Foraging activity and colony thermoregulation of the honey bee *Apis mellifera* following exposure to the organophosphate pesticide chlorpyrifos

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Abstract

Honey bees (*Apis mellifera* L.) are important pollinators and yet, an increasing number of reports suggest honey bee populations worldwide are in decline. Pollinators face many environmental stressors, including exposure to pesticides. Chlorpyrifos is an organophosphate pesticide that is used extensively in New Zealand. This thesis examines whether exposing bees to chlorpyrifos, applied to a forage crop at a concentration recommended by the manufacturer, alters honey bee foraging activity, or colony temperature.

A 1.26ha crop of *Phacelia tanacetifolia* was planted in the Ida Valley of Central Otago, New Zealand, and sprayed with 0.20kg a.i/ha of Lorsban 50EC. The spray event occurred in early January 2017, the height of the austral summer. Honey bee colonies were introduced to the crop either, prior to the spray event or at intervals thereafter. Colonies exposed to pesticide were compared with colonies located on control (untreated) sites.

HiveMind monitors, a satellite based measurement system, were used to provide a real time count of foraging activity and a record of internal hive temperatures in all colonies. Activity and temperature measurements were taken every three hours over a 4 month period.

Significant changes in maximum activity levels and in-hive temperature were identified in all colonies. The results suggest these changes were mainly driven by shifts in forage availability and external temperature over time. There was no clear evidence linking chlorpyrifos exposure to either the decline in activity levels, or to changes in colony temperature. However this may be due to the length of the experiment which may have required a longer study period to observe pesticide effects, or the effects were masked due to other environmental factors. Further work is required to ascertain whether in the longer term, chlorpyrifos alters colony productivity.

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Chapter 1

Introduction

1.1 The honey bee *Apis mellifera*

Bees are a diverse clade of flying insects, with upwards of 17 000 known species in 7 recognised biological families. They are found on every continent apart from Antarctica, and provide important ecosystem services in the form of pollination (Michener, 2000). Bees thrive in a wide range of habitats, with varying adaptations for survival, one of which includes complex social behaviour (Michener, 1974). Social behaviour allows for large colonies to form in which individuals work together for survival. This thesis focusses on the honey bee, *Apis mellifera*, more commonly known as the European honey bee. *A.mellifera* is part of the family Apidae, class Insecta, order Hymenoptera (Gould et al., 1988). They are eusocial insects, with upwards of 60,000 individual bees within a colony through the peak of summer. A colony consists of three castes of bees, workers, drones and generally a single queen (Winston, 1991).

1.1.1 Workers

Worker bees carry out day to day tasks required for colony survival. These jobs include cleaning the hive, caring for the brood, building comb, thermoregulating the hive, undertaker work and foraging (Oster and Wilson, 1979; Robinson, 1992). The workers themselves exhibit age-related divisions of labour, with newly emergent bees (1-2 days

old) cleaning the hive, young workers generally care for brood (nursing) and workers 12-21 days of age build comb, perform undertaker duties and guard the entrance of the hive. After about 3 weeks of performing tasks inside the hive, bees transition into foragers, venturing outside the hive in search of nectar and pollen for storage over the winter. Despite the age-based polyethism, worker bees also display the ability to change between different tasks depending on the needs of the colony (Huang and Robinson, 1996; Shemesh et al., 2010), and are thus capable of significant behavioural plasticity.

1.1.2 Drones

The drones are the males of the colony, existing to mate with the queen (Winston, 1991; Steinhauer et al., 2014). They do not forage, care for young or perform hive duties. Over the summer there is an abundance of drones. Drones will fly in small swarms during the summer, mating with any queen that is on a mating flight. The process of mating will kill the drone in order to transfer sperm to a queen during a mating flight. During the winter months, the drones are not necessary, so the drones are forced from the hive or killed (Winston, 1991).

1.1.3 The queen

The queen is the sole reproductive entity of the hive. She will only make a handful of mating flights early on in her life, providing her with enough sperm to lay eggs for her entire lifespan (Winston, 1991). The queen will lay hundreds of thousands of eggs throughout her life. She does not partake in caring for young or maintenance of the hive, but instead spends her time laying eggs. She also plays a crucial role in regulating the activity of the workers through pheromone control (Winston, 1991). Through a complex mix of chemical compounds, she suppresses the reproductive organs of all female workers, influences the division of labour within the hive, attracts young workers to feed and groom her, attracts drones to her for mating and prevents the rearing of new queens (Winston, 1991).

