of the world relies on this species (Aebi et al., 2011).

Honey bee colonies in the wild European forests used to be placed hundreds of meters apart (Seeley & Smith, 2015). While in the forests of Russia, two colonies were found on trees per square kilometre. Compared to livestock in New Zealand, where for example the stocking rate for cows is 2.7 to 3.7 cows per hectare (Penno, 1999), there is no such recommended stocking rate available for honey bee colonies (Newstrom-Lloyd, 2015). As compared to New Zealand, the hive density in California, US is 3.538 hives/km2 which is similar to the density of hives over the total land area in New Zealand of

2.658 hives/km2 (Newstrom-Lloyd, 2016). Although the density of hives in New Zealand is lower than in California; area wise California is ten times greater in area than New Zealand. Moreover, resource availability and density of hives in an area are factors that mutually affect overcrowding. Therefore, overcrowding in New Zealand is more likely to affect the colonies that are placed with limited resources at different times and locations than what is observed in other countries. For the convenience of beekeepers, hives at apiaries are placed as close as a meter from each other. Compared to feral colonies in the wild, managed hives experienced greater foraging competition, robbing of honey from weak hives in the presence of nectar shortage (Downs & Ratnieks, 2000). Apartfrom this, "drifting" is a common phenomenon where foragers or queens fly back to the neighboringhive returning from a mating flight (Free, 1958). There is also a high risk of transfer of pathogens amongst colonies that are placed close to each other.

In New Zealand, new apiary sites have been placed closed to existing sites that have reached their carrying capacity (MPI, 2017). Some apiary sites have been overstocked in order to obtain more honey production. However, overcrowding of hives is disadvantageous as it leads to lower honey production, increased robbing, loss of colonies, starvation of honey bees and quickly spread diseases and pests to neighboring colonies and sites (Brown et al., 2018).

The data from the Annual Disease Return 2016 NZ Colony loss survey reported colonies lost due to overcrowding by non-commercial beekeepers was 2.37%, commercial beekeepers was 7.44% and large commercial beekeepers was 14.74% (Figure 1.2)(MPI, 2017).

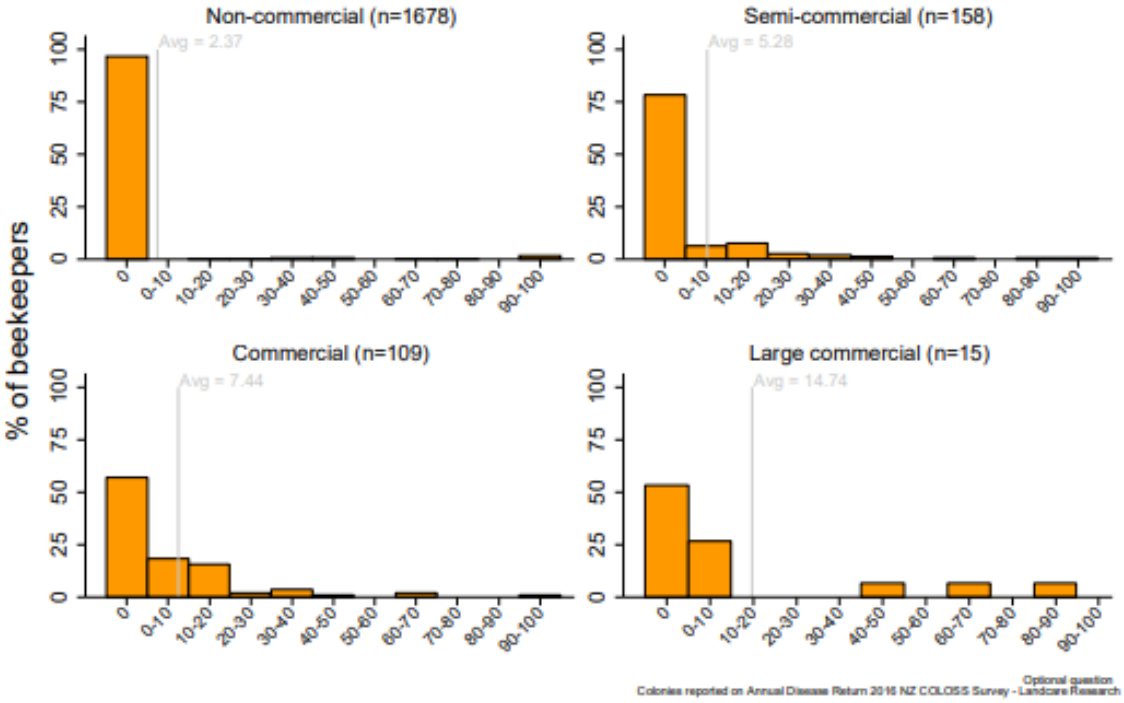

Figure 1.2: The number of apiary sites lost due to overcrowding (2015-2016) (reported by beekeepers in Annual Disease Return 2016 NZ Colony loss survey- Landcare Research)(Brown, 2016)

There is a chance of inter-colony competition due to increased population for various reasons such as foraging, resources, mating, and territoriality that can leave the bees stressed (Newstrom-Lloyd 2015; Wojcik et al. 2018). Moreover, differences in honey production and over-visitation of flowers have been observed when honey bees competed for food resources (Joseph et al. 2020).

Being a eusocial insect, a single honey bee colony may consist of up to sixty thousand workers, five hundred drones, and a queen bee (Anon 2017). Often when the population of a species in an area increases, it gives rise to competition for mates and food resources.

A vast majority of research focuses on various biotic organisms such as mites, mainly varroa mites (*Varroa jacobsoni*) and tarsonemid mites(*Acarapis woodii*); pesticides such as phosmet, infections, and symptoms such as colony collapse disorder (CCD), diseases such as American foulbrood bacterial disease (AFB), deformed wing virus (DWV) and changing climatic conditions that are known to have drastic effectson honey bee colonies(Le Conte et al., 2010; Le Conte & Navajas, 2008; Li et al., 2019). However, stress due to population density has not been explored in New Zealand (Beard, 2015).

Some studies have also observed that honey bee colonies experience stress while competing with surrounding colonies, impacting the native pollinators feeding on the same resources in an area (Mallinger, Gaines-Day, and Gratton 2017; Markwell et al. 1993; Suurd 2020).

1.5 New Zealand's million dollar beekeeping industry

The Western honey-bee is indigenous to Europe, Africa, and the Middle East. However, it has been intentionally introduced to most parts of the world, including New Zealand, to produce honey and improve the pollination of food crops (Dohzono & Yokoyama 2010). Being an agriculturally important pollinator species, these are eusocial insects that live together in colonies (Seeley 2009) and are regarded as the most beneficial pollinators and producers of honey (M. L. Winston 1987).

In New Zealand, the number of apiaries has been increasing rapidly for the past seventy years, leading to the rise of a 550$ million dollar honey industry (Figure 1.3) (Beard, 2015; MPI, 2021).

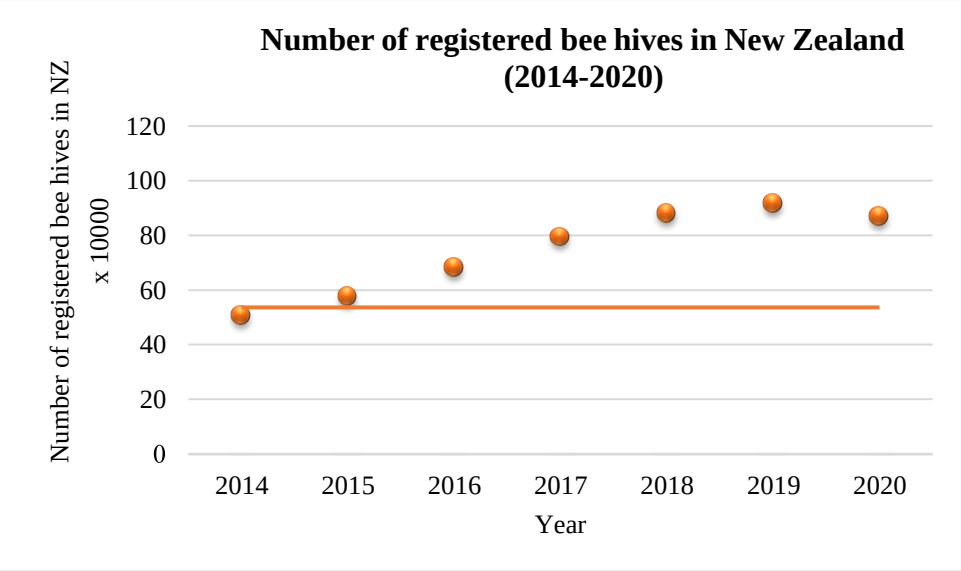

Figure1.3: The number of registered hives in New Zealand from 2014 to 2020. From 5, 07,247 hives[2] registered in 2014, the number of registered hives increased to 8, 69,056 hives[2] in 2020.

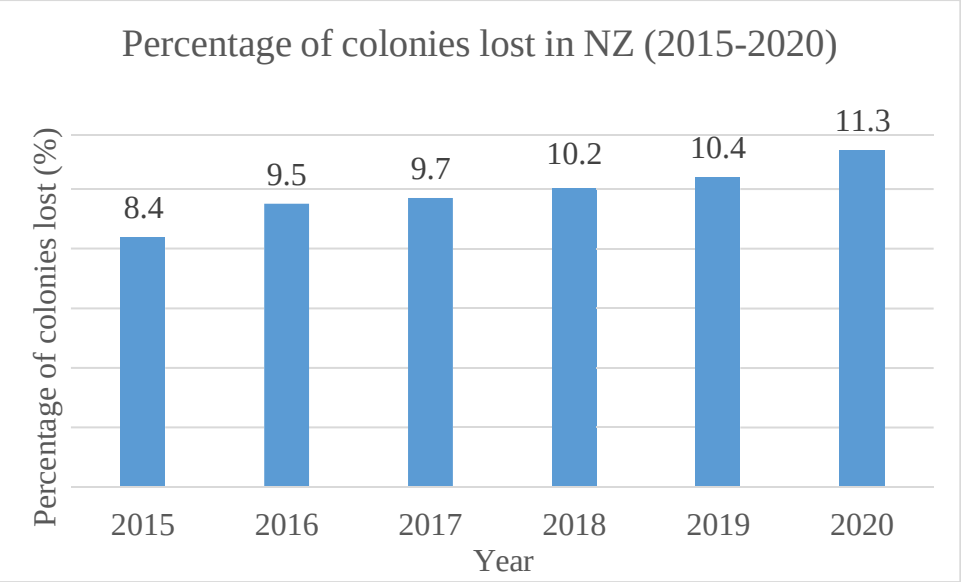

Figure 1.4: The total percentage of managed honey bee colonies lost from 2015-2020 in NewZealand (MPI, 2021)

In recent years, many European and North American countries have reported high rates of mortality affecting their managed honey bee colonies (*Apis mellifera*)(Haubruge et al., 2006; Meixner, 2010; VanEngelsdorp et al., 2012). A similar loss of colonies has been reported in New Zealand (Figure 1.4) (MPI 2020). Despite these losses, there has been an increase in the number of managed honey bee colonies in NZ as the total number of hives has increased threefold of hives present 20 years ago. Alongside these, one of the most concerning issues in New Zealand is the population density of honey bees (Russell, Barron, and Harris 2013). At present, the density of hives over the total land area in New Zealand is 2.658 hives/km^2 (Newstrom-Lloyd 2016). Apart from this, managed apiary sites are being expanded to withhold more hives. This may lead to increased competition between colonies, ultimately leading to stressed honey bees (Newstrom-Lloyd et al 2016). This can have an impact on native pollinators as well as New Zealand's ecosystem (Beard 2015; Markwell, Kelly, and Duncan 1993). Moreover, the ecological implications of managed honey bees on New Zealand's flora and fauna remain poorly known (mainly when there are high hive stocking rates or low seasonal levels of nectar and pollen production)(Beard, 2015).

1.6 Reproductive biology of the Western honey bee

Being eusocial organisms, a honey bee colony is a unit of reproduction consisting of a reproducing queen that gives rise to female workers and male drones (Winston, 1987). Workers carry out different jobs throughout their lifetime, from nurse bees to foragers. Queen bees live inside the hive and are fed by the workers along with the males who have only one function, and that is reproduction (Seeley 2009)**.**

The queen bee lays approximately one thousand five hundred eggs in a day that hatch as larvae. Then these larvae enter the non-feeding pupal stage before emerging as young bees. Fertilized eggs hatch into female workers, and unfertilized eggs hatch into male drones (Figure 1.5) (Winston 1987).

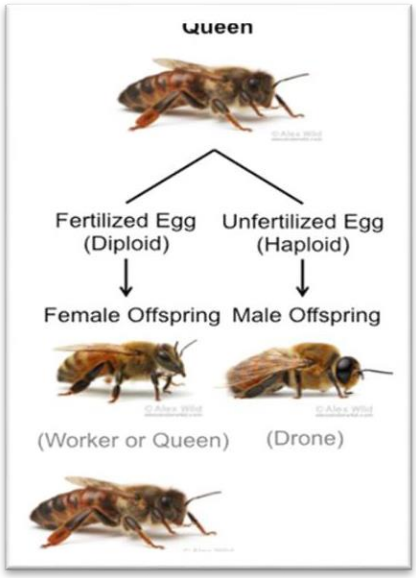

Figure 1.5: Reproductive caste of Western honey bees (*Apis mellifera*) (Mortensen et al., 2013)

A queen bee takes about 16 days to develop into an adult after hatching, while a drone takes 24 days and a worker takes 21 days to mature.

Workers live for four to eight weeks while drones survive up to four months, and the queen bee lives as long as two to three years. The drone's only function is to mate with the queen, tending the brood, building the comb, feeding the queen to foraging (Seeley, 1982).

In this thesis, I will be focusing on identifying markers for western honey bees for early detection of stress due to over-stocking at managed apiary sites.

1.7 Research Aim and Hypothesis

This research aims to answer the following research questions-

1. Does competition lead to an increase in male reproductive output (MRO)?

   If at high density site the number of males that mate with the queen is high, does this competition increase their reproductive output? Moreover, does this lead to males producing more viable sperms to compete against each other and mate successfully with the queen?

2. Is it energetically costly to live in a densely populated area?

   I hypothesize that if foragers are experiencing competition at high density apiary site, they are foraging longer distances to obtain sufficient resources as compared to medium and low densities that do not need to compete due to overpopulation?

3. Can heat shock protein expression be used to measure physiological stress?

   Heat shock proteins are expressed when as organism experiences stress. Are the larvae at high density site experiencing more stress and expressing higher amounts of hsp as compared to the brood at medium and low density sites?

1.8 Research approach

Our research approach includes-

A. Experimental competition trial- This set up will provide samples for all three chapters
B. Lab assessments - These will be conducted for validating a method for detecting hsp70, field trial experiments and testing of MRO samples.

Our research approach includes a combination of experimental competition trial and lab assessments. This study is largely based around an experimental trial where we deliberately set up colonies to be in competition with each other. This set up will provide results for all the research questions.

The thesis is distributed into five chapters. Chapter one (this chapter) introduces the study, Chapter two explains the experimental competition field trial design, Chapter three focuses on validating a method for detecting heat shock proteins (hsp70), Chapter four focuses on the energetic costs of competition (both respiration rate and glycogen stores) from the field trial experiments, and heat shock proteins (hsp70) as a potential measure of physiological stress and chapter five brings in the general discussion.

Chapter 2: Experimental competition trial

2.1 Introduction

In order to assess how competitive stress influences key measures of Western honey bee physiology, I set up an experimental trial that subjected colonies to different population density levels for competition.

The experimental competition trial was designed to investigate differences in physiological measures in Western honey bees (*Apis mellifera*) when managed at varying degrees of competition at the apiary sites.

One of the major concerning issue in the US is their honey bee population declines observed in the almond industry. These almond industry deploy thousands of colonies for pollinating almonds every year (Ratnieks & Carreck, 2010). The suspect behind these colony deaths was overcrowding, poor management practices and lack of forage. Yet, beekeepers lose 30% of their hives every year.

The experimental competition trial was set to lay out low, medium, and high population density conditions faced by managed apiary sites. The density levels in the experimental trial will help observe various stressors that the honey bee population at overcrowded apiary sites face as compared to medium and less populated apiary sites.

All samples for the research objectives of this thesis were collected from this experimental trial.

Twenty-four honey bee colonies were randomly selected as focal colonies from which samples would becollected. A hundred and thirty-six of the colonies were added along with the focal colonies to createa density gradient.

2.2 Timeline of the design

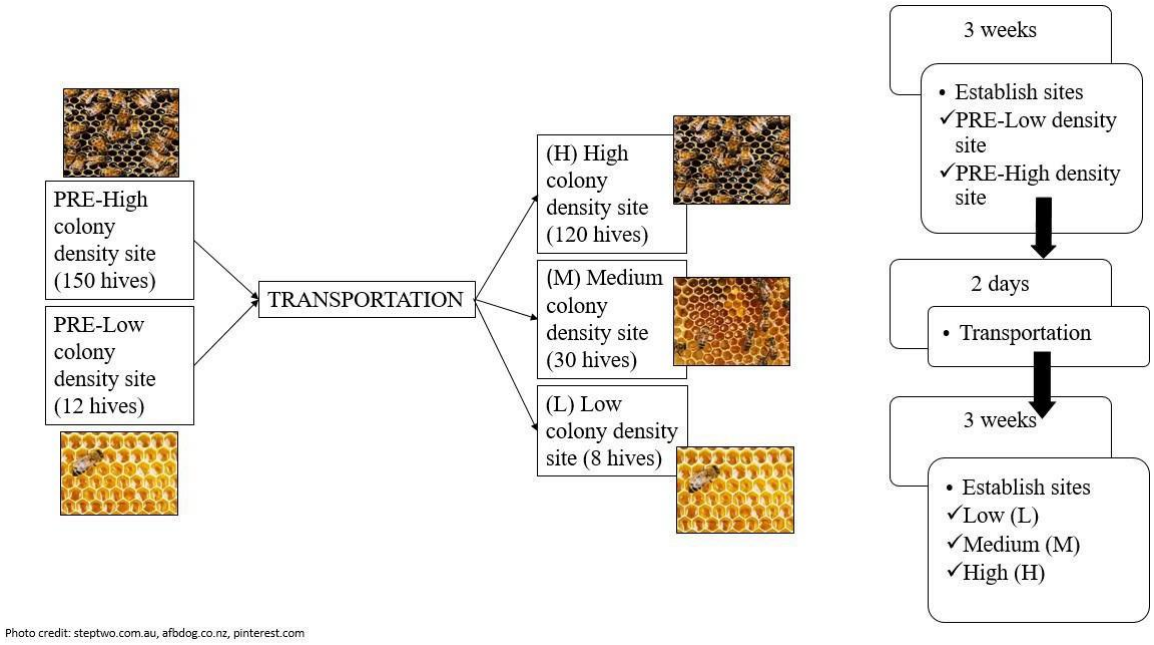

Figure 2.1: Pictorial representation of colony distribution for the experimental competition trial.Photo credits: steptwo.com.au, afbdog.co.nz, pinterest.com

Any colony would have experienced a certain degree of competition considering numerous environmental stressors. As we want to control the competition they are experiencing before our main trial starts, the colonies were placed in a PRE-high-density site while some in PRE-low for a period of three weeks (The colonies are referred with a prefix such as PRE and POST which indicates the apiary density at the timepoint referred to, i.e., before (PRE) and after (POST) transportation). The colonies were maintained in these controlled conditions for three weeks (period for one brood cycle) so that a new generation raised in those conditions would be deployed in POST-high,POST-medium and POST-low density conditions (Figure 2.1).

It was ensured that the selected focal colonies fit the following criteria-

a. the colony was queen right
b. all three stages of brood are visible

c. no apparent signs of diseaseEcrotek Bayvarol© strips were suspended in the hive (for treating susceptible varroa mite infection according to NZ's beekeeping rules and regulations), and drone frames were placed in the hive (for thequeen to lay drone brood).

The hives were distributed across the sites to maintain a density gradient in a manner that eight hives were placed at the low site, 30 hives at the medium site, 120 hives at the high site, and eight hives were at each site were focal colonies for sample collection (Table 2.1). The total number of hives that were included for this trial were based on the number of hives available at Plant and Food Research, Ruakura.

The focal colonies were distinguished with coloureded tapes before the transportation (Figure 2.2). Every hive box was taped with two colours, indicating where it was obtained and which site it would be assigned.

Figure 2.2: Honey bee hives marked with colourful tape to be identified as focal hives

Table2.1: Distribution of colonies for experimental competition trial setup

Density gradient of colony site	No. of colonies from PRE-low	No. of colonies from PRE-high	Others	Total hives
POST-Low	4	4	0	8
POST-Med	4	4	22	30
POST-High	4	4	112	120

In total, this experimental design included 160 hives spread across three locations. Initially, the hives were transported to different locations to establish a pre-high colony density site [(PRE-High) location: 37°51’46”S 175°21’06”E, “Ian”] pre-low colony density site [(PRE-Low), location: 37°46’45”S 175°20’22”E, “Jack”, and 37°48’21”S 175°23’40”E, “Keith”] for four weeks. All three sites were orchards, so had a similar diversity and abundance of floral resource available for the bees. This ensured uniform conditions before the hives were spread across high, medium, and low-density gradients. The hives were distributed across the POST-density sites randomly. These apiary sites were located at a mean distance of 9.6 km and minimum distance of 8 km from each other.

On 17th and 18th December 2020, focal colonies were moved into three different paddocks selected as the three density sites – Keith, Ian, and Jack. The transportation of hives began at 5 am, and a sponge was used to block the hive entrance to avoid drift if it had been done after sunrise when the bees started to forage. These hives were lifted manuallyand placed on three Utes attached with trailers. The sponge was removed once placed at the new site at equal distances.

After four weeks, from 13th to 15th January 2021, colonies were added to the PRE-high and PRE-low sites to maintain density gradient.

These hives were transported to paddocks at Tamahere, Tauwhare, and Ruakura on the 17th of February

2021. The colonies were distributed amongst post-high colony density site [(POST-High) location: 37°46’17”S 175°18’46”E, Ruakura, “POST-High”] (Figure 2.3), post-medium colony density site [(POST- Medium) location: 37°46’45”S 175°20’22”E, Tamahere, “POST-Medium”] and post-low colony density site [(POST-Low) location: 37°45’01”S 175°28’32”E, Tauwhare, , “POST-Low”](refer to Table 2.1).

Figure 2.3: POST-high density site consisting of a hundred and twenty hives

2.3 Collection of samples

Samples were collected on the following days T-1, T+1, T+21, T+35 days, wherein T is the day the hives were transported to POST density sites. (Table 2.1).

Table 2.2: Collection of samples on the four sampling days

ID	Samples collected			Days from first collection(T-1)	Date of collection
	Drones	**Foragers**	**Larvae**		
T-1	Yes	Yes	Yes	0	16/02/2021
T+1	No	Yes	Yes	1	18/02/2021
T+22	Yes	Yes	Yes	22	10/03/2021
T+36	Yes	No	No	36	25/03/2021
	No	Yes	Yes		26/03/2021

Depending upon the objectives of this thesis, honey bee larvae are collected for hsp70 analysis, foragers for energetics, and drones for male reproductive output, as seen in Table 2. The samples were collected on the day prior to transportation, i.e., T-1;a day after transportation, i.e., T+1; on completion of first life cycle after transportation, i.e., T+22 and, on completion of the second life cycle after transportation, i.e., T+36.

Larvae for hsp: Honey bee larvae were used to determine heat shock protein 70. Larvae were collected off the frames and set into culture plates for further analysis. Sixteen larvae from each focalhive were collected on four sampling days, such as on T-1(a day before transportation of hives); T+1(larvae collected after the hives have been transported), and T+22 (larvae emerged from eggs laidby the queen after transportation of hives) (Figure 2.4)

. The culture plated were placed in polystyrene box filled with dry ice in order to flash freeze the larvae. The larvae samples were analysed at Pollination Laboratory, Plant and Food Research Centre,Ruakura.

Figure 2.4: Collecting larvae for hsp70 analysis

Foragers for energetics: Foragers were collected to determine the amount of glycogen present at four different time points. Newly emerged foragers were collected on T-1(a day before transportation of hives); T+1(foragers shall be collected after transportation of hives), and T+22 (Forager adults emerged from eggs laid by the queen after transportation of hives). The newly emerged bees from brood frames were hand-picked and placed in universal bottles immediately stored in a polystyrene box filled with dry ice to flash freeze for storage purposes. These samples were analysed at the Applied Surgery and Metabolism Laboratory, The University of Auckland.

Drones for MRO: 10 drones from each hive were collected on T+1, T+22 (twenty-one days after transportation as drone larvae require twenty-one to twenty-four days to mature); fourteen days after, i.e., T+36 (adult drones emerging from pupae). They were immediately analysed at the Pollination Laboratory, Plant and Food Research Centre, Ruakura.

2.4 Outcomes

For MRO, there was not a significant amount of drones in each of the hives during the sampling days. Instead of sampling every hive, we decided to have mixed samples from each density site.

Therefore, DCAs (Drone congregation areas) were identified by Dr. Ashley Mortensen, and drones were captured on the 25th of March 2021, using net drone traps attached to a helium balloon at every

site (Figure 2.5)(Mortensen & Ellis, 2014).

Figure2.5: Capturing drones in a net at a DCA at POST-high density site

The traps were set up at the same time at every site. Plastic jars with sugar syrup-filled syringes were kept ready to carry the drones to the laboratory for analysis. Five workers were placed in the jars to assist the drones. Every site had five plastic jars for collecting drones. As the drones were trapped in the net, they were lowered, and ten were collected into every jar.

In total, 150 drones were collected to analyse sperm production, motility, and sperm volume.

The drone had to be held at its thorax with the head facing downwards, pressed along the side of the thorax, which brought about eversion and ejaculation (Laidlaw, 1977). Among the 150 drones, only a few were active and mature to obtain the semen by external stimulation. The rest of the drones had to be discarded as no ejaculate was obtained or the drones were weak and immature. The drone ejaculate obtained was placed on a hemocytometer under a light microscope for observation.

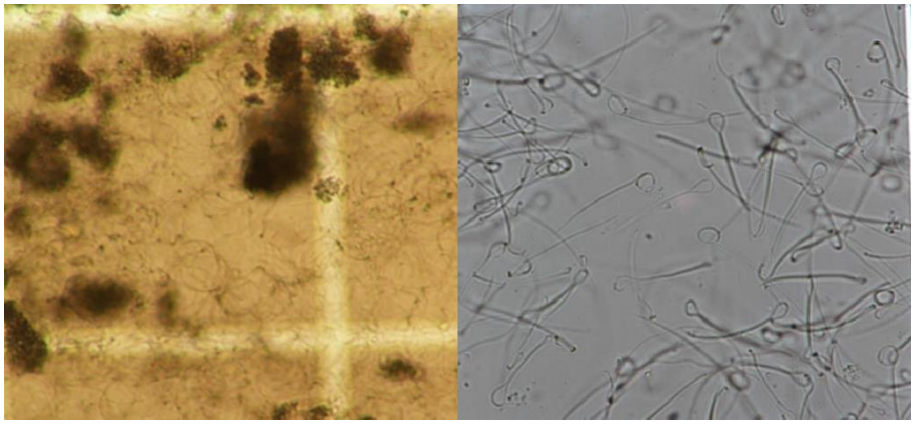

Figure2.6: (A) motile sperm, not-diluted, (B) non-motile sperm, 20% dilution

Figure 2.6 A has rounded sperm that were viable, whereas sperm in Figure 2.6 B are non-viable (Yaniz et al., 2020).

Another attempt to obtain drones and queens with the objective to analyse their glycogen storage across three density sites was planned for the spring season (October-November 2021) but had to be cancelled due to the Covid-19 lockdown.

The next chapter involves the field trial experiments that answer our research questions and analyse potential markers of stress.

Chapter 3: Validating heat shock proteinhsp70 detection method using Human hsp70 ELISA kit

3.1 Introduction

This section provides an overview of validating a methodology to detect heat shock proteins in honey bee larvae using Human hsp70 ELISA kit.

Heat shock proteins (hsp) are a highly conserved family of proteins that are expressed when an organism experiences stress (Hartl, 1996). The expression of heat shock protein induces a response mechanism at the cellular level that helps the organism survive in adverse conditions (King & MacRae, 2015; Ma & Haddad, 1997; Matz et al., 1996; Nagao et al., 1990).

Depending upon their size and function, six hsp gene families fall in the range of small (10-27 kilo Dalton or kD weight) to large (90-110 kD) and are expressed in response to stress(Feder & Hofmann, 1999). In insects, hsps are distributed into four groups: the small heat shock proteins (shsp), hsp60, hsp70, and hsp90. Apart from shsp that act independently of ATP to prevent denaturing of substrate proteins; other groups are hsp60, hsp70 and hsp90. These other groups are ATP dependent and aid the folding of proteins, degradation and disaggregation processes, and cell localization. Their expression influences an organism's cellular metabolism, synthesis of proteins, and cell signaling pathways (Clare & Saibil, 2013; Sakano et al., 2006).

hsp70 is a principal protein of the heat shock protein family and is referred to as a sensitive indicator of stress in many organisms (Gibney et al., 2001; Pyza et al., 1997). In contrast to other bioindicators of stress such as vitellogenin (helps monitor targeted stress response at organ and system level), hsp70 expression is a general bioindicator which is sensitive to both biotic and abiotic stressors (*e.g.*, Maradonna *et al*. 2007).

3.2 Detection of hsp70

Various antibodies have been tested for detecting conserved heat shock protein 70 and heat shock

protein chaperone 70. These have been used to detect protein expression of the hsp70 family when organisms are exposed to stress (Feder & Hofmann, 1999). ELISA (Enzyme-linked Immunosorbent Assay) was developed in early 1971 (Aydin, 2015). This testuses antibodies that bind to target antigens and detect the expression of various hormones and viruses.ELISA has been previously used to detect hsp70 in various insects and yields more precise results than other techniques such as Western Blotting. In a study conducted to detect hsp70 in blue mussel *Mytilusedulis*, provided evidence for ELISA as being more sensitive as compared to western blotting when detecting differences in levels of hsp70 expression.(e.g.,Pempkowiak et al. 2001). ELISA test can detect stress proteins, including hsp70, at critically lower concentrations than those detected by techniques such as SDS- PAGE and Western blotting. (Yu et al. 1994, Vanickova et al. 1995, Pyza etal. 1997).

Cross-species antibodies have been used to detect hsp70. This is evident in the case of detecting honey bee protein band at 70kDa (kilo-dalton) region using monoclonal antibodies of mouse with bovine protein of hsp70 family through Western blotting(Chacon-Almeida et al., 2000).The mouse antibody was able to immunologically identify two protein bands at 70kDa region that indicated homology of heat shock proteins across species. These proteins have been conserved by evolution and are present in all organisms (Matz et al., 1996).

Human hsp70 ELISA kit (Thermo Fischer^{TM}) is an Enzyme-linked Immunosorbent Assay for quantitative detection of human hsp70. We specifically used this kit as it has not been validated for insects. The main aim of this study is to experimentally determine whether this kit could be used to detect heat shock protein 70 expressions in honey bees.

Honey bee larvae were chosen for this study to control the developmental age of the samples and were reared in the same environmental conditions.

3.3 Method description

Sample collection and preparation

The larvae were obtained from the experimental trial at four subsequent time points: T-

1,T+1,T+22,T+36.Three random colonies were selected for sampling from the four time points.

In order to determine whether a pooled sample of larvae would provide a detectable amount of hsp70 protein expression, the following samples were prepared-

Sample 1: One larval head

Sample 2: Three larval heads

Sample 3: One whole larva

Larvae were placed on ice to prevent desiccation. The larval head was cut off using a sterile scalpel and were transferred to 2mL Eppendorf tubes. 600µl of the pro-prep solution was added to each tube. A micro-pestle was used per tube to pestle the larval heads to homogenize the tissues. These tubes were held at -20°C for 30minutes. The tubes were centrifuged in Eppendorf™ 4515R at 13,000 RCF (4°C) for 5 minutes. The supernatant was aspirated into labelled tubes and stored at -20°C until required for measurement of protein concentration.

3.3.1 Protein assay- PRO-MEASURE™ method

The samples were diluted in three ratios as 1:20, 1:50 and 1:70 distilled H_2O. The standards were prepared in 9 tubes. 100µL of distilled H_2O was pipetted into each tube. A serial dilution was 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 H_2O as diluent. A blank was prepared by adding 100µL of distilled H_2O. 1000µL of PRO-MEASURE™ 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.

3.3.2 ELISA- detection of hsp70

A Human hsp70 ELISA kit was used to quantify hsp70. The test protocol in the Human hsp70 ELISA Kit manual was followed with modifications.

The following kit contents had to be prepared as per the user manual in the kit-

1. Wash buffer (use within 30 days): as per the number of samples
2. Assay buffer (use within 30 days): as per the number of samples
3. Biotin-conjugate (use within 30 minutes): Needed to be diluted with assay buffer in 1:100 dilution
4. Streptavidin-HRP (use within 30 minutes): Needed to be diluted with assay buffer in 1:100 dilution
5. Human hsp70 standard (use immediately): reconstituted to the volume stated on the vial.

The number of microplate strips required was determined and placed on the provided holder. Wash buffer and Assay buffer were prepared according to the number of strips used. The microplate was washed with approximately 400µL of wash buffer in each well twice. The solutions were removed by flicking the plate over in the sink. The microplate was dabbed on a paper towel to remove excess wash buffer and kept upside down until the following reagents were prepared to be added within 15 minutes of washing. The Human hsp70 standard was reconstituted by adding 260µL of distilled water and kept aside for 30 minutes.

Standards were prepared in serial dilution by following the "external standard dilution" protocol present in the Human hsp70 ELISA kit. For sample and blank preparation, 100µL of sample diluent was added to blank wells and 50µL to sample wells, respectively. 50µL of the sample was added to each well (Biological replicates were valued over technical replicates as the kits were costly and limited). The plate was covered with an adhesive film and fixed on a microplate shaker for 2 hours at room temperature.

The biotin-conjugate was prepared as per requirements, i.e. 15 minutes before the two-hour incubation period. After incubation, the plate contents were emptied into the sink, and the plate was washed six times with wash buffer solution and 100µL biotin-conjugate was immediately added to all wells. The plates were then covered with adhesive film and left on a microplate shaker for an hour at room temperature. Streptavidin-HRP was prepared 15 minutes before completing an hour of the incubation period. The plate contents were emptied into the sink, and the plate was washed six times. 100µL of

freshly prepared streptavidin-HRP was added to all wells. The plates were covered with an adhesive film and left on a microplate shaker for 30 minutes at room temperature. The plate contents were emptied into the sink, and the plate was washed six times. 100µL of TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution was added to all wells and again plates were covered with an adhesive film and left in the dark for 30 minutes at room temperature. The absorbance was then measured at intervals of 15 minutes at 450nm within an hour of adding the TMB Substrate. 100µL of stop solution was added to each well after sufficient development of a dark blue colour (Figure3.1). The absorbance was measured at 450nm using a FLUOstar® Omega plate reader (FLUOstar® Omega, BMG Labtech) before an hour had elapsed.

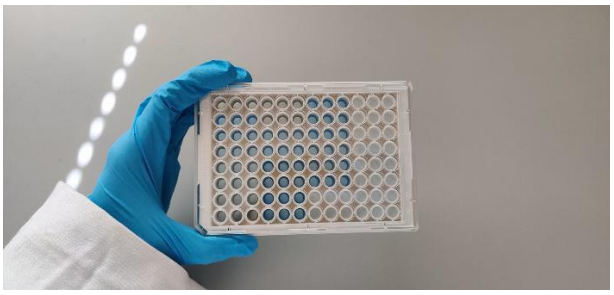

Figure 3.1: ELISA plate with blue colour developed after detecting of hsp70 in the samples.

3.3.3 Output

The Human hsp70 ELISA kit works in detecting western honeybee hsp70 and can help to quantify it to a certain extent.

The values obtained from the standard curve provide evidence for the kit being able to detect hsp70 expression. However, comparing these results with other data sets is difficult at this early stage as proper quantification has not been Figured out using this method.

Further steps in the development of this method would be using control samples that are not exposed to stress and stressed samples.

Chapter 4: Field trial experiments

4.1 Energetics

4.1.1 Respiration in old foragers

4.1.1.1 Introduction

Overstocking honey bee hives at a single site might be beneficial for managing apiaries, but it may be disadvantageous for the honey bee population (Newstrom-Lloyd, 2015; Seeley & Smith, 2015). Compared to widespread feral colonies, honey bee colonies situated at an over-populated apiary site tend to experience inter-colony competition while foraging (Crane, 1990).

Honey bee workers have to take on various tasks as a part of their development, beginning as nurse bees and ending as foragers (Winston, 1987). Foragers have the most crucial and high-risk task of searching for pollen and nectar to feed the hive. Some studies show that various stressors such as parasites, colony disturbance, and low hive reserves push workers to start foraging at an early stage (Perry et al., 2015). A high death rate has been documented in precocious forgers (Amdam, 2011). The task of foraging introduces an additional level of complexity and stress (Wolf & Schmid-Hempel, 1989). A colony that is under external stress, such as competition for resources in an overstocking situation, might show signs of stress in the energetics of foragers.

As a key component of energetics, respiration in insects has been a topic of interest for metabolic physiologists since the early 1670s (Woodworth, 1932). The majority of studies on insect respiration are focused on a respiratory pattern called discontinuous gas-exchange cycle (DCG) studied in ants, beetles, and termites. (Hetz & Bradley, 2005; Klok & Chown, 2005; Lighton, 1996). Research on the subject has been mostly restricted to comparing temperature and age as the only crucial driving factors of respiratory patterns in insects (Bernd & 1979; Klok & Chown, 2005).