As a study species, the honey bee has been useful in understanding the dynamics of social insect colonies and the health of terrestrial ecosystems. Social insects require a

high level of coordination between colony members if the colony is to survive. If a colony is failing it can be an indicator of a problem within the colony, but also the state of the surrounding ecosystem. Through observations of foraging levels, thermoregulation ability, mortality levels, pollutants within pollen, honey stores, number of larvae and the workers, it is possible to assess the health of the surrounding ecosystem (Celli and Maccagnani, 2003).

1.2 Coordinated activity

1.2.1 Thermoregulation

The ability to work together is of paramount importance to the survival of honey bee colonies. One important example is the honey bees' ability to regulate colony temperature. A colony can face a wide range of external temperatures, ranging anywhere from sub-zero temperatures, to temperatures above 40°C, depending on their location. This extreme range of temperatures would be very difficult for an individual bee to survive, which is why the adaptation of colony thermoregulation is a survival benefit to honey bees (Seeley, 2009). Thermoregulation of an entire hive allows for faster warming of thoracic temperatures for foraging (Heinrich, 1979), optimal temperatures for brood production (Jones et al., 2005; Tan et al., 2012), and protection from any varying extremes of external temperature.

Honey bees have the ability to regulate the internal temperature within the colony through a variety of behavioural mechanisms. Behaviours range from clustering and shivering flight muscles to warm up specific areas of the colony, to fanning their wings to cool down the colony (Heinrich, 1979; Kronenberg and Heller, 1982; Fahrenholz et al., 1989; Stabentheiner et al., 2010). Cycles of fanning and clustering are correlated with metabolic rate and activity levels within a colony (Kronenberg and Heller, 1982). As external temperature decreases, clustering increases within a colony, and conversely, as temperature increases an increase of locomotor activity and fanning occurs (Kronenberg and Heller, 1982). A large amount of clustering also occurs on comb that contains brood, which is crucial for keeping brood at optimal temperatures for development. Brood require temperatures within the 33-36°C range for proper development (Stabentheiner

et al., 2010). Colonies with brood over the summer period will regulate their internal hive temperature to an average of 35.5°C (Fahrenholz et al., 1989). In the winter the average temperature within a cluster falls to 21°C, however this varies with the external temperature (Fahrenholz et al., 1989). The main mechanism of heat production within the hive is shivering of thoracic flight muscles, which is performed by bees above 2 days old (Stabentheiner et al., 2010). If a colony is unable to thermoregulate, it may signal greater problems for a colony as a whole. Thermoregulation is crucial not only for rearing brood (Jones et al., 2005), but also for overwinter survival, and can be used as an indirect measure of colony health (Meikle et al., 2017).

1.2.2 Foraging behaviour

Foraging is essential for honey bees as it provides the necessary food and water for a colony to survive. The foragers of the hive are tasked with collecting resources for the colony, including water, nectar, pollen and resin (Winston, 1991). There are two classes of forager bees; scouts and "reticent bees" (Van Nest and Moore, 2012). Scouts search for food and relay this information back to reticent bees who then forage based on this information (Van Nest and Moore, 2012). Information on the position of the forage is passed through the waggle dance (Von Frisch, 1967), passing information such as the quality of forage and its direction and distance from the colony (Biesmeijer and Seeley, 2005; Riley et al., 2005). The ability to communicate provides reduced energy consumption for foragers as only a small number of the foragers have to search for viable food sources.

The decision of when to go out and forage is determined by time of day, temperature, colony needs and genetic traits (Hunt et al., 1995; Huang and Robinson, 1996; Abou-Shaara, 2014). Onset and offset of foraging has been observed to be dependent on multiple factors such as region, weather, light and forage availability (Joshi and Joshi, 2010). External temperature plays an important role in the onset of foraging, and also the amount of bees foraging at any one time. Onset of foraging has been observed to occur at a mean ambient temperature as low as 6.5°C (Tan et al., 2012), however Joshi and Joshi (2010) found the mean ambient temperature value to be closer to 16°C, with the lowest levels of foraging occurring at or below 10°C (Joshi and Joshi, 2010). Tan et al. (2012) observed the highest foraging activity at an ambient external temperature of

approximately 20°C, while Blažytė-Čereškienė et al. (2010) report a significant decline in foraging activity at temp above 43°C, with foraging levels dropping as low as those observed at 10°C by Joshi and Joshi (2010). Temperature is clearly an important factor affecting foraging activity.