An increase in respiration rate is known as a stress response in animals (Johnson & White, 2009) and has been referred to as an indicator of stress in insects (Kestler, 1991; Lima et al., 2016). The highest energy expenditure is incurred during flight by insects, equating to higher metabolic rates (Kammer & Heinrich, 1978). The metabolic rates of insects are best analysed by investigating respiration because CO_2 is a by-product of every cell's activity in the body (Woodworth, 1932). In the case of pollinators such as honey bees, they use aerobic carbohydrate oxidation while flying; therefore, CO_2 emission and consumption of oxygen provides a correspondent (1:1 ratio) measure of oxygen consumption and measure of metabolic rate (Rothe & Nachtigall, 1989). This section investigates the amount of CO_2 expired by foragers amongst focal hives in POST- density environments to detect differences in respiration rate and determine whether this was associated with the density conditions they were experiencing, as a potential measure of competitive stress.

4.1.1.2 Aims and Hypothesis

I hypothesize that honey bees that are experiencing stress due to resource competition at an apiary site would forage more frequently and over longer distances than non-stressed bees in order to collect the pollen or nectar they are seeking. This would cause them to adapt to a higher rate of respiration compared to the bees at moderately and low populated sites.

This chapter compares respiratory rates amongst the POST-high, POST-medium, and POST-low density sites (see Chapter 2 for detail on the experimental design), by measuring the amount of CO_2 expired by individual foragers at the three sites.

4.1.1.3 Materials and methods

4.1.1.3.1 Sample collection

For measuring the rate of respiration in honey bees (*Apis mellifera*), foragers from focal hives placed at each of the three sites were captured between March 2021 and April 2021. The returning foragers were caught using an insect vacuum catcher (Spider Vac, KATCHA^{TM}) after temporarily obstructing the hive entrance with a piece of sponge.

I collected ten returning foragers from four out of the eight focal hives at each of the three sampling sites to reduce the number of samples. This data contains 40 honey bees from three sampling sites, i.e., 120 honey bees in total. I placed the foragers in a plastic jar (Clear Round Wide-Mouth 6 oz Plastic Jars; ULINE, Wisconsin, United States; Model no.: S-12753B-W) and fit the plastic jar cap with a syringe filled with sugar syrup (50% w/v), (Figure 4.1). These were brought to the lab and maintained at 26°C in the incubator. The honey bees were sampled for respiration rate within three days of capturing.

Figure 4.1: Honey bee foragers in a plastic jar with sugar-filled syringe

4.1.1.3.2 Setting up the CO_2 measuring system

I used an industrial-grade CO_2 measuring sensor (ExplorIR®-M 20% CO2 Sensor, CM-40531 Development Kit) for measuring the amount of CO_2 produced by honey bees. A plastic cover was 3D printed to hold the sensor and adapt the sensor so that it would fit the opening of a plastic cream bottle (Figure 4.2). The sensor also measures humidity temperature and atmospheric pressure, and the software reports the CO_2 in parts per million or percent.

The CO_2 sensor is connected to the laptop via USB cable, and the output of the CO_2 sensor is digitized and read by running Gaslab Version 2.3.1.3 software. The CO_2 sensor was conFigured to atmosphericCO_2 (410 ppm) before each measurement in the software's calibration tab. The software displayed a real-time graph while recording humidity, CO_2, atmospheric pressure, and temperature in the measuring chamber at 1 s intervals. The temperature in the chamber was between the range of 20°C to 27°C depending on the surrounding temperature in the lab. Data were saved as .csv files and these were exported and analysed using Excel.

The measuring chamber (500ml plastic cream bottle) was re-oxygenated at the beginning of every CO_2 measurement for two minutes using an aquarium air bubbler (Aquarium air pump STELLAR W-40 (230-240V ~ 50Hz, 3W). Holes were punched into the measuring chamber at an equal distance from the top and bottom, and two pipette tips were inserted. The pipette tips faced opposite directions to facilitate rotation of the air and were glued into the holes using hot glue. The pipette tip near the mouth of the measuring chamber was labelled as an inlet and the one at the bottom as an outlet. For logging the forager's respiratory activity, the CO_2 sensor was fit on the mouth of the measuring chamber. Between re-oxygenations, the pipette tips were sealed with blu tac to prevent any leakage of gas.

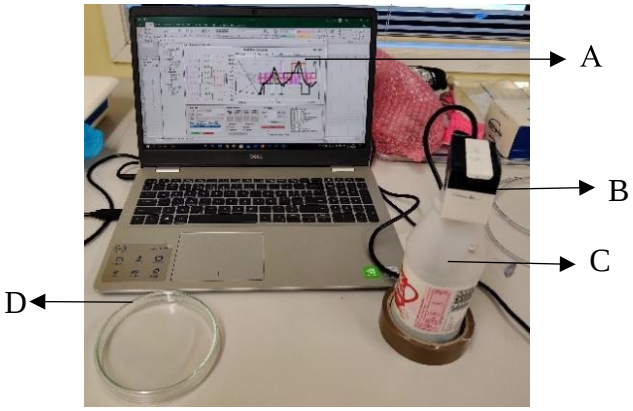

Figure 4.2: Set-up for measuring CO_2 respiration by forager honey bees, showing: (A) Real- time graph by Gaslab software, (B) CO_2 sensor, (C) CO_2 measuring chamber, and (D) a petridish for weighing the honey bee.

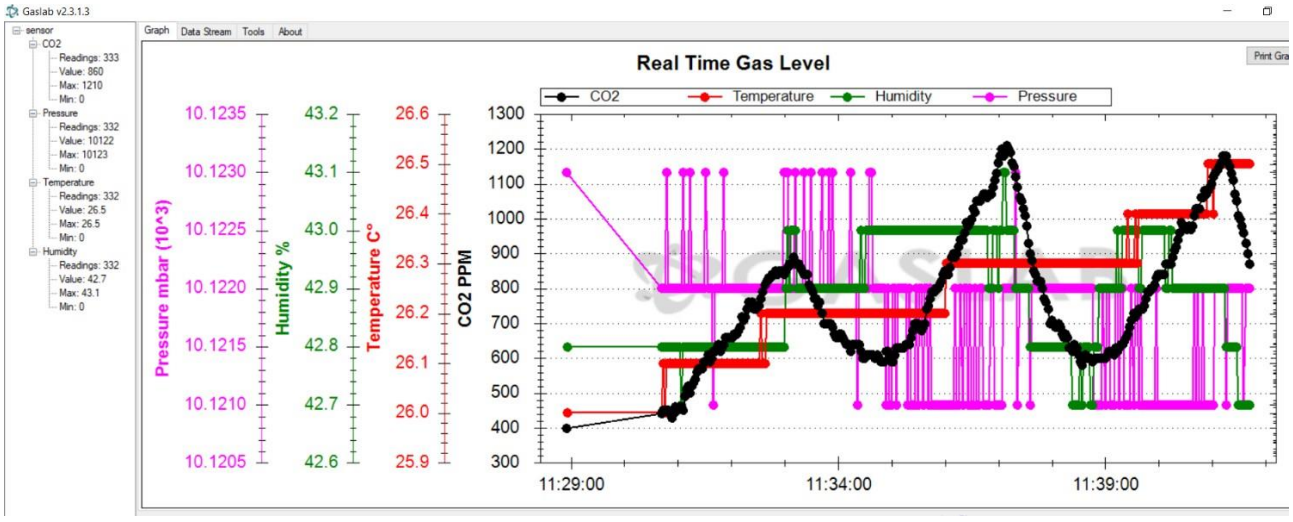

Figure 4.3: Real-time graph displaying output of respiring forager in the measuring chamber inamount of CO_2 (ppm) per second.

The honey bee was then transferred into the measuring chamber (Figure 4.2) using blunt forceps. The CO_2 production of the honey bee was logged in real-time on the software (Figure 4.3).

After placing the honey bee in the measuring chamber, I initially re-oxygenated the measuring chamber for two minutes, after which the Gaslab software was run.

I re-oxygenated the measuring chamber by attaching the air bubbler through the inlet pipette chamber every two minutes allowing the accumulated CO_2 to exit through the lower pipette outlet. In order to minimize errors, the measuring chamber was re-oxygenated every two minutes to obtain three peaks of CO_2 accumulation. A total of 10-minute reading of every individual honey bee was recorded.

The fresh mass of the honey bee was determined immediately after by placing the honey bee in a petri dish on a weighing scale. This method was repeated for all 120 honey bees. The rate of respiration was estimated by performing calculations in Microsoft Excel 2016 and RStudio software R version 3.6.3 for statistical analysis.

4.1.1.4 Calculations

In order to calculate the amount of CO_2 expired by a forager, the 10-minute Gaslab reading was exported as a ".csv" file. The data from this file was copied onto an excel sheet. A scatter graph with straight lines and markers was plotted using the data. The three curves were plotted onto three individual graphs and slope was determined using the formula

$$Y = mx + c$$

The mean of the three curves was calculated, resulting in a value, (b) in parts per million (ppm) moles of CO_2/sec. The formula used to convert the amount of CO_2 expired in ml of CO_2/min/gm body weight units is

$$A = (b * 60 \text{ seconds}/ 1000000) * (\text{volume of the chamber}) / \text{weight of the forager}$$

Where A is the amount of CO_2 expired (ml of CO_2/min/gm) by an individual forager.

The data calculated in Rstudio did not include negative or 0 values.

4.1.1.5 Results

The mean amount of CO_2 expired by foragers at each POST-density site are shown in the Figure 4.4, Figure 4.5 and Figure 4.6.

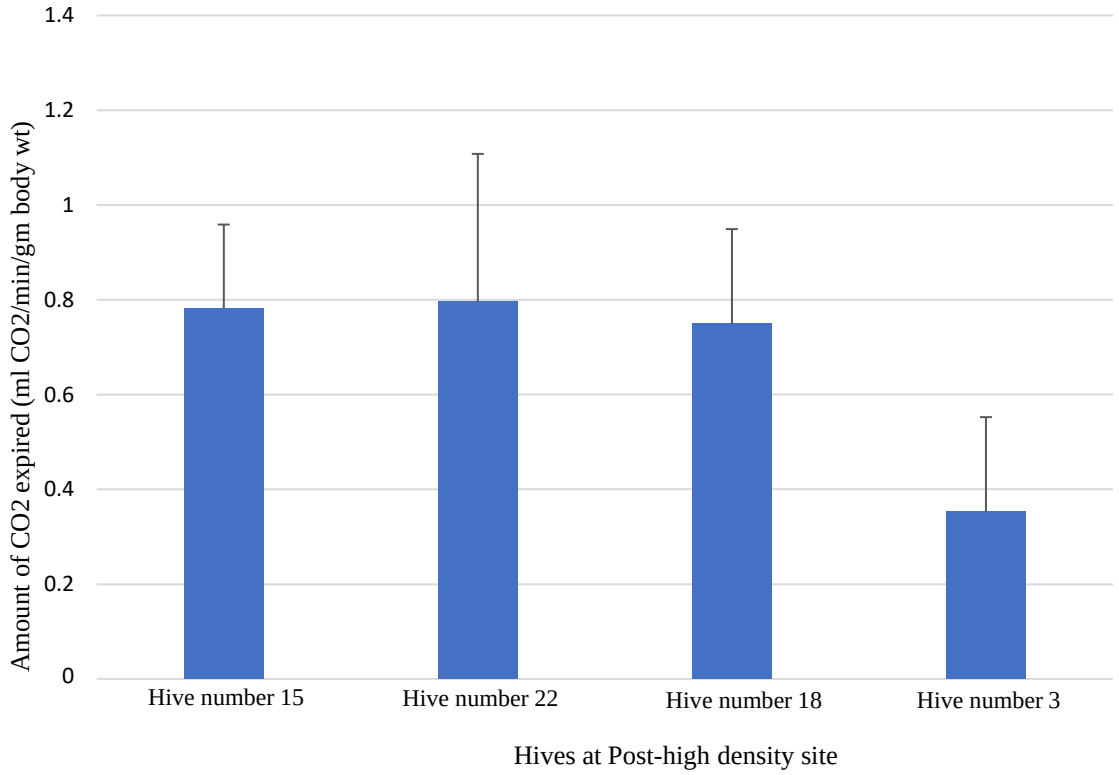

Figure 4.4: Mean amount of CO_2 expired by foragers at POST-high density site (ml CO_2/min/gm body wt). The error bars represent standard error calculated for each hive independently.

At POST-high density site, seven foragers from hive number 3 did not survive, therefore, were excluded from the data.

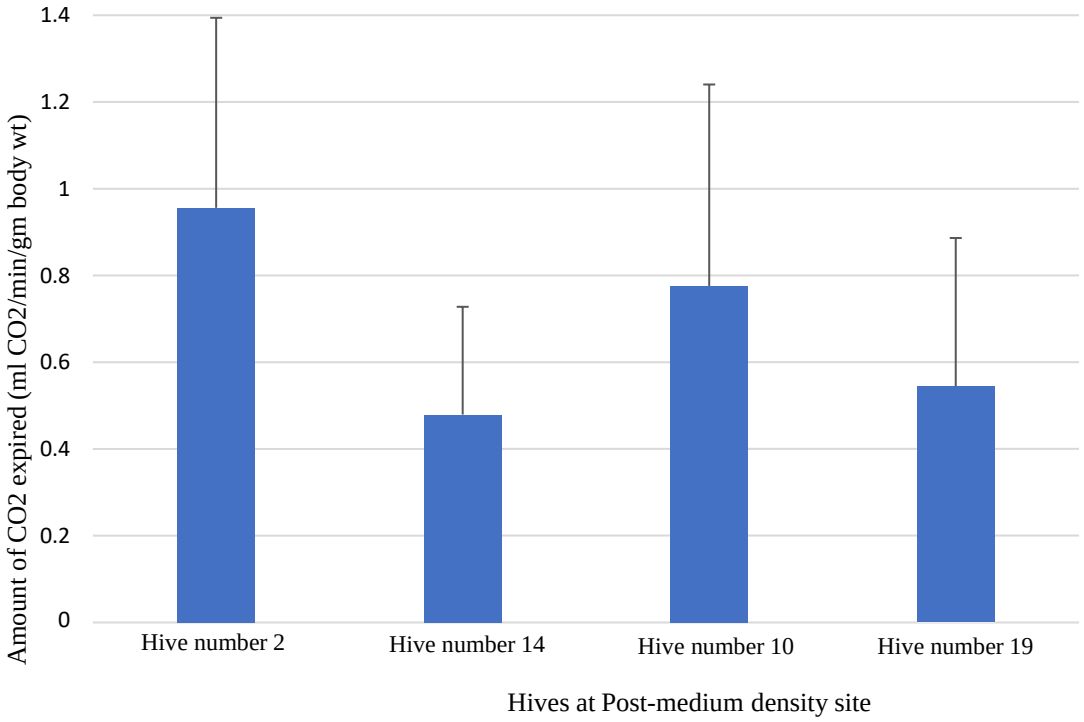

Figure 4.5: Mean amount of CO_2 expired by foragers at POST-medium density site (ml CO_2/min/ gm body wt). The error bars represent standard error calculated for each hive independently.

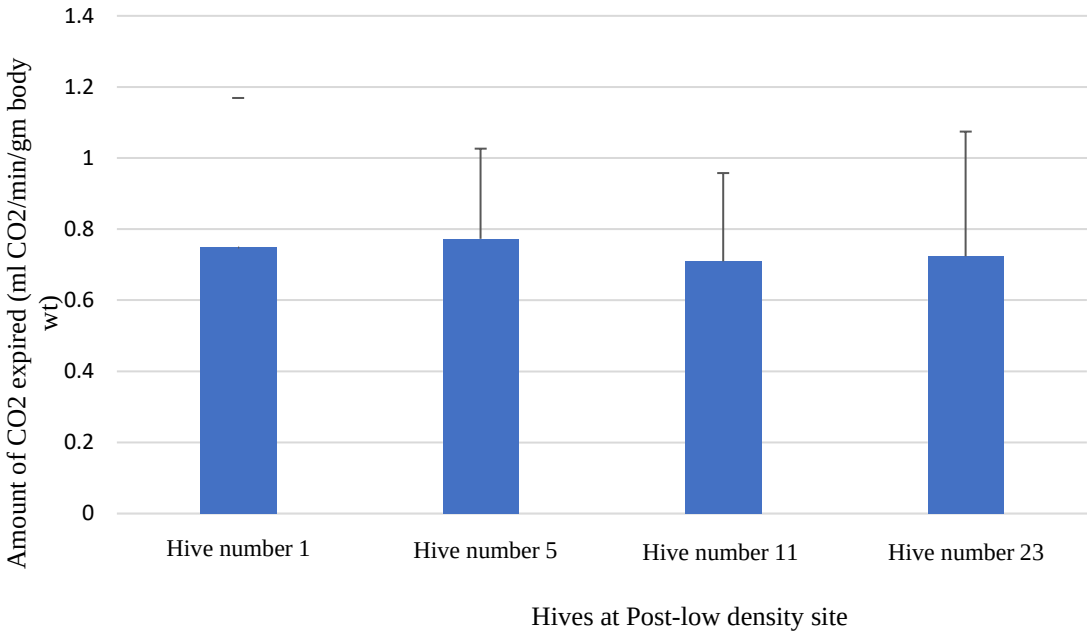

Figure 4.6: Mean amount of CO_2 expired by foragers at POST-low density site (ml CO_2/min/ gm body wt). The error bars represent standard error calculated for each hive independently.