The decision for a honey bee to collect a particular resource is based on genetic traits (Hunt et al., 1995), collective colony needs (Fernández and Farina, 2005), individual decisions and sucrose response thresholds (Pankiw and Page Jr, 2000). Depending on available floral resources, the particular resources collected may have an optimal time for collection. Forager bees have an incredible sense of time, allowing them to remember when the best time is to visit particular plant species (Silva et al., 2013). High levels of pollen collection have been observed in the morning, with a reduction in collection in the afternoon (Reyes-Carrillo et al., 2007). High levels of nectar foraging have been observed in the afternoon compared to the morning (Pernal and Currie, 2001), however, these timings may be dependent on the floral species available. Preferences for particular food sources have been documented on various occasions, where foragers will show a preference for individual plant species, even flying over other viable sources to visit the desired forage (Mayer and Lunden, 1988; Chittka et al., 1999; Fohouo et al., 2008; Sushil et al., 2013). Honey bee foragers can fly great distances in search of forage (Beekman and Ratnieks, 2000; Hagler et al., 2011). However the average distance travelled by forager honey bees has been shown to be colony dependent. If the colony is large, it is more likely that foragers will travel further (Beekman et al., 2004). Maximum foraging distance of a honey bee can be as far as 5983 m (Hagler et al., 2011), and is heavily dependent on colony strength, the food resource and season (Abou-Shaara, 2014).

1.2.3 Rhythmicity of honey bee activity

Honey bees display two distinct forms of rhythmicity throughout their life. Initially nurse bees show arrhythmicity while caring for the brood and carrying out in hive-duties. Arrhythmicity is the state of showing no overt daily rhythms, with the ability to be active at all times of the day (Bloch et al., 2002). Arrhythmic behaviour is beneficial in certain biological settings, for example, when brood needs to be cared for around the clock (Bloch et al., 2002). With the transition to foraging, honey bees undergo a change in rhythmicity, switching to circadian rhythmicity (Bloch and Robinson,

2001; Eban-Rothschild and Bloch, 2012). A circadian rhythm is defined as a 24 hour oscillating pattern that is endogenous and entrainable (Saunders, 2002). This rhythm is governed by a circadian system, which is made up of a circadian clock, photoreceptors and an output system (Tomioka and Matsumoto, 2010). The circadian clock generates the 24 hour oscillation rhythm, the photoreceptors are necessary for the clock to synchronise light-dark cycles (LD), and the output system transfers the information from the clock to tissues around the body, enabling regulation of their rhythmicity 9(Saunders, 2002; Dunlap et al., 2004; Tomioka and Matsumoto, 2010). The major synchronising agents (known as zeitgebers) for entraining of circadian clocks are light and temperature (Fuchikawa et al., 2016). Light is thought to be the most important zeitgeber, as entrainment to light and dark cycles are commonplace throughout the natural world (Kronfeld-Schor et al., 2013). Temperature is also thought to be important, especially within cave-dwelling animals who have no contact with LD cycles (Eban-Rothschild and Bloch, 2012; Fuchikawa et al., 2016).

1.3 The importance of the honey bee *Apis mellifera* as a pollinator

Bees are the most important guild of pollinators as they visit more than 90% of the leading 107 global crop types (Klein et al., 2007). Out of all species of bee, *Apis mellifera* is the most commonly managed pollinator species, used to enhance agricultural production and capable of increasing yield in some crops by up to 96% (Klein et al., 2007). The honey bee's native range originally spanned from Central Asia, up to Southern Scandinavia and across to Africa. However over the past 400 years their range has increased greatly, reaching across most of the globe due to human transportation (Crane, 2013). The modern day use of honey bees as crop pollinator, and the demand for the honey they produce, have led humans to introduce the honey bee to most continents (Whitfield et al., 2006). The management of honey bee colonies has always faced difficulties, but as time has gone on, threats to honey bee colonies have become more pronounced. Threats affecting colony survival and health include agricultural intensification (Banaszak, 1992; Steffan-Dewenter et al., 2002), immunosuppression (Alaux et al., 2010), parasites (Le Conte et al., 2010), pesticides

(Goulson et al., 2015; Johnson et al., 2010) and climate change (Settele et al., 2016). These threats do not just work in isolation, rather interactions between stressors are thought to be the driving factor behind pollinator decline (Potts et al., 2010). A further concern is the massive increase in agricultural demand for managed pollinators. According to Aizen and Harder (2009), pollinator stocks are now unable to keep up with the levels of demand necessary for today's agricultural needs.