The mean amount CO_2 expired by foragers belonging to POST-high density site is 0.76 CO_2/min/ gm body wt, for POST- medium it is 0.70 CO_2/min/ gm body wt and for POST- low it is 0.74 ml CO_2/min/ gm body wt. The differences between the three density sites were tested using analysis of variance (ANOVA) to determine the confidence with which the hypothesis could be accepted or rejected (Figure 4.7) and performed a Tukey honestly significant differences (HSD) (Figure 4.8) mean comparison test using RStudio software R version 3.6.3

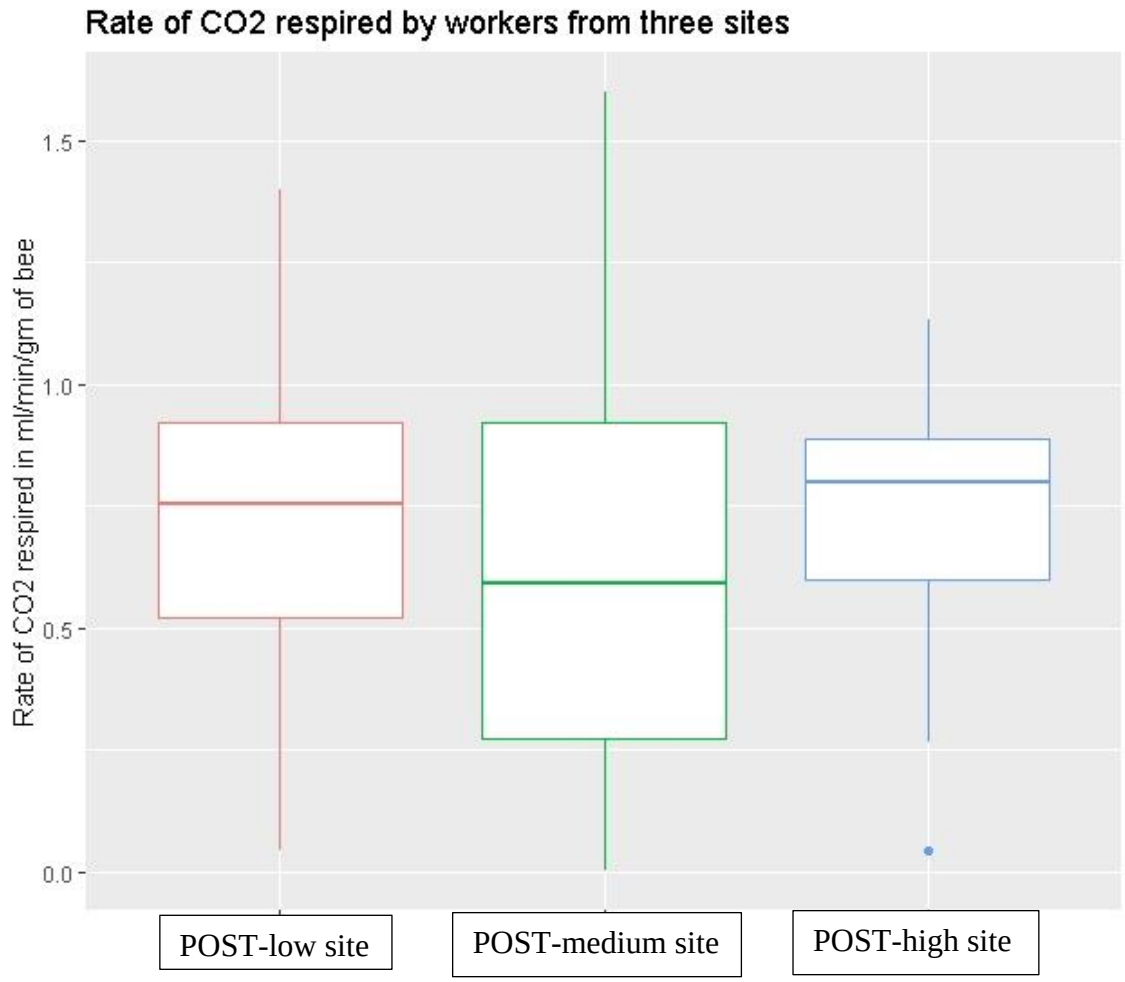

Figure 4.7: One-way ANOVA plot from POST-high, POST-medium and POST-low site

Table **4.1: One-way ANOVA statistical analysis result. "Df" means the degrees of freedom in the source. "Sum sq" means the sum of squares due to the source. "Mean Sq" means "the mean sum of squares due to the source." F value means the *F*-statistic. P means the *P*-value.**

Source	Df	Sum Sq	Mean Sq	F value	P
Hive sites	2	0.243	0.121	**0.999**	**0.372**
Residuals	101	12.278	0.122		

One-way ANOVA P value resulted to be insignificant as P value is > 0.05. After one-way ANOVA the post-hoc Tukey (HSD) analysis was run and used for multiple comparison of the sites paired in groups as shown in table 3.

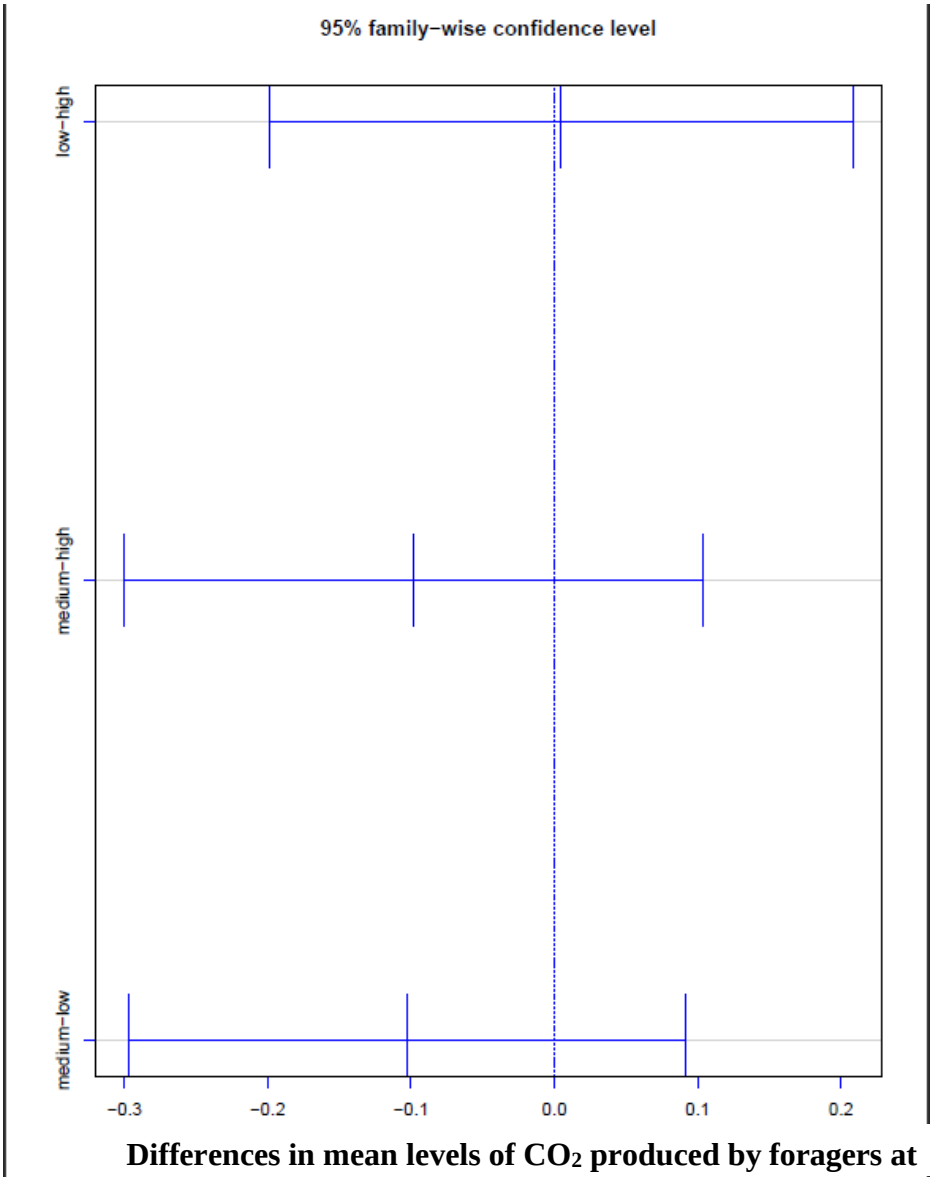

Figure 4.8: Tukey (HSD) graph showing the mean difference for each pair of sites; low-high,medium-high, medium low. Overlapping mean levels of CO_2 expired by foraging honey beesfrom the three sites indicate there is likely no difference between them.

Table 4: Tukey (HSD) result obtained from RStudio. The values of "diff" column on the table are the mean difference between the groups. "lwr" giving the lower end point of the interval, "upr" giving the upper end point and "p adj" giving the p-value after adjustment for the multiple comparisons.

	diff	lwr	upr	p adj
low-high	0.005	-0.198	0.208	**0.998**
medium-high	-0.098	-0.300	0.103	**0.481**
medium-low	-0.103	-0.297	0.091	**0.419**

The Tukey (HSD) analysis calculated by the data in ANOVA has resulted in an insignificant P value (P value>0.05). The p adj value for low-high is 0.998, medium-high is 0.481, for medium-low is 0.419 (table 4). This indicates that there is no difference between the mean of respiration rate for the three groups. Therefore, there is no evidence that the population densities and competitive stress had any effect on the respiratory rate of honey bees.

4.1.1.6 Conclusion

Variation in the amount of CO_2 expired by foragers (CO_2/min/ gm body wt) amongst hives at the three sites is observed to be minimal. On further analysis, one-way ANOVA and Tukey (HSD) provide evidence that no variation was observed in the amount of CO_2 expired by foragers belonging to the three density sites.

This could be due to incorrect measurements of the respiration rate using the CO_2 meter system, human error or a general variability in the samples due to a smaller sample size. Thus, I will be rejecting the hypothesis that foragers experiencing stress due to overpopulation tend to showcase an increased respiratory rate. The respiratory rate is possibly conserved in insects (Lighton,1996).

Moreover, every flying insect does require a certain amount of energy in order to fly. Insects, when faced by adverse environmental conditions, are unable to adapt beyond the capacity of the flight muscle structure. The muscle's aerobic capacity restricts the insect's flight and foraging ability (Suarez et al., 2005). Furthermore, beyond a specific respiration rate would lead to muscle loss and affect their ability to fly (Buck, 1962).

Nevertheless, the CO_2 measuring system was able to detect carbon di-oxide expired by foragers in the measuring chamber (CO_2 in ppm (parts per million)). But, calculated respiratory rates are quite high compared to other papers (Allen, 1959; Blanke & Lensing, 1989). Although, precise measurements would be possible with the help of flow cytometry which is generally used in respirometry studies. Flow cytometry can help erase trace amounts of CO_2 accumulated and provide a more sensitive measuring system. . In fact, studies have measured metabolic rates during hovering using respirometry chambers and also during forward flight in relatively large wind tunnels and modern gas analyser system such as differential infrared carbon di-oxide gas analyser (DIRGA) (Harrison & Fewell, 2002; Kovac et al., 2007).

However, measuring the resting metabolic rates of these foragers and comparing it with their active respiration rate could provide more evidence to the study in determining whether the colonies experienced stress during the experimental competition trial.

The next section is set to determine the amount of glycogen content stored in honey bees and will continue to explore the relationship between metabolism and population density. Although this shows that have similar metabolic rates. Glycogen analysis provides a different view.

The next section is built on another vital aspect of energetics that is the amount of energy, here glycogen stored in bees.

4.1.2 Glycogen storage in newly emerged bees

4.1.2.1 Introduction

This chapter questions: does competition at a densely populated area make it energetically costly for honey bees to sustain their colony and honey production? To do so this section investigates the glycogen reserves of newly emerged bees exposed to inter-colony competitive stress at three differently populated apiary sites.

The diet of honey bee colony is rich in sugars as their metabolism is based on carbohydrates rather than lipids (Hepburn et al., 1979; Leta et al., 1996). Carbohydrates are the main energy source for honey bees, and are mostly obtained through pollen and nectar collected by foragers (Seeley, 1982). Worker honey bees at the nurse stage initially consume mostly protein rich pollen and then go on to consume nectar and honey, providing them sufficient energy for flight (Crailsheim et al., 1992).

Glycogen is present in every insect's body in varying amounts(Steele, 1982). It serves as a metabolic energy reserve. Glycogen is a polymer made up of glucose units. Glycogen in muscles acts as a carbohydrate energy reserve. Other energy sources are available such as lipids and trehalose, trehalose acts an osmolyte while it is present as trehalose in fat bodies and organs. The trehalose can also be oxidized for energy, and is used for adaptation to cold or used to form chitin(Pimentel et al., 2017).

During flight, insects such as honey bees utilize glycogen, while larger insects such as locusts tend to use lipid reserves (e.g. locusts) for releasing energy (Mayer & Candy, 1969). Flying insects such as bees, express enzymes of glycolysis at some of the highest levels measured in animals as their flight muscle contraction rates are quite high. At that rate, oxidation of lipids is not possible, nor do bees form lactate.

Adipokinetic hormone stimulates the breakdown of lipids, it also increases glycogen phosphorylase activity in the fat body to help metabolize fats in insects (van Marrewijk et al., 1986). Glycogen phosphorylase acts through the process of phosphorylysis to break down glycogen by cleaving the terminal glucosyl residues from the glycogen polymers and making free glucose 6-phosphate immediately available for glycolysis and activity (Steele, 1982). The adipokinetic hormone is increased

when insects are exposed to general stress. Such as when locusts (*Schistocerca gregaria*) are exposed to insecticides that induce oxidative stress and in firebugs (*Pyrrhocoris apterus*) that that were exposed to mechanical stress (Candy, 2002; Kodrik, 2008; Kodrík & Socha, 2005). This would indicate glycogen stores are accessed in stressful times.

Honey bees that are exposed to competitive stress, display an increase in metabolic demand (Even et al., 2012). To meet high metabolic demands, they likely rapidly metabolize their energetic reserves. After emerging from a cell, the newly emerged bee slowly starts feeding on pollen as its hypopharyngeal glands, body fat, organs, and muscles start developing (Winston, 1987). However, the growth and nutrition of bees is dependent on the state of the colony, sufficient pollen reserves, quality of brood rearing and the queen, the supply of nectar and pollen by the foragers (Levin & Haydak, 1951). All these factors play a role on the lifespan of worker bees and in building a sustainable colony (Haydak, 1970).

For this section, newly emerged bees were selected to study whether the amount of stored glycogen was affected due to stress and competition levels at three density sites. Newly emerged bees were collected on T-1 day, T+1 day, T+22 days, and T+36 days.

The experimental competition trial provided all the samples at subsequent time points for comparing the amount of stored glycogen in newly emerged bees.

4.1.2.2 Aims and Hypothesis

When honeybee colonies are experiencing competitive stress, the amount of stored glycogen in newly emerged workers may be depleted. In comparison, the colonies at medium density site and low density site that are not experiencing high levels of competitive stress are predicted to display moderate and normal levels of stored glycogen. If glycogen reserves of bees living at high density sites is depleted to an extent more than the ones at medium and low density sites, this may indicate that they are using up more energy, making it costly to live in a densely populated area.

The main aim of this study is to estimate the amount of stored glycogen in newly emerged bees from twenty-four focal colonies and compare it amongst other sites.

4.1.2.3 Materials and methods

I collected the samples and quantified protein, free glucose, and total glucose to determine stored glycogen per bee and hive-site at four different time points.

4.1.2.3.1 Sample collection

Newly emerged bees are a day old and were identified by hair on their head and an overall pale yellow body colour (Seeley, 1982). Ten newly emerged honey bees were collected from the brood frames of each focal hives on four different time points; T-1, T+1, T+22, T+36, making it a total of 960 honey bees.They were placed in universal glass bottles and stored in dry ice to flash freeze them. The honey beeswere then stored in a -80°C freezer at Plant and Food Research Centre, Ruakura. The stored samples were transported to be analysed at ASML (Applied Surgery and Metabolism Lab), The University of Auckland.

4.1.2.3.2 Quantitation of protein and glycogen

A pilot trial was conducted by randomly selecting 40 honey bees (as seen in Table 5) from T+1 to determine dilution factors and evaluate unknown concentrations obtained. The protein concentrations act as a baseline for calculating glycogen concentration per body weight of the bee samples.

Table 4.2: Honey bee samples selected for the pilot trial

Focal hive id number (T+1)	Honey bee ID number				
J22	1	2	3	5	8
J24	1	4	5	7	8
I6	2	5	6	8	10
I8	1	2	5	9	10
I9	1	2	4	7	10
I11	2	3	4	5	8
I12	1	2	4	8	10
K16	1	2	4	5	8

4.1.2.3.3 Preparing sample homogenate:

The honey bees were placed on an ice top to remove hair and wings to prevent desiccation. While the honey bee was held with soft forceps, a scalpel was used to shave off the hair on the head and thorax. The wings were pinched off with sharp forceps, and the honey bee was placed in labelled 2mL Eppendorf tubes (Eppendorf Safe-Lock sterile microcentrifuge tubes) after weighing individually. 1000µl of PBS solution was added to the Eppendorf tube. Stainless steel beads were added, and each tube was placed in the adapter and securely clamped into TissueLyser II (Qiagen) for 10 minutes to rapidly homogenize the sample. The stainless steel beads were removed, and the homogenate was stored in the -80°C freezer until further use.

4.1.2.3.4 Quantitation of total protein

Preparation of microplate assay for protein quantitation:

Diluted bovine serum albumin (BSA) standards and working reagent were prepared following the

microplate procedure as stated in Thermo scientific™ Pierce™ BCA Protein Assay Kit user guide. 5µL of homogenate was added to Nunc™ MicroWell™ 96-Well Microplates (Thermo Scientific™) instead of adding 25µL of the sample (homogenate) as stated in the protocol. The plates were placed on a plate shaker for 30 seconds at room temperature for 30 minutes. The absorbance was measured at 562nm by SpectraMax iD3 Multi-Mode Microplate Reader. Using Excel, the amount of protein was calculated in mg/mL of a honey bee.

4.1.2.3.5 Quantitation of glycogen

Sample homogenates were taken out of the freezer and placed on ice (To prevent the samples from desiccating, the tubes were kept on ice in polystyrene boxes until microplate assay was prepared). Two sets of 2mL Eppendorf tubes were labelled for each total glucose assay and free glucose assay; one for preparing diluted homogenate and the other for preparing the assays. 200µl of homogenate and 50 µl of perchloric acid (PCA) were added to 2mL Eppendorf tubes. The tubes were vortexed and left on ice for 10 minutes.

4.1.2.3.6 Preparation of homogenate for free glucose quantitation

100 µl of homogenate was pipetted into a new set of 2mL Eppendorf tubes, 50 µl KHCO3 and 10 µl of amyloglucosidase (AMG) were added to each tube. These tubes were incubated for 2.5 hours at 37°C on Eppendorf ThermoMixer® C. These tubes were centrifuged at 10,000 rpm for 5 minutes at 4°C.

4.1.2.3.7 Preparation of homogenate for free glucose quantitation

100 µl of homogenate was pipetted into a new set of 2mL Eppendorf tubes, and 60 µl of $KHCO_3$ was added. These tubes were kept on ice until centrifuged at 10,000 rpm for 5 minutes at 4°C.

Preparation of standards:

Six glucose standards were prepared in the following concentrations- S1 (30mM), S2 (15mM), S3 (7.5mM), S4 (3.75mM), S5 (1.875mM), S6 (0.9375mM) in 50mL centrifuge tubes.

Preparation of microplate assay:

D-Glucose HK Assay procedure (HK/G6P-DH Format) was followed (ignored provided standards and solution 4), and samples were loaded in duplicates on the microplate. The assay template was noted down, and the microplate was placed on a shaker for three minutes. The absorbance was measured at 562nm using SpectraMax iD3 Multi-Mode Microplate Reader

4.1.2.4 Calculations

The total protein present in the samples was calculated using the protein assay standard curve. The standard curve was used to estimate the unknown concentration of samples based on the known values of the serially diluted standards.

Microplate values obtained from the plate reader were extracted from the .csv file onto excel sheets. The average of duplicates was calculated, and an average of blank was subtracted from each value. Unknown concentration (x) was determined by using the linear equation for the line graph -

$$y=mx + c$$

Where m and c are constants; x is the absorbance value obtained, y represents the unknown concentration. The amount of protein was calculated in µg/mL of a honeybee after dividing the value by the weight of the honeybee.

The unknown concentration for free glucose and total glucose was calculated similarly in g/L. The glycogen content in each sample was calculated by subtracting absorbance measured for free glucose from the absorbance of the samples that have been digested with amyloglucosidase for total glucose. The glycogen value obtained was divided by the weight of the honeybee and converted to µg/mg of bee units. These values were grouped according to the site they belonged to and were averaged.

To minimize the error, excel statistical and data analysis tools were used to calculate standard deviation and standard error and plotted on graphs.