1.4 Observed trends in *Apis mellifera* populations over time

Reports of large scale colony loss of managed honey bees are not a recent development, with some reports dating back over 160 years in the United States (Underwood and Vanengelsdorp, 2007). While there are many possible factors influencing the collapse of a colony, the most unusual is "colony collapse disorder"(Evans et al., 2009). The term "colony collapse disorder (CCD)" appeared in 2009 (Evans et al., 2009), used to describe the phenomenon of a large disappearance of workers from a hive, leaving behind young workers, intact food stores and the queen (Evans et al., 2009). Furthermore, nest robbers delay their invasion of such hives, which is yet to be explained (Dainat et al., 2012). There are also other factors that losses can be attributed to, such as parasites and pesticides. The term "colony failure" is now used most commonly to describe the decline in honey bee populations that have been observed in North America, Europe and Australia (Underwood and Vanengelsdorp, 2007).

A review by Meixner et al. (2010) observed that between 1947 and 2008, the US saw a 61% decline in managed honey bee colonies (5.9 million colonies down to 2.3 million); while Europe exhibited a 29% decrease between 1961 to 2007 (21 million colonies down to 15.5 million). Over the last 50 years a handful of countries within Europe such as Finland and Spain have seen increases as high as 50%; whereas other countries such as Sweden have seen large decreases, with 75% loss. Large scale surveys of managed colony loss in the United States have been carried out over the past decade (Hayes Jr et al., 2008; Vanengelsdorp et al., 2012; Kulhanek et al., 2017), with 10 years worth of reports on winter losses, and 5 years on summer losses. The highest rate of colony loss was in 2012/2013, with 45% colony loss over winter (Steinhauer et al., 2014), and the

most recent survey published showed 40.5% colony loss in 2015/2016 (Kulhanek et al., 2017). Despite the high levels of colony loss in the United States, between 2006 and 2016 the number of colonies increased from 2.39 million to 2.59 million. The increase in colony numbers is due to replacement of lost hives, through the splitting of stronger existing colonies into multiple new colonies (Meixner et al., 2010). While these regional losses underlie an overall trend of pollinator decline, globally the number of managed colonies have increased by 45% since 1961 (Aizen et al., 2008; Aizen and Harder, 2009). Despite the global increase in managed colonies, the global increase in crops dependent on pollination services has grown by 3-fold between 1961 and 2006 (Aizen et al., 2008), which outweighs the observed colony increases over this time (Aizen and Harder, 2009). This may lead to large losses in agricultural productivity if pollination services provided by animals are lost (Figure 1.1).

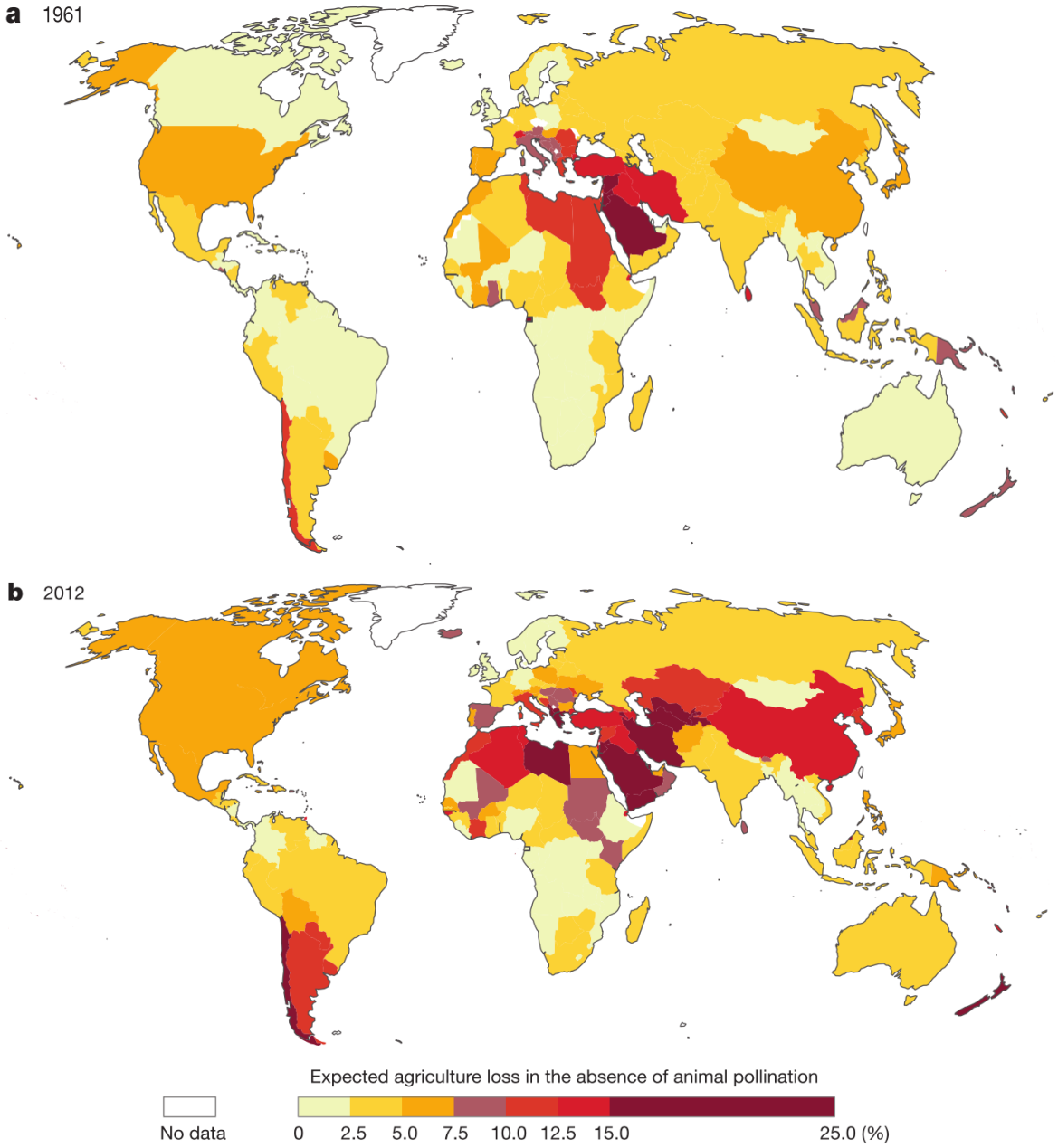

Figure 1.1: Global projected loss of agriculture without animal pollinators (from Potts et al. (2016)). (a) Projection from 1961. (b) Projection from 2012. Figure based on the FAO dataset (http://faostat.fao.org/). Data values are estimated at the country level. The distribution of agricultural land, crop differences and dependence on pollinators are spatially heterogeneous within countries.

1.5 Populations of *Apis mellifera* in New Zealand

While studies focussing on pollinator decline are scarce within New Zealand, a similar survey to Kulhanek et al. (2017) was carried out in New Zealand looking to quantify colony losses over winter in 2016 (Brown, 2017). A large number of beekeepers (2179) responded, reporting on 275,356 colonies, which is 40% of all production colonies in New Zealand. From their estimates, about 10% of colonies are lost over winter yearly. However the total number of colonies in New Zealand increased by 20% between March 2015 and June 2016. While the colony numbers are increasing, this is not a good indicator of overall bee health. Monitoring of the rate of colony loss provides a more accurate measure of how the environment is affecting the health of managed bee populations (Meixner et al., 2010). New Zealand may be in a dangerous position if a decline in pollinators occurs, due to the high levels of endemism within its flora and fauna, yet a very high proportion of introduced naturalised plants (52%) (Newstrom-Lloyd et al., 2013). This suggests most native pollinators may not be well equipped to pollinate introduced species, and introduced pollinators may be ill-equipped to pollinate native species. Therefore any locally extinct pollinator species are unlikely to be replaced due to New Zealand's isolation as an island nation. New Zealand has a low diversity of native pollinators, and until bumble bees and honey bees were introduced, had no native counterpart to the large social bees on other continents (Donovan, 2007). The introduction of *Apis mellifera* and other pollinators to New Zealand allowed for increased productivity within the agricultural industry (Newstrom-Lloyd et al., 2013). This indicates that a decline in either native or introduced pollinators could have major consequences on New Zealand's agricultural industry and native flora.