4.1.2.5 Results

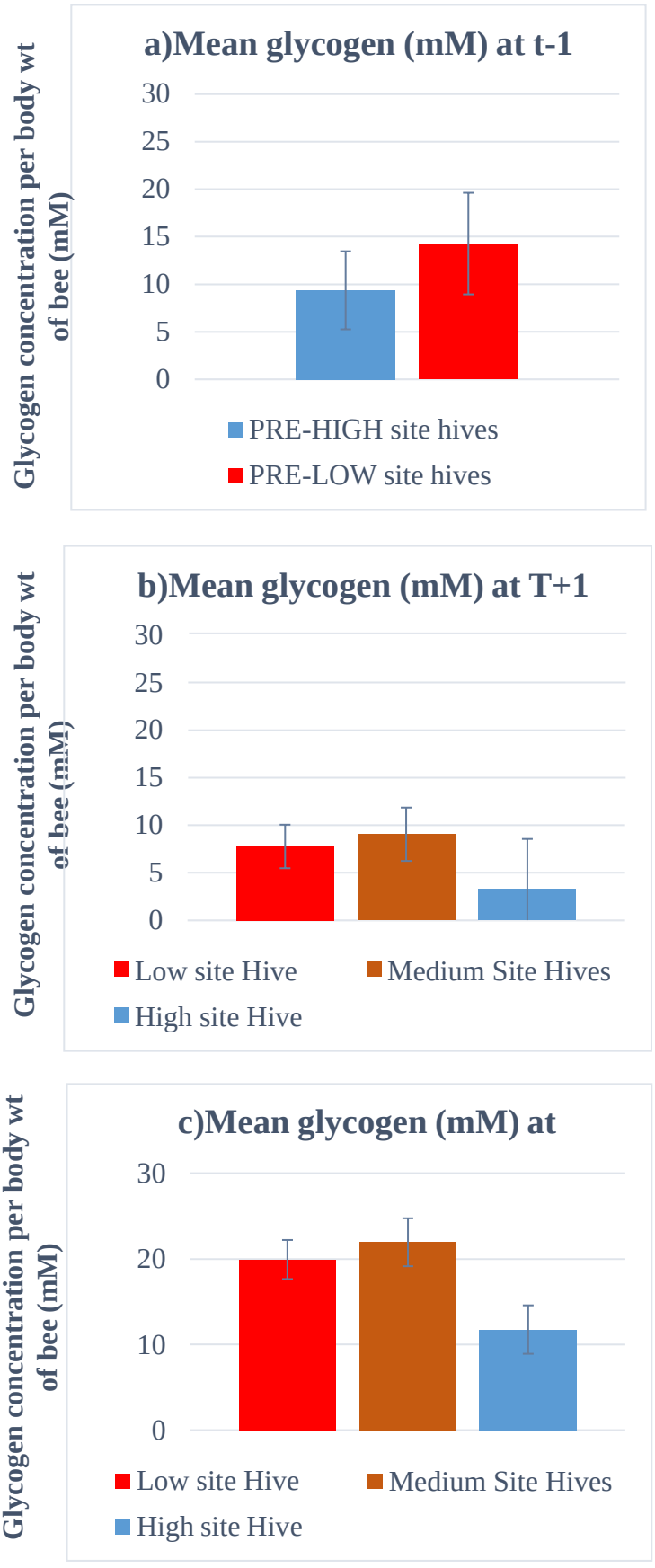

Figure 4.9: Mean amount of glycogen (mM) at a) T-1 day for PRE- high and PRE-low sites, andfor POST-high, POST-medium and POST-low density site hives b) T+1 day, c) T+22 day

The amount of glycogen (mM) in bees decreases on T+1 day but increases furthermore on T+22 for low-density and medium density hives. Whereas, for high density hives, it drops on T+1and an increase is seen on T+22 but is much less as compared to low and medium density hives (Figure 4.9).

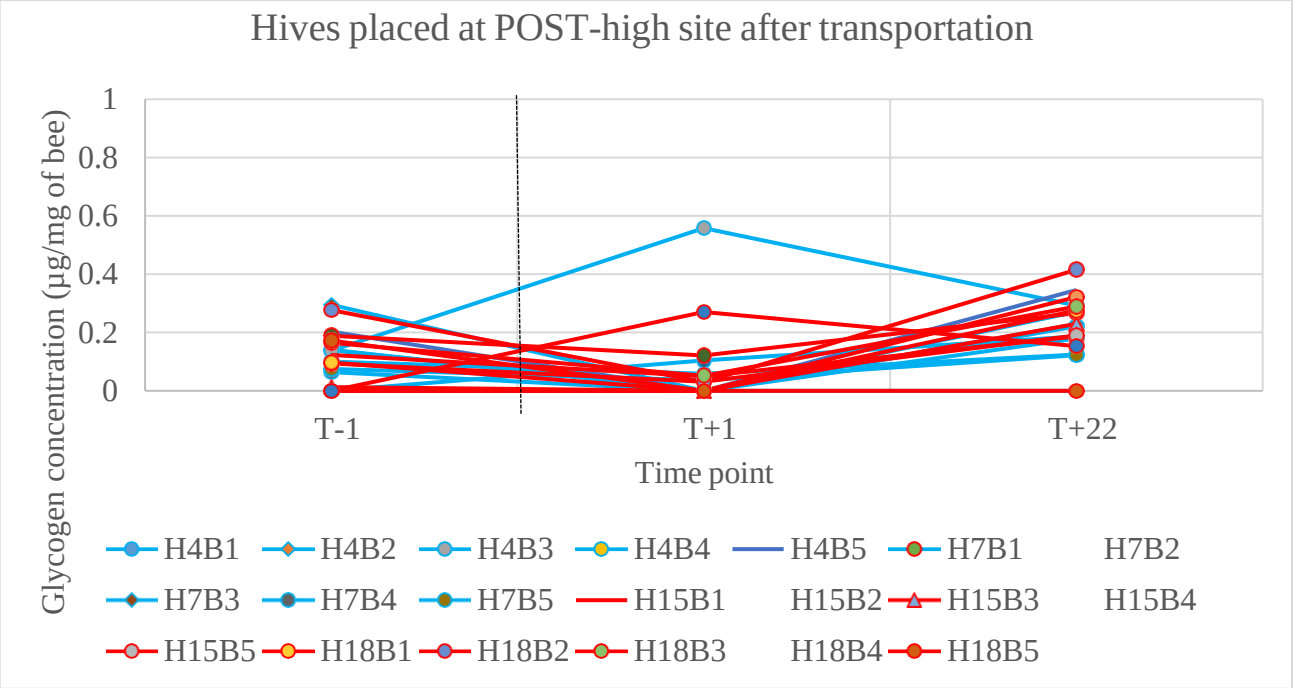

Figure 4.10 The graph shows glycogen concentration (µg/mg of bee) of each bee from four hives that were placed at POST-high density site to see the effect of competitive stress on their glycogen stores. The blue colour lines are for the bees from the hives that were placed at the PRE-High density site and the red coloured lines are for the hives that were placed at the PRE-low density site prior to transportation. The dotted black line represents transportation day.

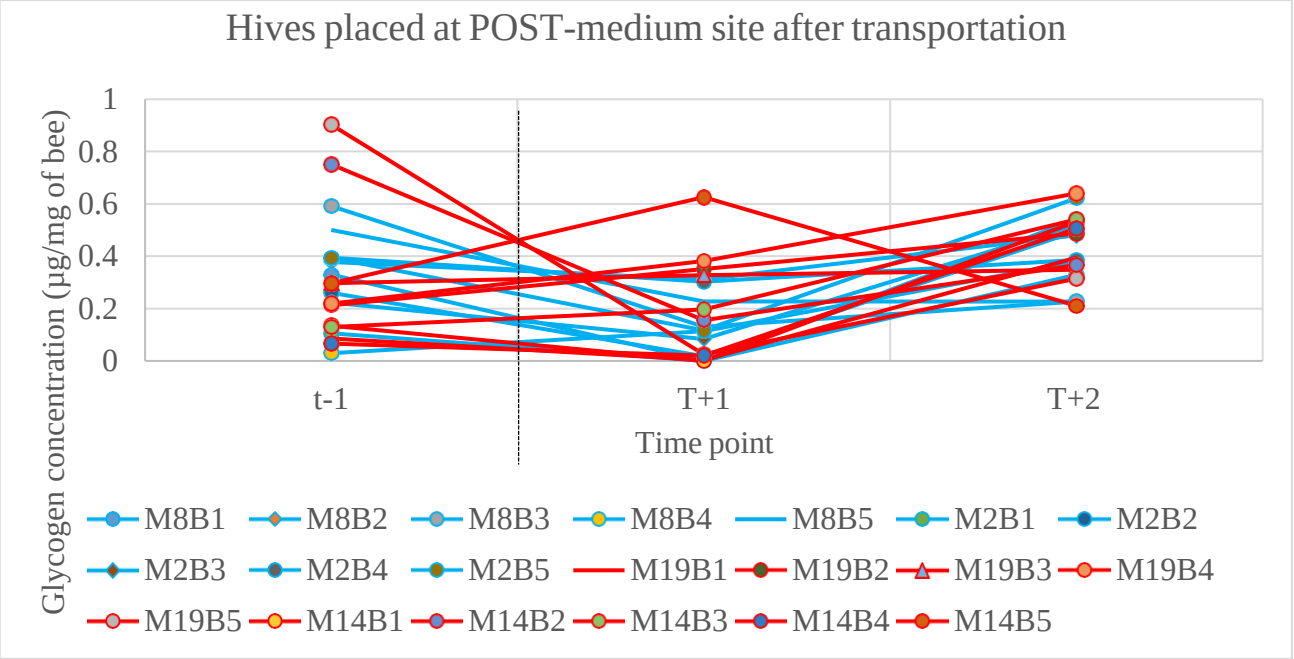

Figure 4.11: The graph shows glycogen concentration (µg/mg of bee) of each bee from four hives that were placed at POST-medium density site to see the effect of competitive stress on their glycogen stores. The blue colour lines are for bees from the hives that were placed at the PRE-High density site and the red coloured lines are for the hives that were placed at the PRE-low density site prior to transportation. The dotted black line represents transportation day.

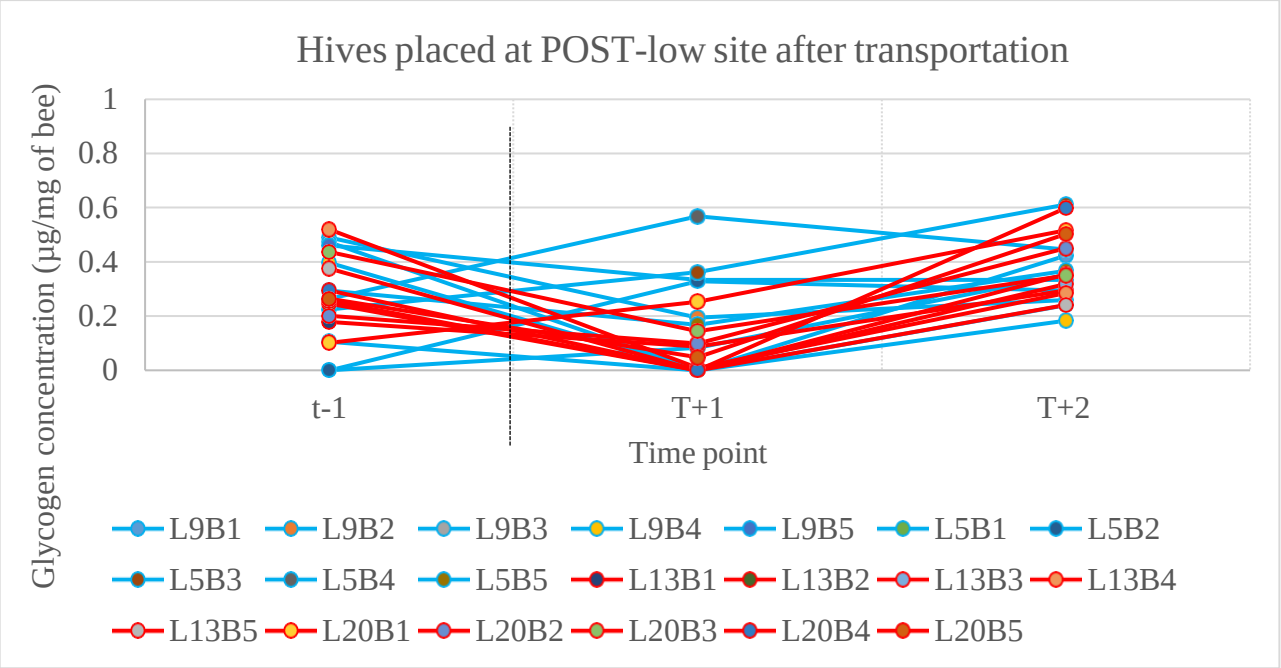

Figure 4.12: The graph shows glycogen concentration (µg/mg of bee) of each bee from four hives that were placed at POST-low density site to see the effect of competitive stress on their glycogen stores. The blue colour lines are for the bees from the hives that were placed at the PRE-High density site and the red coloured lines are for the hives that were placed at the PRE-low density site prior to transportation. The dotted black line represents transportation day.

The overall comparison of the three Figures (Figure 4.10-4.12) demonstrate that bees from hives at pre-High density site show a drop in glycogen levels on T+22. Whereas, bees from hives situated at medium density site thatconsisted both hives from PRE-high and PRE-low showed an increase from T+1 to T+22.

4.1.2.6 Conclusion

The trend seen in Figure 4.9 demonstrates that glycogen reserves of bees at high density site reduces more as compared to the ones at medium and low density site throughout the experimental competition trial.

The results provide evidence for depleted glycogen reserves of honey bees that were placed at high density site. In fact the hives that were placed at PRE-high density site and then transported to POST-

high density site have the lowest amount of glycogen concentration of all other bees.

Moreover, the amount of glycogen content in the sampled bees is comparable to other studies that tested levels of glycogen depletion due to diets mostly (Mogren et al.; Smart et al.).

Although bee responses are specific and adaptive to stress, they tend to entail a reduction of energetic metabolism (Kunieda et al., 2006). On broader note, glycogen reserves do deplete in newly emerge bees that faced competition at high density site. Thus, glycogen reserves can be used as a potential marker of stress in honey bees. Although in depth research is required further.

4.2 Investigating heat shock proteins (hsp) – Can hsp expression be used as a potentialmeasure of physiological stress?

4.2.1 Introduction

Honey bee colonies at overstocked apiary sites are experiencing competition and population density-related stress (Akratanakul, 1986; Komasilova et al., 2021; Seeley & Smith, 2015). However, how do larvae physiologically react to such external stressors? Moreover, is there a way to detect and use these physiological responses as indicators of stress?

This section focuses on how honey bee larvae respond to competition-related stress on a cellular level. It investigates the upregulation of heat shock protein 70 (hsp70) and whether the expression of this protein can be used as an indicator of stress. Therefore, hsp70 could help detect stress at an early stage and enable more effective population density management at an apiary level to maximize honey production and the overall honey bee wellbeing.

Exposure to stress over time impedes organismal function down to the cellular level (Morimoto, 1998). This can disrupt protein structure, leading to misfolding or unfolding and even aggregation, causing a cascade of biochemical reactions that can lead to cell apoptosis. These disruptions can be averted by the transcription of highly conserved heat shock genes (Morimoto, 1993).

Heat shock proteins (hsps) represent a group of proteins expressed by organisms in response to stresses. hsps were defined as “highly conserved molecular chaperones that help fold denatured or new proteins when an organism experiences stress” (Feder & Hofmann, 1999; Georgopoulos &

Welch, 1993). The name "heat shock proteins" can be deceptive as these proteins are upregulated not only on exposure to heat but, numerous stressors such as, starvation, toxic pollutants and anoxia (King & MacRae, 2015).

Amongst all the hsps, heat shock protein 70 (hsp70) has been the most widely researched and acknowledged as a "sensitive biomarker" for toxicants and detecting stress in animals and cell culture lines compared to other cellular markers such as oxidative regulation, DNA damage and cell cycle regulation (Farcy et al., 2009; Gibney et al., 2001; Maradonna & Carnevali, 2007; Pempkowiak et al., 2001). Stress protein concentrations in cells increase within minutes of exposure to environmental, physical or chemical stressors (Hranitz et al., 2009). Analysis and quantitation of stress proteins immediately after exposure to suspected stress is advantageous as stress proteins are susceptible and have a shorter response time than other measures such as growth rate(Pyza et al., 1997) and can serve as an efficient indicator of the stress response(Hranitz et al., 2009).

Mouse cell lines exposed to heavy metals expressed hsp70 in a ratio relative to the level of toxicity of metals and their corresponding lethal dosage (LC50) (Gibney et al., 2001). Hsp70 expression has also been used to detect general stress in marine species like the Black Goby, an Atlantic ray-finned fish, when exposed to certain pollutants, expressed hsp70 as the primary stress response (Maradonna & Carnevali, 2007). Studies on species such as *Drosophila melanogaster* show that under anoxia-like conditions, hsp70 is upregulated (Feder & Hofmann, 1999). When fruit fly larvae were exposed to heat, an increase in hsp70 expression was observed along with impaired nervous system functioning and weakened wing muscles as adults. Meanwhile, rodents express hsp70 even at critically low temperatures.

The cell signaling pathway during a stress response that gives rise to hsp70 upregulation is similar to that observed in vertebrates through to single-celled organisms such as yeast(Beere, 2004; Morimoto, 1998), and this includes honey bees (Feder & Hofmann, 1999). An increase in stress response by upregulation of has been reported after honey bees were exposed to stressors such as heat, toxic

substances, bacterial infections, and parasites (Gregorc & Bowen, 1999; Gregorc et al., 2004; Lipiński & Żółtowska, 2005; Scharlaken et al., 2008).

This study investigates the expression of hsp70 due to competitive stress by the larvae. Honey bee larvae were specifically chosen for this study to explore hsp70 expression, as they are more sensitive to stress than adults (Hranitz & Barthell, 2003; King & MacRae, 2015; Ma & Haddad, 1997; Zhao et al., 2010). Bee larvae were collected from the experimental competition trial set-up across PRE and POST density sites at four time points.

4.2.2 Aims and Hypothesis

This section aims to determine hsp70 expression in honey bee larvae that are experiencing stress in order to use it as a physiological indicator of stress. The hypothesis is that honey bee larvae experiencing competition-related stress will upregulate of hsp70. Highest hsp70 expression will occur at the site with highest competition and medium and low levels of expression in larvae at medium and low density sites respectively.

4.2.3 Methods Study Design

Twenty-four focal hives were distributed equally across two initial sites; high density site (PRE high with twelve focal hives) and low density sites (two PRE low sites with six focal hives each). These hives were kept in the initial site for three weeks and then transported. A day before transporting these hives, samples were collected i.e., on T-1 day. These hives were distributed across three sites with additional hives that helped maintain a density gradient: high (120 colonies), medium (30 colonies) and low colony density (8 colonies). The samples were then collected at three subsequent time points.

4.2.3.1 Collection of honey bee larvae

Honey bee larvae were collected to investigate and compare the expression of heat shock proteins (hsp70) across PRE and POST density sites at four time points.

At each time point, sixteen larvae from each of four focal hives (two PRE-high and two PRE-low), across all three sites (high, medium and low density) were collected, making it a total of 1536 larvae. These were collected on all four time points- T-1 day, T+1 day, T+22 days and T+35 days.