1.6 Pesticides

Review papers from Staveley et al. (2014) and Goulson et al. (2015) explored possible reasons for the decline in honey bees globally. Staveley et al. (2014) believe the main factors driving the reduction in colony survival is a combination of the parasitic *Varroa* mite and pathogens. However they also state that pesticides may bring an added stressor that further weakens colonies. The conclusion from Goulson et al. (2015) is

similar, stating that the low levels of pesticides colonies face in agricultural settings may be enough to exacerbate the effects of parasites on infected colonies. Foragers regularly come into contact with pesticides while foraging, especially within agricultural settings (Alburaki et al., 2017), which can lead to possible lethal or sublethal effects (Cutler et al., 2014). Pesticide links to colony failure are often downplayed, relegated to a secondary contributing factor (Genersch, 2010; Staveley et al., 2014). Despite this assessment there are still many studies suggesting the adverse effects of pesticides at sublethal doses (Cutler et al., 2014; Sánchez-Bayo et al., 2016). The present study focuses on the organophosphate, chlorpyrifos.

1.7 Chlorpyrifos

1.7.1 Mode of action

Chlorpyrifos (O, O-diethyl O-3,5,6-trichloro-2-pyridinyl phosphorothioate) is a common organophosphate that has been used in over 100 countries worldwide, and is still available for public use in most, including in New Zealand (Aktar et al., 2009; Solomon et al., 2014). Organophosphates are a group of commonly used pesticides that were popularised in the 1970s after the banning of organochlorides such as DDT and dieldrin. Organophosphates are toxic to animals through their ability to inhibit acetylcholinesterase (Fukuto, 1990; Williamson et al., 2013). Acetylcholinesterase is an enzyme responsible for the hydrolytic degradation of the neurotransmitter acetylcholine into two inactive products, choline and acetic acid (Fukuto, 1990). Acetylcholine is involved in transmitting signals across synaptic junctions within the nervous system and at neuromuscular junctions (Fukuto, 1990). In layman's terms, acetylcholine is a chemical that is released by the nervous system, involved, among other things, in the activation of muscles. When acetylcholinesterase is blocked, acetylcholine is no longer broken down into its inactive components. Instead a rapid build-up of this neurotransmitter occurs at the neuromuscular junction (Fukuto, 1990; Williamson et al., 2013). This in turn causes a repeated and uncontrolled stimulation of the nerve fibres or muscles because of the build-up of acetylcholine in the synaptic cleft. This is known as a cholinergic crisis, which can lead to muscle spasms, eventual loss of motor function and if the

concentration is high enough, death (Fukuto, 1990; Williamson et al., 2013). Chlorpyrifos' mode of action is similar for both targeted and non-targeted organisms (Reigart, 2009). This can lead to poisoning of non-target species, including humans (Reigart, 2009). In the past pesticides were considered "safe" for pollinators up to their lethal dose 50% (LD50). An LD50 is the concentration at which 50% of an exposed test sample dies. According to the safety data sheet (SDS) for chlorpyrifos, honey bees have an oral LD50 of 0.36 micrograms/bee and a contact LD50 of 0.070 micrograms/bee. However more studies are finding sublethal effects occurring at concentrations far below the LD50 of most pesticides (Desneux et al., 2007). Desneux et al. (2007) define a sublethal effect as "an effect (physiological or behavioural) on individuals that survive an exposure to a pesticide".