To sample the larvae, a brood frame with capped brood was taken out and knocked down on absorbent lab benchtop paper placed on a hard surface. The medium-sized larvae that fell out were picked up with soft forceps and placed individually into wells of 48-well sterile culture plates (48-well, flat bottom, Nunc™ Cell-Culture Treated Multidishes, Thermo Scientific™) (Figure 4.13). Each culture platewas secured with Parafilm® and placed in a polystyrene box filled with dry ice to prevent larvae fromdesiccation. These plates were transported to Plant and Food Research, Ruakura, and stored at -80°C.

Figure 4.13: Collecting honey bee larvae from a focal hive.

4.2.3.2 Dissecting honey bee larvae

Sterile scalpel and sharp forceps were used to cut off the larval head. Forceps and scalpel were dipped in ethanol flamed between dissecting each larva. Three larval heads were transferred to 2mL Eppendorf tubes. 600µl of the pro-prep solution was added to each tube. A micro-pestle was used per tube to pestle the larval heads to homogenize the tissues. These tubes were held at -20°C for 30minutes. The tubes were centrifuged in Eppendorf™ 4515R at 13,000 RCF (4°C) for 5 minutes. The supernatant was aspirated into labeled tubes and stored at -20°C until required for measurement of protein concentration.

4.2.3.3 Protein assay- PRO-MEASURE™ method

The samples were diluted in a ratio of 1:70 by pipetting 98.6µL of distilled H_2O and 1.4µL supernatant. The standards were prepared in 9 tubes. 100µL of distilled H_2O was pipetted into each tube. A serial dilution was 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 H_2O as diluent. A blank was prepared by adding 100µL of distilled H_2O. 1000µL of PRO-MEASURE™ 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.

4.2.3.4 ELISA- detection of hsp70

A Human hsp70 ELISA kit was used to quantify hsp70. The test protocol in the Human hsp70 ELISA Kit manual was followed with modifications (Refer to Chapter II). The number of microplate strips required was determined and placed on the provided holder. Wash buffer and Assay buffer were prepared according to the number of strips used. The microplate was washed with approximately 400µL of wash buffer in each well twice. The solutions were removed by flicking the plate over in the sink. The microplate was dabbed on a paper towel to remove excess wash buffer and kept upside down until the following reagents were prepared to be added within 15 minutes of washing. The Human hsp70 standard was reconstituted by adding 260µL of distilled water and kept aside for 30 minutes. Standards were prepared in serial dilution by following the "external standard dilution" protocol present in the Human hsp70 ELISA kit. For sample and blank preparation, 100µL of sample diluent was added to blank wells and 50µL to sample wells, respectively. 50µL of the sample was added to each well (Biological replicates were valued over technical replicates as the kits were costly and limited). The plate was covered with an adhesive film and fixed on a microplate shaker for 2 hours at room temperature.

The biotin-conjugate was prepared as per requirements, i.e. 15 minutes before the two-hour incubation period. After incubation, the plate contents were emptied into the sink, and the plate was washed six

times with wash buffer solution and 100µL biotin-conjugate was immediately added to all wells. The plates were then covered with adhesive film and left on a microplate shaker for an hour at room temperature. Streptavidin-HRP was prepared 15 minutes before completing an hour of the incubation period. The plate contents were emptied into the sink, and the plate was washed six times. 100µL of freshly prepared streptavidin-HRP was added to all wells. The plates were covered with an adhesive film and left on a microplate shaker for 30 minutes at room temperature.

The plate contents were emptied into the sink, and the plate was washed six times. 100µL of TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution was added to all wells and again plates were covered with an adhesive film and left in the dark for 30 minutes at room temperature. The absorbance was then measured at intervals of 15 minutes at 450nm within an hour of adding the TMB Substrate. 100µL of stop solution was added to each well after sufficient development of a dark blue colour. The absorbance was measured with an excitation at 450nm and emission at 650nm using a FLUOstar® Omega plate reader(FLUOstar® Omega, BMG Labtech) before an hour had elapsed.

4.2.4 Calculations

Absorbance values obtained at the two wave lengths were corrected by subtracting absorbance read at primary wave length (450nm) from reference wave length (650nm). A standard curve was obtained by using the corrected values and known standard concentrations plotted on a scatter graph in Excel. The concentration of samples was determined using the standard line equation,

$Y = mx + c$

4.2.5 Results

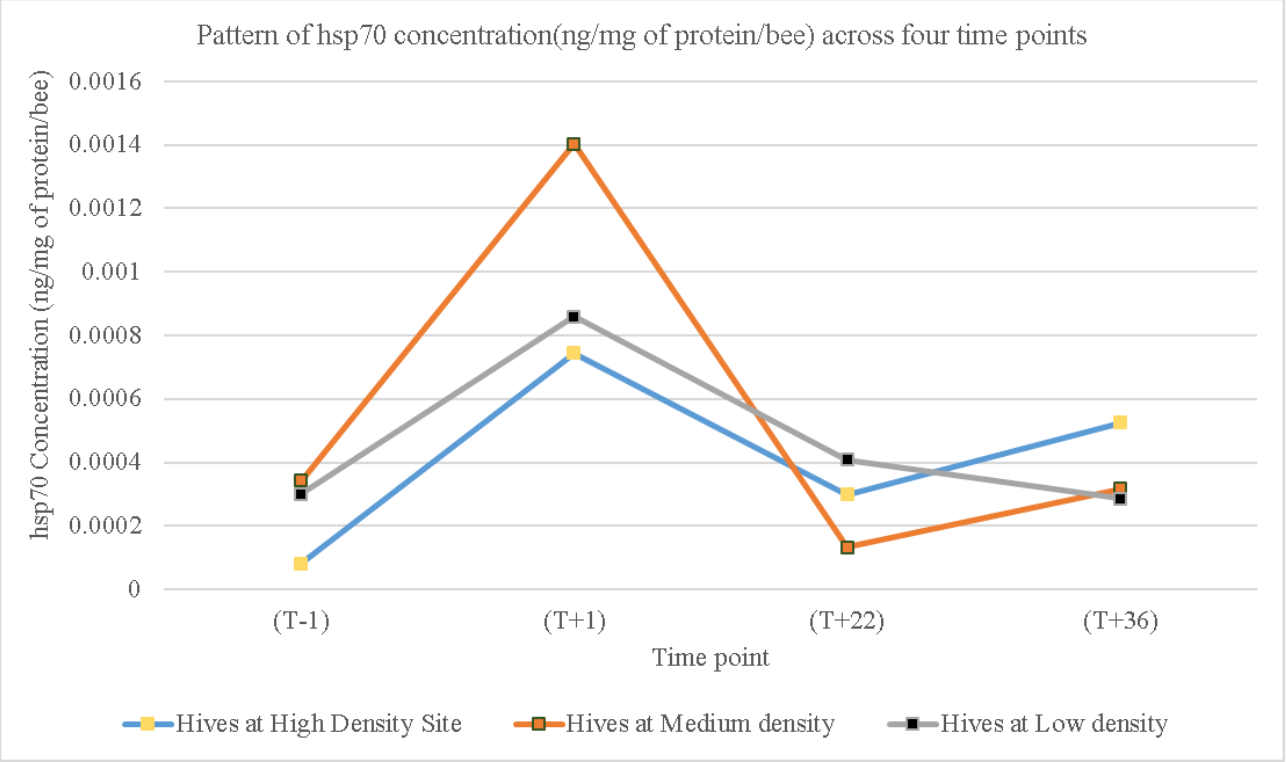

Figure 4.14: The line graph shows the pattern of hsp70 concentration across the three density sites on four different time points.(T-1, T+1, T+22, 1+36)

The mean hsp70 concentrations (ng/ml) in larvae sampled across density sites over the four timepoints are shown in Figure 4.15- Figure4.18.The analysis of hsp70 concentration across four timepoints shows a significant rise in hsp70 concentration over time but, with being highly

affected by the movement of hives (Figure 4.14).

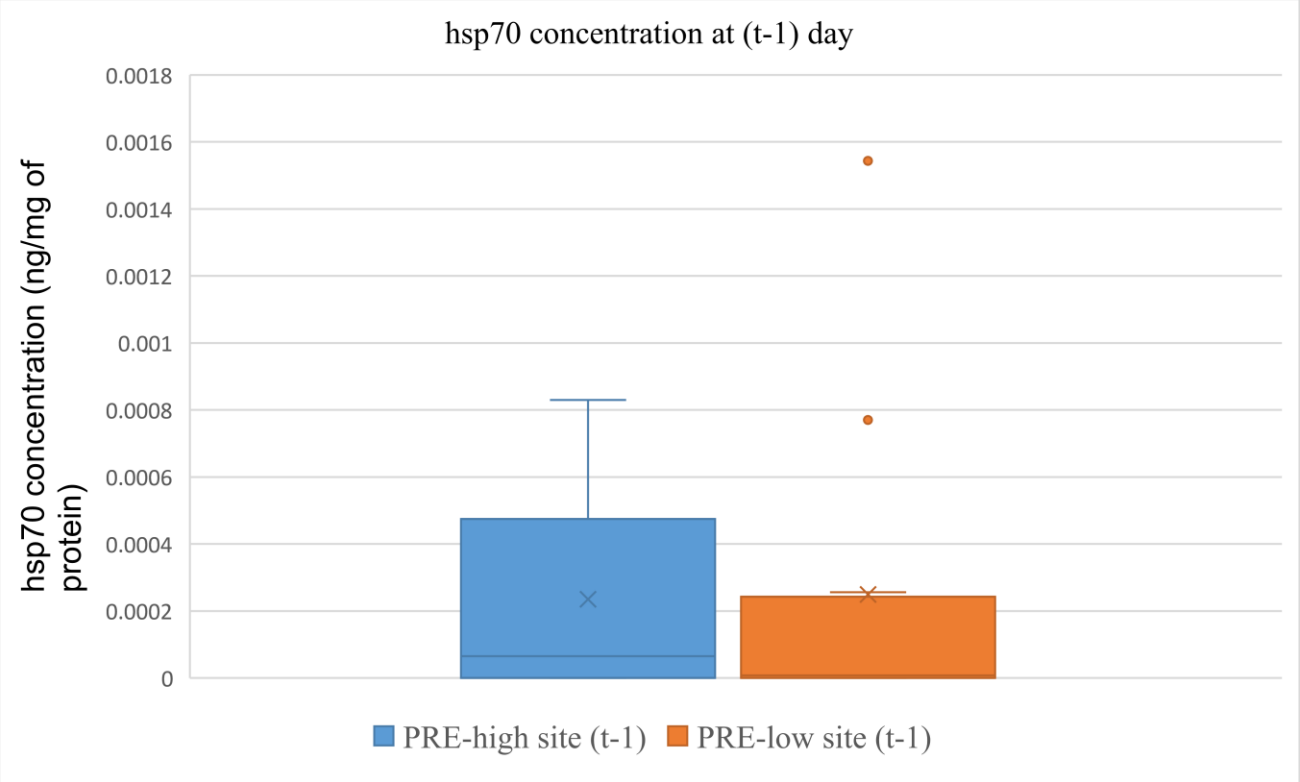

Figure 4.15: Box and whisker plot of hsp70 concentration in larva on the day prior to transportation (T-1) at PRE-high and PRE-low sites, showing median (line), 25th and 75th percentiles (box), maximum and minimum (whiskers), outliers (dots) and means (X).

On (T+1): The PRE-high site hsp 70 concentration (mean = 0.0004 ng/ml of protein) was higher than followed by PRE-low density site (mean = 0.0002 ng/ml of protein) (Figure 4.15).

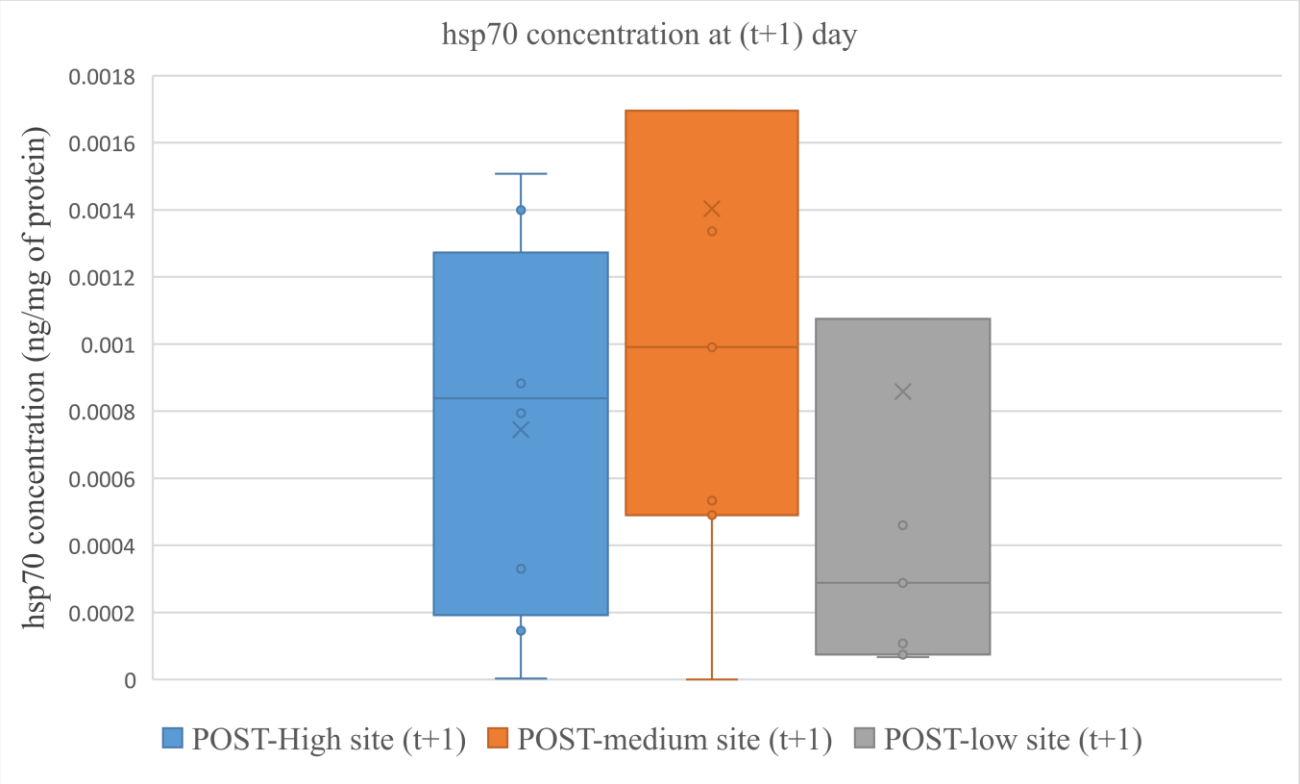

Figure 4.16: Box and whisker plot of hsp70 concentration in larva on the day after transportation (T+1) at POST-high,POST-low and POST-medium density sites, showing median (line), 25th and 75th percentiles (box), maximum and minimum (whiskers), outliers (dots) and means (X).

On (T+1): All the three density sites demonstrated an increase in hsp70 concentration from (T-1) to (T+1). The POST-medium site hsp 70 concentration (mean = 0.0012 ng/ml of protein) was the highest followed by POST-high density site (mean = 0.0006 ng/ml of protein) and POST-low densitysite (mean = 0.0004 ng/ml of protein)(Figure 4.16).

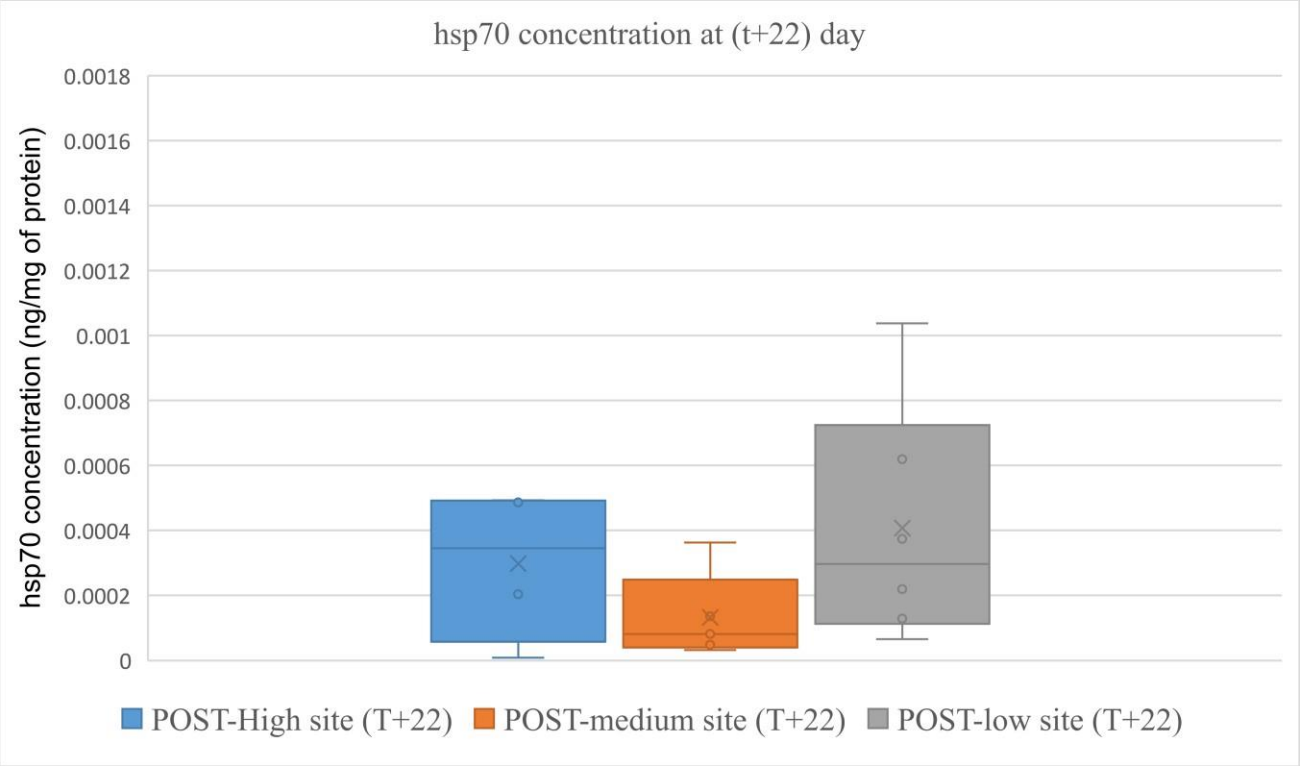

Figure 4.17: Box and whisker plot of hsp70 concentration in larva on twenty-two days after transportation (T+1) at POST-high,POST-low and POST-medium density sites, showing median (line), 25th and 75th percentiles (box), maximum and minimum (whiskers), outliers (dots) and means (X).

On (T+22): The hsp70 concentration decreases across all three sites decreases from (T+1) to (T+21) with the lowest being POST-medium site (mean = 0.00007 ng/ml of protein) followed by POST-high site (mean = 0.00015 ng/ml of protein) and POST-low site (mean = 0.00031 ng/ml of protein) (Figure 4.17).