1.7.2 Honey bee exposure to chlorpyrifos

Chlorpyrifos is used primarily as a contact pesticide (Solomon et al., 2014), for control against foliar pests such as aphids, beetles, caterpillars, leafhoppers, mites and scale. Chlorpyrifos is applied at a variety of concentrations depending on which pests are being targeted and which crop is being sprayed. These concentrations range from 200/mL/h.a. - 2400/mL/h.a (SDS Lorsban 50EC). Chlorpyrifos is sold under multiple different labels, including Lorsban, Dursban, Empire20 and Equity, and produced by multiple different manufacturers around the world. It is commonly found in a liquid concentrate formula, which is sprayed directly on crops. It is usually applied through tractor boom and aerial application. Chlorpyrifos has short to moderate persistence in the environment (Solomon et al., 2014), with primary mechanisms of dissipation including volatilisation (Mackay et al., 2014), photolysis (Racke, 1993), abiotic hydrolysis (Katagi et al., 2002) and microbial degradation (Racke, 1993). Volatilisation is the main dissipation pathway from foliage in the 12 hours after application, but this is reduced as the formulation is adsorbed into the foliage or soil and photolysis occurs (Mackay et al., 2014). Chlorpyrifos is a non-systemic pesticide, which means chlorpyrifos is not uptaken by the roots or absorbed into the foliage of the plant, therefore it is not redistributed throughout the plant itself (Tomlin et al., 2009). With systemic pesticides, the pesticides are uptaken into the plant itself, which gives the added benefit of long term protection from soil invertebrates and sucking insects (Elbert et al., 1990).

However a downside of using systemic pesticides is the production of contaminated nectar and pollen, which can have adverse effects on pollinators (Rortais et al., 2005). Any nectar and pollen collected from these plants likely has small traces of pesticides within, potentially at levels high enough to cause sublethal effects (Rortais et al., 2005).

There are multiple routes of chlorpyrifos exposure for pollinators, such as direct contact with the pesticide on foliage and soil, through aerial spray drift (dust and aerosols), ingestion through pollen, nectar, and to a lesser extent water (Cutler et al., 2014). Although chlorpyrifos is a non-systemic pesticide, it is still possible for chlorpyrifos to be present within both nectar and pollen, through direct spray contact of the chemical onto the nectaries and anthers (Cutler et al., 2014), however the nectaries are usually not exposed on most plants (Willmer, 2011), so exposure through nectar is thought to possess minimal threat to pollinators (Cutler et al., 2014). The act of pollination and foraging is not the only way for bees to come into contact with pesticides, as there are also secondary routes of exposure. For honey bees, pesticides can be brought back into the hive by foragers, and pesticide contaminated food can be fed to larvae and young workers (Chauzat et al., 2006; Bernal et al., 2010). Workers inside the hive may be at higher risk from contaminated pollen compared to foragers as they consume it daily, with nurse bees consuming up to 65mg of pollen over 10 days (Rortais et al., 2005). Nurse bees also process pollen into bee bread to feed to larvae (DeGrandi-Hoffman et al., 2013; Cutler et al., 2014). This process involves unpacking the pollen balls brought into the hive by foragers, and mixing the pollen with saliva and honey using the mandibles and tongue (Dietz, 1975). However the process of creating bee bread has been observed to reduce the concentration of the pesticide, lowering the concentration over three-fold (DeGrandi-Hoffman et al., 2013). Pesticides have been shown to contaminate the colony matrix as well, moving between frames through recycling of drawn comb containing pesticides (Wu et al., 2011). These secondary routes of exposure leave all life stages at risk of sub-lethal pesticide effects.

1.7.3 Chlorpyrifos usage in New Zealand

Chlorpyrifos is one of the most frequently used organophosphate pesticides in New Zealand (Jackson, 2010), applied to kiwifruit, avocados, wheat and roses, but also used widely in other agricultural ventures. Pesticides have been used for the past

60 years in NZ, allowing for the intensification of pastoral lands which would not have been possible without pesticides (Jackson, 2010). In 2004 approximately 1278 tonnes of active pesticide ingredients from all pesticide classes were applied in NZ. In 2010 there were 28 insecticidal active ingredients in over 60 commercial products registered for control on pasture and forage crops (Jackson, 2010). While chlorpyrifos is authorised for use in America and the majority of Europe, it is banned in Denmark, Finland, Latvia, Lithuania, Sweden and Yemen (Watts, 2013). In 2006, chlorpyrifos was placed on a priority list for reassessment in New Zealand, but is yet to have sufficient evidence to outright ban the substance. Chlorpyrifos has also been found within managed bee colonies within New Zealand. A study carried out in the Otago region, New Zealand, examined levels of chlorpyrifos within 17 different apiaries throughout the region. Chlorpyrifos was detected at 17% of the sites, with concentration levels varying from 35-286 pg/bee (Urlacher et al., 2016). This provides evidence that New Zealand colonies have varying levels of chlorpyrifos within them, albeit below LD50 concentrations.