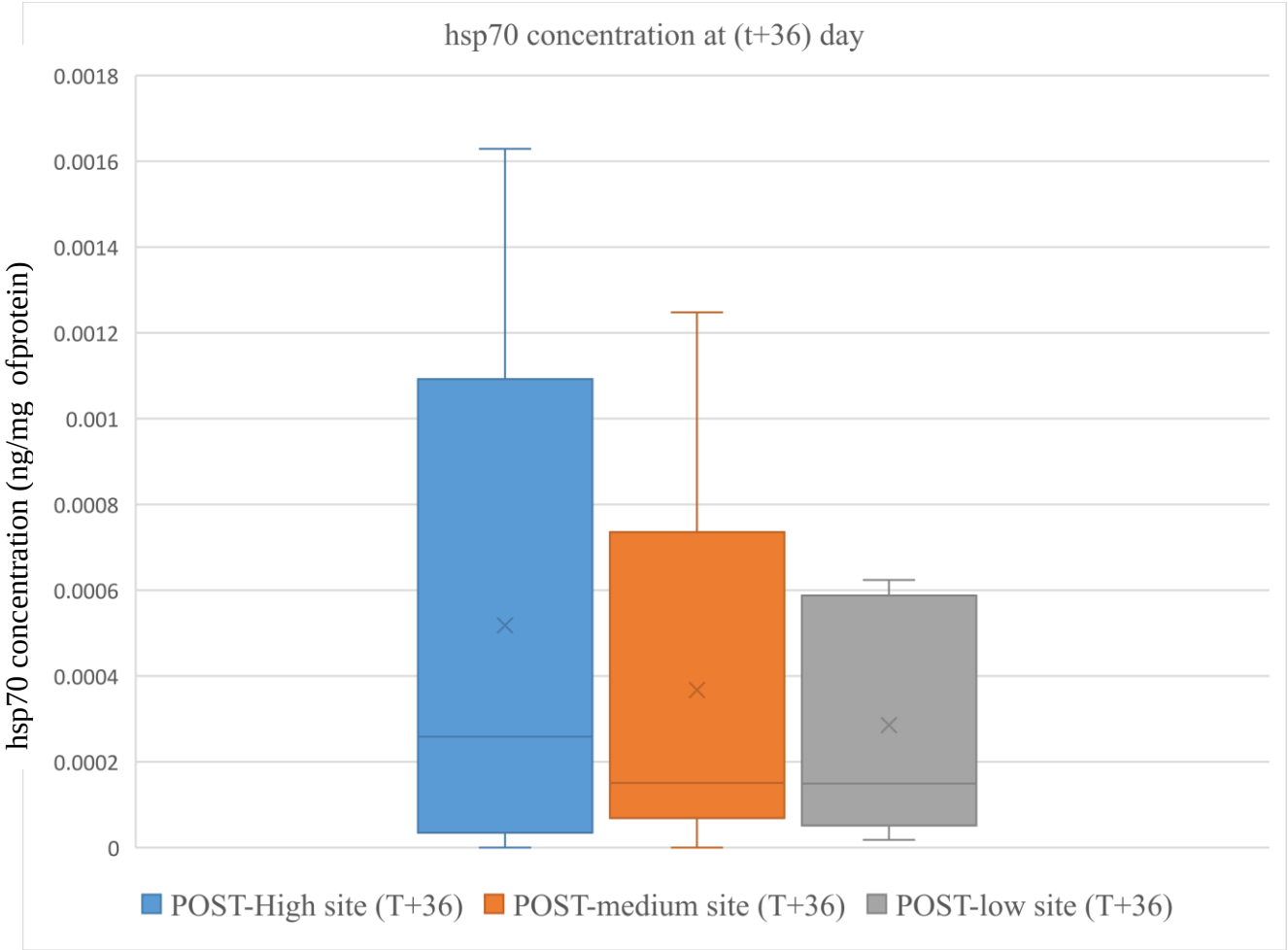

Figure 4.18: Box and whisker plot of hsp70 concentration in larva on thirty-six days after transportation (T+1) at POST-high,POST-low and POST-medium density sites, showing median (line), 25th and 75th percentiles (box), maximum and minimum (whiskers), outliers (dots) and means

(X).

On (T+36): POST-high density site (mean = 0.0005 ng/ml of protein) and POST-medium density site (mean = 0.0003 ng/ml of protein) showed an increase in hsp70 concentration on (T+36) while POST- low density site (mean = 0.0002 ng/ml of protein) drops further.

There was no difference in hsp70 concentration in larvae samples from high or low density sites before the hive movement (Two-sample Student's t-test, P=0.06). The movement of hives had a significant effect on hsp70 concentration (Two-sample Student's t-test, P=0.01). There was no difference in how hsp70 concentration in hives at high density as opposed to hives at low density responded to movement, from T-1 to T+1 (T-test, P=0.77, Figure 4.15 – 4.16). There was no difference in hsp70 concentration in hives at POST-high density, POST-low density and POST-medium density sites on T+22(single-factor ANOVA, P=0.292) and T+36 days (single-factor ANOVA, P=0.735, Figure 4.17 - 4.18).

4.2.6 Conclusion

Heat shock protein expression increased exponentially after transportation. This indicates that transportation acted as a stressor rather than the inter-colony competition which induced hsp70gene expression.

Further statistical analysis provides evidence for proving that hsp70 expression increased for both PRE-high and PRE-low hives after transportation. However, there was no significant difference in the expression of hsp70 at either T+22 or T+36.

There have been studies that investigated the hsp70 gene expression due to transportation as a stressor and found a similar spike in the expression of this stress protein (Jones et al., 2005; Melicher etal., 2019; Oldroyd, 2007).

Although this results in rejecting the hypothesis that larvae at the high-density site would express the highest amount of hsp70 due to inter-colony competition, there is an increase in hsp70 expression at all sites on T+36 day.

Furthermore, there are still many unanswered questions about the method we used to detect hsp70. Even though it provides sensitive detection of hsp70, it does not provide a quantitative value that can be compared to other studies. Further work is required to establish a quantitative analysis of hsp70 expression at different apiary density sites.

Chapter 5: General Discussion

5.1 Discussion

This chapter concludes the thesis by summarizing the findings around the three research questions and explaining how these findings contribute to the field. It will also lay out limitations and a foundation for further research.

This study aimed to investigate physiological markers of stress in Western honey bees (*Apis mellifera*). In recent times, overstocking or crowding of honey bees at an apiary site has become a concerning issue in New Zealand (MPI, 2021; Newstrom-Lloyd, 2015). Although there is an increase in the number of honey bee colonies, the honey bee population declines and lower honey production have beenreported lately (Beard, 2015). Therefore, high population density might be inducing stress on the colonies stacked at the apiary site affecting honey bee health and pollination (Seeley & Smith, 2015). Recognizing stress at an early stage can help beekeepers to better manage their apiaries and the overall honey bee population's well-being. Therefore, this study aims to identify potential markers of stress that can help recognise stress caused due to overstocking at an early stage. Thus, for this study, we investigated three areas where stress might affect honey bee physiology as the precursor for developing a marker of stress that beekeepers could use to manage overstocking.

Our trial experimentally established three apiary sites with different degrees of inter-colony competition: highly populated (120 hives), moderately (30 hives), and less (8 hives) populated apiary sites. The research aimed to analyse respiration rate, glycogen stores, heat shock proteins and male reproductive output in honey bees as potential indicators of stress.

While conducting experiments for male reproductive output, out of the 150 drones collected, only 15 drones were mature enough to collect ejaculate to test sperm mobility and viability. The techniques followed to test mobility were limited by human error and the age of the drones. Thus, we could not collectany substantial data on the male reproductive output.

I found that the respiration rate measured in honey bees across the three density sites did not appear to be affected by competitive stress, unlike hypothesized in this study. Moreover, honey bees rapidly

increase their respiration rate for the duration of flight and lower back to a resting state rapidly once they land. This is possible as they depend on immediate glucose oxidation depending on metabolic demands. It is a possible that respiration capacity has limited capacity to vary given that resting metabolic rate generally tracks with maximal metabolic rate (Weibel & Hoppeler, 2005), and a specific amount of power is required for flight. Therefore, metabolic rate may be largely fixed.

However, glycogen stores were more depleted at the high density site than medium and low density sites when measured on T+36 day of the experimental competition trial. It has been shown that newly emerged workers, when exposed to stress, are most likely to use up their glycogen reserves to meet their high metabolic demands (Even et al., 2012). The larvae sampled at this time point were entirely raised in the experimental conditions of the trial (including high competition at the high density site), and the majority of nurse bees feeding them would also have developed during these trial conditions (Seeley, 1982). This study suggests that glycogen stores in bees could provide an indicator of competitive stress if the colonies had been experiencing this stress for a sufficient time period.

The movement of hives was associated with an increase in hsp70 expression, demonstrating that the expression of this gene can be a useful measure of stress in honey bees as it is in other organisms (Ma & Haddad, 1997; Maradonna & Carnevali, 2007; Matz et al., 1996; Sakano et al., 2006). However, there was no evidence of an association between colony density and hsp70 expression, suggesting that this is not a useful way to measure stress in honey bees across the range of densities explored in this trial.

5.2 Significance

Being able to recognise stress at an early stage is necessary and beneficial for effective management of its effects on the colony health and honey production (Barron, 2015). Furthermore, if it can be identified that honey bees are competing for floral resources before these resources are depleted, this might provide a means to manage the impact of honey bees on native ecosystems that might otherwise be negatively impacted by large honey bee populations. Moreover, numerous stressors that affect the

Colonies' health indirectly affects their pollination services used for agriculture as per current global reports.

Since 1970's researchers have used ELISA's, western blotting, quantitative PCR and other techniques to analyse heat shock proteins in honey bees and other organisms (Farcy et al., 2009; Hranitz & Barthell, 2003; Hranitz et al., 2010; Pempkowiak et al., 2001; Sahebzadeh & Lau, 2017). Human hsp70 ELISA was never used before to detect honey bee heat shock proteins. However, this study explored heat shock proteins in honey bee larvae and successfully detected honey bee hsp70 using cross-species antibodies (Human hsp70 ELISA kit). This methodology may be of assistance to improve our understanding of transportation as a potential stressor.

Analysis of glycogen reserves of newly emerged larvae provides critical evidence for the bees having experienced stress due to overcrowding at high density site as compared to low and medium density sites. A practical progression of this work is to measure glycogen reserves of the drones, larvae and the queen across a range of overcrowded apiary sites. This shall help provide a broad picture of the level of stress experienced by honey bee castes and the colony as a whole. The glycogen reserves of honey bees can be used as a marker of stress and be applied to determine how stressed an apiary site is.

5.3 Limitations

The scope of this study was limited in terms of the sample size of the experimental trial. Perhaps, measuring stress at higher colony density apiary sites might be empirical to identify efficient and reliable markers of stress.

An additional uncontrolled factor is whether the stress experienced by the honey bees is competitive stress or not. Heat shock protein analysis resulted in transportation being the stressor rather than inter-colony competition.

The methodology used for assessing male reproductive output had shortcomings due as I was not able to replicate the described methods because time constraints due to covid-19 disruptions and the

seasonal life cycle of honey bees made it challenging to carry out sampling in their natural setting at commercial apiary sites. As they would be experiencing various levels of competition, which would have helped us see whether their results would correlate with our experimental trials.

5.4 Future research

In terms of the markers considered for this study, what is now needed is investigating heat shock proteins and glycogen reserves of all honey bee castes in high-density site colonies. This needs to be carried out over a longer period to make the markers more reliable as well as to shed light on effects of these stressors at overcrowded apiary sites. A further study could explore other potential behavioural markers of competitive stress due to overpopulation, such as aggression and robbing (Tran et al., 2014; Willingham et al., 2000).

Moreover, a colony carrying capacity of apiary sites need to be devised as seen in dairy and poultry management practices in New Zealand (Newstrom-Lloyd, 2015). With the help of this research and available models, we could estimate the threshold of the number of honey bee colonies that can co-exist without stressing the honey bees and having a negative impact on native flora and fauna.

Bibliography

Abou-Seif, M., Maier, V., Fuchs, J., Mezger, M., Pfeiffer, E.-F., & Bounias, M. (1993). Fluctuations of carbohydrates in haemolymph of honeybee (Apis mellifica) after fasting, feeding and stress. *Hormoneand Metabolic Research*, *25*(01), 4-8.

Aebi, A., Vaissière, B., VanEngelsdorp, D., Delaplane, K., Roubik, D., & Neumann, P. (2011). Back to the future:Apis versus non-Apis pollination—a response to Ollerton et al. *Trends in Ecology & Evolution*, *27*, 142-143. https://doi.org/10.1016/j.tree.2011.11.017

Akratanakul, P. (1986). *Beekeeping in Asia* (Vol. 4). Food & Agriculture Org.

Allen, D. M. (1959). Respiration Rates of Larvae of Drone and Worker Honey Bees, Apis mellifera L. *Journal ofEconomic Entomology*, *52*(3), 399-402. https://doi.org/10.1093/jee/52.3.399

Amdam, G. V. (2011). Social context, stress, and plasticity of aging. *Aging cell*, *10*(1), 18-27.

Armstrong, D. P. (1991). Aggressiveness of breeding territorial honeyeaters corresponds to seasonal changesin nectar availability. *Behavioral Ecology and Sociobiology*, *29*(2), 103-111.

Austad, S. N. (1983). A game theoretical interpretation of male combat in the bowl and doily spider (Frontinellapyramitela). *Animal Behaviour*, *31*(1), 59-73.

Aydin, S. (2015). A short history, principles, and types of ELISA, and our laboratory experience withpeptide/protein analyses using ELISA. *Peptides*, *72*, 4-15. https://doi.org/10.1016/j.peptides.2015.04.012

Balderrama, N., Núñez, J., Guerrieri, F., & Giurfa, M. (2002). Different functions of two alarm substances in thehoneybee. *Journal of Comparative Physiology A*, *188*(6), 485-491.

Barron, A. B. (2015). Death of the bee hive: understanding the failure of an insect society. *Current opinion ininsect science*, *10*, 45-50.

Beard, C. (2015). Honeybees (Apis mellifera) on public conservation lands. *Online http://www. doc. govt.nz/Docu ments/science-and-technical/honeybees-on-public-conser vation-lands. pdf*.

Beere, H. M. (2004). "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. *J CellSci*, *117*(Pt 13), 2641-2651. https://doi.org/10.1242/jcs.01284

Bernd, & , H. (1979). Thermoregulation of African and European honeybees during foraging, attack, and hiveexits and returns. *Journal of Experimental Biology*, *80*(1), 217-229.

Bicker, G., & Menzel, R. (1989). Chemical codes for the control of behaviour in arthropods. *Nature*, *337*(6202),33-39.

Blanke, M., & Lensing, W. (1989). Measurement of Metabolic Activity of the Honeybee by Assessing Respiration. *Journal of Apicultural Research*, *28*(3), 131-135. https://doi.org/10.1080/00218839.1989.11100834

Bordier, C., Suchail, S., Pioz, M., Devaud, J. M., Collet, C., Charreton, M., Alaux, C. (2017). Stress response
in honeybees is associated with changes in task-related physiology and energetic metabolism. *Journal of Insect Physiology*, *98*, 47-54. https://doi.org/https://doi.org/10.1016/j.jinsphys.2016.11.013

Brown, P., Newstrom-Lloyd, L. E., Foster, B. J., Badger, P. H., & McLean, J. A. (2018). Winter 2016 honey bee colony losses in New Zealand. *Journal of Apicultural Research*, *57*(2), 278-291. https://doi.org/10.1080/00218839.2018.1430980

Brown, P. N.-L., Linda E; Foster, Barry J; Badger, Paul H; McLean, John A. (2016). Winter 2016 honey bee colony losses in New Zealand. *Taylor & Francis*, S9. https://doi.org/https://doi.org/10.6084/m9.figshare.5882533.v1

Buck, J. (1962). Some physical aspects of insect respiration. *Annual Review of Entomology*, *7*(1), 27-56. Candy, D. (2002). Adipokinetic hormones concentrations in the haemolymph of Schistocerca gregaria,
measured by radioimmunoassay. *Insect Biochemistry and Molecular Biology*, *32*(11), 1361-1367.

Chacon-Almeida, V. M. L., Simoes, Z. L. P., & Bitondi, M. M. G. (2000). Induction of heat shock proteins in the larval fat body of Apis mellifera L. bees. *Apidologie*, *31*(4), 487-501. <Go to

ISI>://WOS:000088975900003
Chrousos, G. P., & Gold, P. W. (1992). The Concepts of Stress and Stress System Disorders: Overview of Physical and Behavioral Homeostasis. *JAMA*, *267*(9), 1244-1252. https://doi.org/10.1001/jama.1992.03480090092034
Clare, D. K., & Saibil, H. R. (2013). ATP-driven molecular chaperone machines. *Biopolymers*, *99*(11), 846-859.https://doi.org/10.1002/bip.22361Collins, A. M. (2000). Relationship between semen quality and performance of instrumentally inseminated honey bee queens. *Apidologie*, *31*(3), 421-429.
Crailsheim, K., Schneider, L. H. W., Hrassnigg, N., Bühlmann, G., Brosch, U., Gmeinbauer, R., & Schöffmann, B.(1992). Pollen consumption and utilization in worker honeybees (Apis mellifera carnica): Dependenceon individual age and function. *Journal of Insect Physiology*, *38*(6), 409-419. https://doi.org/https://doi.org/10.1016/0022-1910(92)90117-V
Crane, E. (1990). *Bees and beekeeping: science, practice and world resources*. Heinemann Newnes.
Dowds, B. M., & Elwood, R. W. (1985). Shell wars II: the influence of relative size on decisions made during hermit crab shell fights. *Animal Behaviour*, *33*(2), 649-656.
Downs, S. G., & Ratnieks, F. L. (2000). Adaptive shifts in honey bee (Apis mellifera L.) guarding behavior supportpredictions of the acceptance threshold model. *Behavioral Ecology*, *11*(3), 326-333.
Even, N., Devaud, J.-M., & Barron, A. B. (2012). General stress responses in the honey bee. *Insects*, *3*(4), 1271-1298. https://doi.org/http://dx.doi.org/10.3390/insects3041271
Ewald, P. W., & Carpenter, F. L. (1978). Territorial responses to energy manipulations in the Anna hummingbird. *Oecologia*, *31*(3), 277-292.
Farcy, E., 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 & Chaperones*,*14*(4), 371-380. https://doi.org/10.1007/s12192-008-0091-8
Farooqui, T. (2012). Review of octopamine in insect nervous systems. *Open access insect physiology*, *4*, 1. Feder, M. E., & Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response:
evolutionary and ecological physiology. *Annu Rev Physiol*, *61*, 243-282. https://doi.org/10.1146/annurev.physiol.61.1.243
Free, J. (1958). The drifting of honey-bees. *The Journal of Agricultural Science*, *51*(3), 294-306.
Georgopoulos, C., & Welch, W. (1993). Role of the major heat shock proteins as molecular chaperones. *AnnualReview of Cell Biology*, *9*(1), 601-634.
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-lethalconcentrations. *Biomarkers*, *6*(3), 204-217.
Gregorc, A., & Bowen, I. D. (1999). In situ localization of heat-shock and histone proteins in honey-bee (Apis mellifera L.) larvae infected with Paenibacillus larvae. *Cell Biology International*, *23*(3), 211-218.
Gregorc, A., Pogacnik, A., & Bowen, I. (2004). Cell death in honeybee (Apis mellifera) larvae treated with oxalicor formic acid. *Apidologie*, *35*(5), 453-460.
Harrison, J. F., & Fewell, J. H. (2002). Environmental and genetic influences on flight metabolic rate in the honey bee, Apis mellifera. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *133*(2), 323-333. https://doi.org/https://doi.org/10.1016/S1095-6433(02)00163-0
Hartl, F. U. (1996). Molecular chaperones in cellular protein folding. *Nature*, *381*(6583), 571-580.
Haubruge, E., Nguyen, B. K., Widart, J., Thomé, J.-P., Fickers, P., & De Pauw, E. (2006). Le dépérissement de l'abeille domestique, Apis mellifera L., 1758 (Hymenoptera: Apidae): faits et causes probables. *Notes Fauniques de Gembloux*, *59*(1), 3-21.
Havard, T., Laurent, M., & Chauzat, M.-P. (2019). Impact of stressors on honey bees (Apis mellifera; Hymenoptera: Apidae): Some guidance for research emerge from a meta-analysis. *Diversity*, *12*(1), 7.

Haydak, M. H. (1970). Honey bee nutrition. *Annual Review of Entomology*, *15*(1), 143-156.
Heller, H. C. (1971). Altitudinal zonation of chipmunks (Eutamias): interspecific aggression. *Ecology*, *52*(2), 312-319.
Hepburn, H., Cantrill, R., Thompson, P., & Kennedi, E. (1979). Metabolism of Carbohydrate, Lipid and Protein During Development of Sealed Worker Brood of of the African Honeybee. *Journal of Apicultural Research*, *18*(1), 30-35.
Hetz, S. K., & Bradley, T. J. (2005). Insects breathe discontinuously to avoid oxygen toxicity. *Nature*, *433*(7025),516-519.
Hranitz, J., & Barthell, J. F. (2003). Heat shock protein 70 during development of the leafcutting bee, Megachilerotundata (Hymenoptera: Megachilidae). *Southwestern Entomologist*, *28*, 97-103.
Hranitz, J. M., Abramson, C. I., & Carter, R. P. (2010). Ethanol increases HSP70 concentrations in honeybee (Apis mellifera L.) brain tissue. *Alcohol*, *44*(3), 275-282. https://doi.org/10.1016/j.alcohol.2010.02.003
Hranitz, J. M., Barthell, J. F., Thorp, R. W., Overall, L. M., & Griffith, J. L. (2009). Nest Site Selection Influences Mortality and Stress Responses in Developmental Stages of Megachile apicalis Spinola (Hymenoptera: Megachilidae). *Environmental Entomology*, *38*(2), 484-492. https://doi.org/10.1603/022.038.0223
Johnson, E. C., & White, M. P. (2009). 31 - Stressed-Out Insects: Hormonal Actions and Behavioral Modifications. In D. W. Pfaff, A. P. Arnold, A. M. Etgen, S. E. Fahrbach, & R. T. Rubin (Eds.), *Hormones, Brain and Behavior (Second Edition)* (pp. 1069-1097). Academic Press. https://doi.org/https://doi.org/10.1016/B978-008088783-8.00031-0
Jones, J. C., Helliwell, P., Beekman, M., Maleszka, R., & Oldroyd, B. P. (2005). The effects of rearing temperature on developmental stability and learning and memory in the honey bee, Apis mellifera. *Journal of Comparative PhysiologyA,191*(12),1121-1129. https://doi.org/10.1007/s00359-005-0035-z
Kammer, A. E., & Heinrich, B. (1978). Insect Flight Metabolism. In J. E. Treherne, M. J. Berridge, & V. B. Wigglesworth (Eds.), *Advances in Insect Physiology* (Vol. 13, pp. 133-228). Academic Press. https://doi.org/https://doi.org/10.1016/S0065-2806(08)60266-0
Kestler, P. (1991). Cyclic CO2 release as a physiological stress indicator in insects. *Comparative biochemistry and physiology: C: Comparative pharmacology and toxicology*.
King, A. M., & MacRae, T. H. (2015). Insect Heat Shock Proteins During Stress and Diapause. *Annual Review ofEntomology*, *60*(1), 59-75. https://doi.org/10.1146/annurev-ento-011613-162107
Klok & Chown (2005). Temperature-and body mass-related variation in cyclic gas exchange characteristics and metabolic rate of seven weevil species: broader implications. *Journal of Insect Physiology*, *51*(7), 789-801.
Klok & Chown, (2005). Temperature- and body mass-related variation in cyclic gas exchange characteristics and metabolic rate of seven weevil species: Broader implications. *Journal of Insect Physiology*, *51*(7), 789-801. https://doi.org/https://doi.org/10.1016/j.jinsphys.2005.03.007
Kodrik, D. (2008). Adipokinetic hormone functions that are not associated with insect flight. *Physiological Entomology*, *33*(3), 171-180.
Kodrík, D., & Socha, R. (2005). The effect of insecticide on adipokinetic hormone titre in the insect body. *Pest Management Science: formerly Pesticide Science*, *61*(11), 1077-1082.
Koffler, S., Meneses, H. M., Kleinert, A. D. P., & Jaffe, R. (2016). Competitive males have higher quality sperm in a monogamous social bee. *BMC Evolutionary Biology*, *16*, Article 195. https://doi.org/10.1186/s12862-016-0765-2
Komasilova, O., Komasilovs, V., Kviesis, A., & Zacepins, A. (2021). Model for finding the number of honey bee colonies needed for the optimal foraging process in a specific geographical location. *PeerJ*, *9*, e12178.https://doi.org/10.7717/peerj.12178
Kononenko, N. L., Wolfenberg, H., & Pflüger, H. J. (2009). Tyramine as an independent transmitter and a precursor of octopamine in the locust central nervous system: an immunocytochemical study. *Journalof Comparative Neurology*, *512*(4), 433-452.

Kovac, H., Stabentheiner, A., Hetz, S. K., Petz, M., & Crailsheim, K. (2007). Respiration of resting honeybees Journal ofInsect Physiology, 53(12), 12501261. https://doi.org/https://doi.org/10.1016/j.jinsphys.2007.06.019

Kunieda, T., Fujiyuki, T., Kucharski, R., Foret, S., Ament, S., Toth, A., . . . Kage, E. (2006). Carbohydrate metabolism genes and pathways in insects: insights from the honey bee genome. *Insect Molecular Biology*, *15*(5), 563-576.

Kvetnansky, R., Sabban, E. L., & Palkovits, M. (2009). Catecholaminergic systems in stress: structural andmolecular genetic approaches. *Physiological Reviews*, *89*(2), 535-606.

Laidlaw, H. H. (1977). *Instrumental Insemination of Honey Bee Queens: Pictorial Instructional Manual*. Dadant.https://books.google.co.nz/books?id=Nq0cAQAAMAAJ

Le Conte, Y., Ellis, M., & Ritter, W. (2010). Varroa mites and honey bee health: can Varroa explain part of thecolony losses? *Apidologie*, *41*(3), 353-363. https://doi.org/10.1051/apido/2010017

Le Conte, Y., & Navajas, M. (2008). Climate change: impact on honey bee populations and diseases. *RevueScientifique et Technique*, *27*(2), 485-497, 499-510.

Leta, M., Gilbert, C., & Morse, R. (1996). Levels of hemolymph sugars and body glycogen of honeybees (Apismellifera L.) from colonies preparing to swarm. *Journal of Insect Physiology*, *42*(3), 239-245.

Levin, M. D., & Haydak, M. H. (1951). Seasonal variation in weight and ovarian development in the workerhoneybee. *Journal of Economic Entomology*, *44*(1), 54- Li, J., Heerman, M. C., Evans, J. D., Rose, R., Li, W., Rodríguez-García, C., . . . Chen, Y. (2019). Pollen reverses decreased lifespan, altered nutritional metabolism and suppressed immunity in honey bees (Apis mellifera) treated with antibiotics. *J Exp Biol*, *222*(Pt 7). https://doi.org/10.1242/jeb.202077

Lighton, J. R. (1996). Discontinuous gas exchange in insects. *Annual Review of Entomology*, *41*(1), 309-324.

Lima, M. A. P., Martins, G. F., Oliveira, E. E., & Guedes, R. N. C. (2016). Agrochemical-induced stress in stingless bees: peculiarities, underlying basis, and challenges. *Journal of Comparative Physiology A*, *202*(9), 733-747. https://doi.org/10.1007/s00359-016-1110-3

Lipiński, Z., & Żółtowska, K. (2005). Preliminary evidence associating oxidative stress in honey bee drone broodwith Varroa destructor. *Journal of Apicultural Research*, *44*(3), 126-128.

Ma, E., & Haddad, G. G. (1997). Anoxia regulates gene expression in the central nervous system of Drosophila melanogaster. *Brain Research. Molecular Brain Research*, *46*(1-2), 325-328. https://doi.org/10.1016/s0169-328x(97)00074-0

Maradonna, F., & Carnevali, O. (2007). Vitellogenin, zona radiata protein, cathepsin D and heat shock protein70 as biomarkers of exposure to xenobiotics. *Biomarkers*, *12*(3), 240-255.

Matz, J. M., LaVoi, K. P., Moen, R. J., & Blake, M. J. (1996). Cold-induced heat shock protein expression in rat aorta and brown adipose tissue. *Physiol Behav*, *60*(5), 1369-1374. https://doi.org/10.1016/s0031- 9384(96)00220-x

Mayer, R. J., & Candy, D. J. (1969). Changes in energy reserves during flight of the desert locust, Schistocerca gregaria. *Comparative Biochemistry and Physiology*, *31*(3), 409-418. https://doi.org/10.1016/0010- 406x(69)90022-x

Meixner, M. D. (2010). A historical review of managed honey bee populations in Europe and the United Statesand the factors that may affect them. *Journal of Invertebrate Pathology*, *103*, S80-S95.

Melicher, D., Wilson, E. S., Bowsher, J. H., Peterson, S. S., Yocum, G. D., & Rinehart, J. P. (2019). Long-distance transportation causes temperature stress in the honey bee, Apis mellifera (Hymenoptera: Apidae). *Environmental Entomology*, *48*(3), 691-701. https://doi.org/10.1093/ee/nvz027

Mogren, C. L., Benítez, M.-S., McCarter, K., Boyer, F., Lundgren, J. G., & Cooke, S. *Diverging landscape impacts on macronutrient status despite overlapping diets in managed (Apis mellifera) and native (Melissodesdesponsa) bees*. https://doi.org/10.1093/conphys/coaa109

Morimoto, R. (1993). Cells in stress: transcriptional activation of heat shock genes. *Science*,

259(5100), 1409- 1411.
Morimoto, R. I. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes & Development*, *12*(24), 3788-3796.
Mortensen, A. N., & Ellis, J. D. (2014). Scientific note on a single-user method for identifying drone congregation areas. *Journal of Apicultural Research*, *53*(4), 424-425.
Mortensen, A. N., Schmehl, D. R., & Ellis, J. (2013). European honey bee-Apis mellifera. *European Honey Bee-Apis Mellifera. Entomology and Nematology Department*.
MPI. (2017). New Zealand Colony Loss Survey Report –2016. https://www.mpi.govt.nz/dmsdocument/16711-new-zealand-colony-loss-survey-report-2016
MPI. (2021). Report on the 2020 New Zealand Colony Loss Survey [Report].
Nagao, R. T., Kimpel, J. A., & Key, J. L. (1990). Molecular and cellular biology of the heat-shock response.
Advances in Genetics, *28*, 235-274.
Neven, L. G. (2000). Physiological responses of insects to heat. *Postharvest Biology and Technology*, *21*(1), 103- 111.
Newstrom-Lloyd, L. (2015). Managing manuka for carrying capacity and competition. *New Zealand Beekeeper*,*23*, 18-19.
Newstrom-Lloyd, L. (2016). Bees without borders: What is the limit? *New Zealand Beekeeper Journal*, 22-27.
Oldroyd, B. P. (2007). What's killing American honey bees? *PLoS Biology*, *5*(6), e168.
Pempkowiak, J., Radłowska, M., Bełdowski, J., Kupryszewski, G., Wysocki, J., & Głos´ nicka, R. (2001). Westernblotting versus ELISA detection of stress proteins induced in the blue mussel Mytilus edulis exposed to cadmium and tributyltin. *Biomarkers*, *6*(6), 400-405.
Penno, J. (1999). Stocking rate for optimum profit. *South Island Dairy Event*, *1*, 25-43.
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. Pimentel, A. C., Montali, A., Bruno, D., & Tettamanti, G. (2017). Metabolic adjustment of the larval fat body inHermetia illucens to dietary conditions. *Journal of Asia-Pacific Entomology*, *20*(4), 1307-1313. https://doi.org/https://doi.org/10.1016/j.aspen.2017.09.017
Pyza, E., Mak, P., Kramarz, P., & Laskowski, R. (1997). Heat shock proteins (HSP70) as biomarkers inecotoxicological studies. *Ecotoxicol Environ Saf*,*38*(3),244-251. https://doi.org/10.1006/eesa.1997.1595
Ratnieks, F. L., & Carreck, N. L. (2010). Clarity on honey bee collapse? *Science*, *327*(5962), 152-153.
Riddiford, L. (1980). Interaction of ecdysteroids and juvenile hormone in the regulation of larval growth and metamorphosis of the tobacco hornworm. *Progress in ecdysone research, JA Hoffmann, editor*.
Rothe, U., & Nachtigall, W. (1989). Flight of the honey bee. *Journal of Comparative Physiology B*, *158*(6), 739-749. https://doi.org/10.1007/BF00693012
Roussel, E., Carcaud, J., Sandoz, J.-C., & Giurfa, M. (2009). Reappraising social insect behavior through aversive responsiveness and learning. *PLoS ONE*, *4*(1), e4197.
Sahebzadeh, N., & Lau, W. H. (2017). Expression of heat-shock protein genes in Apis mellifera meda (Hymenoptera: Apidae) after exposure to monoterpenoids and infestation by Varroa destructor mites(Acari: Varroidae). *European Journal of Entomology*, *114*, 195-202. https://doi.org/10.14411/eje.2017.024
Sakano, D., Li, B., Xia, Q., Yamamoto, K., Fujii, H., & Aso, Y. (2006). Genes Encoding Small Heat Shock Proteins of the Silkworm, Bombyx mori. *Bioscience, Biotechnology, and Biochemistry*, *70*(10), 2443-2450. https://doi.org/10.1271/bbb.60176
Scharlaken, B., de Graaf, D. C., Goossens, K., Peelman, L. J., & Jacobs, F. J. (2008). Differential gene expressionin the honeybee head after a bacterial challenge. *Developmental & Comparative Immunology*, *32*(8), 883-889.
Schmolz, E., Lamprecht, I., & Schricker, B. (1995). A method for continuous direct calorimetric

measurements of energy metabolism in intact hornet (Vespa crabro) and honeybee (Apis mellifera) colonies. *Thermochimica Acta*, *251*, 293-301.
Schoener, T. W. (1983). Field experiments on interspecific competition. *The american naturalist*, *122*(2), 240-285.
Seeley, T. D. (1982). Adaptive significance of the age polyethism schedule in honeybee colonies. *Behavioral Ecology and Sociobiology*, *11*(4), 287-293. https://doi.org/10.1007/BF00299306
Seeley, T. D., & Smith, M. L. (2015). Crowding honeybee colonies in apiaries can increase their vulnerability tothe deadly ectoparasite Varroa destructor. *Apidologie*, *46*(6), 716-727. https://doi.org/10.1007/s13592-015-0361-2
Selye, H. (1950). Stress and the general adaptation syndrome. *British medical journal*, *1*(4667), 1383. Selye, H. (1956). *The stress of life*.
Smart, M. D., Otto, C. R. V., & Lundgren, J. G. *Nutritional status of honey bee (Apis mellifera L.) workers acrossan agricultural land-use gradient*. https://doi.org/10.1038/s41598-019-52485-y
Steele, J.E. (1982). Glycogen phosphorylase in insects. *Insect Biochemistry*, *12*(2), 131-147. https://doi.org/https://doi.org/10.1016/0020-1790(82)90001-4
Stratakis, C. A., & Chrousos, G. P. (1995). Neuroendocrinology and pathophysiology of the stress system.
Annals of the New York Academy of Sciences, *771*(1), 1-18.
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.
Suarez, R. K., Darveau, C.-A., Welch, K. C., Jr., O'Brien, D. M., Roubik, D. W., & Hochachka, P. W. (2005). Energy metabolism in orchid bee flight muscles: carbohydrate fuels all. *Journal of Experimental Biology*, *208*(18), 3573-3579. https://doi.org/10.1242/jeb.01775
Tran, M. V., O'Grady, M., Colborn, J., Van Ness, K., & Hill, R. W. (2014). Aggression and food resource competition between sympatric hermit crab species. *PLoS ONE*, *9*(3), e91823.
Van Marrewijk, W. J. A., van den Broek, A. T. M., & Beenakkers, A. M. T. (1986). Hormonal control of fat-body glycogen mobilization for locust flight. *General and Comparative Endocrinology*, *64*(1), 136-142. https://doi.org/https://doi.org/10.1016/0016-6480(86)90039-0
VanEngelsdorp, D., Caron, D., Hayes, J., Underwood, R., Henson, M., Rennich, K., Lee, K. (2012). A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee InformedPartnership. *Journal of Apicultural Research*, *51*(1), 115-124
Veenstra, J. A. (2009). Does corazonin signal nutritional stress in insects? *Insect Biochemistry and MolecularBiology*, *39*(11), 755-762.
Weibel, E. R., & Hoppeler, H. (2005). Exercise-induced maximal metabolic rate scales with muscle aerobic capacity. *Journal of Experimental Biology*, *208*(9), 1635-1644. https://doi.org/10.1242/jeb.01548
Willingham, R., Klopchin, J., & Ellis, J. (2000). Robbing Behavior in Honey Bees. *Behavioral Ecology*, *11*(3), 326-333.
Wilson, E. E., & Wolkovich, E. M. (2011). Scavenging: how carnivores and carrion structure communities. *Trends Ecol Evol*, *26*(3), 129-135. https://doi.org/10.1016/j.tree.2010.12.011
Winston, M. L. (1987). *The biology of the honey bee / Mark L. Winston*. Harvard University Press.
Wolf, T. J., & Schmid-Hempel, P. (1989). Extra loads and foraging life span in honeybee workers. *The Journalof Animal Ecology*, 943-954.
Woodworth, C. E. (1932). Some Effects of Reduced Atmospheric Pressure Upon Honey Bee Respiration. *Journal of Economic Entomology*, *25*(5), 1036-1042. https://doi.org/10.1093/jee/25.5.1036
Yaniz, J. L., Silvestre, M. A., & Santolaria, P. (2020). Sperm quality assessment in honey bee drones. *Biology- Basel*, *9*(7), Article 174. https://doi.org/10.3390/biology9070174
Zhao, L., Becnel, J. J., Clark, G. G., & Linthicum, K. J. (2010). Expression of AeaHsp26 and AeaHsp83 in Aedes aegypti (Diptera: Culicidae) Larvae and Pupae in Response to Heat Shock

Stress. *Journal of Medical Entomology*, *47*(3), 367-375. https://doi.org/10.1093/jmedent/47.3.367