1.8 Aims of this study

This study aims to determine whether chlorpyrifos, applied to a forage crop under normal field conditions at an appropriate concentration, and following recommended spraying guidelines, affects honey bee foraging activity levels, colony thermoregulation or colony resilience.

This study has 3 main goals:

1. To determine whether the number of bees entering or leaving the colony can be monitored successfully over a full season using a HiveMind activity monitoring system.

2. To monitor maximum activity levels of *Apis mellifera* foragers and to compare activity in colonies exposed to chlorpyrifos, with levels of activity in control colonies

3. To determine whether internal temperature (i.e. hive thermoregulation) is compromised in colonies exposed to chlorpyrifos.

1.8.1 Hypotheses

Based on the literature, it is hypothesised that (a) the activity of forager bees will be negatively affected by exposure to chlorpyrifos, and (b) that colonies exposed to chlorpyrifos will be unable to thermoregulate as effectively as control colonies.

This study was undertaken as part of a collaborative research programme with Dr Kim Hageman (Department of Chemistry, University of Otago), in which the effects of a chlorpyrifos spray event were examined on:

- Honey bee activity levels and thermoregulation (current investigation)
- Accumulation of chlorpyrifos in the bodies of foragers, nurse bees, larvae and within the hive matrix (Sue Heath, PhD student, Department of Zoology, Otago)
- Volatilisation and spread of chlorpyrifos (Supta Das, PhD student, Department of Chemistry, Otago)
- Chlorpyrifos levels within soil and foliage (Maddy Taylor, MSc student, Department of Chemistry, Otago)

Chapter 2

Methods

2.1 Colony sites

2.1.1 The colonies

The colonies used for this experiment were housed in standard 10-frame Langstroth hives. The brood supers contained 10-frames, while any honey supers contained 9 frames. All hives had a queen excluder between the top brood box and the honey supers. Prior to the start of the experiment, colonies were equalised in strength through internal assessment of the hives, however this did not include matching frame for frame. The equalisation looked at a combination of food stores and amount of brood, which lead to swapping frames from stronger hives into weaker hives. All hives were requeened prior to the onset of the experiment, using same sister queens from a single queen breeder.

By the start of the experiment (19th December), all colonies had two full size brood supers, and between 1-3 three quarter size honey supers. Additional honey supers were removed until all hives only had one super by the time of the spray event (8th January). All hives were checked for queen presence weekly throughout the experiment. A single hive was found to be queenless during the experiment, however this hive did not have a HiveMind monitor attached so did not affect this experiment. All colonies were regularly checked for new queens and supercedure cells. If found, supercedure cells

were squashed using a hive tool. Prior to the start of the experiment, all hives were treated with 4 strips of Bayvarol (1st October) per brood box, to reduce presence of *Varroa* mites.

2.1.2 Control site

Two months prior to the onset of the experiment, 30 honey bee colonies were established and placed in a field of abundant vipers bugloss (*Echium vulgare*) and thyme (*Thymus vulgaris*), at a holding site at Golden Road, Otago, New Zealand (45°11'09.26S – 169°24'18.66 E), 50 km from the treatment site. Each colony was numbered and randomly placed into treatment groups. Hives were colour coded according to treatment, ready for introduction to the experimental site at specific intervals after the spray event (see Table 2.1). These colonies provided data on the initial control site up till the 14th of January 2017. One set of five colonies (Treatment group 1) was not present on the initial control site, but rather was established on the treatment site prior to the spray event (see Table 2.1).

Figure 2.1: Control site at Golden Road, Otago, with colonies waiting to be transferred to the experimental site. The two front hives have HiveMind monitors attached, circled in red.

2.1.3 Treatment site

Five colonies were established on the 10th of December at the treatment site (Marshall's cottage, Otago; 45°13'03.25S – 169°42'13.66E; Table 2.1). This group (treatment 1) provided background readings for activity, internal temperature and external temperatures prior to the spray event on the treatment site. The treatment site was in the middle of a large dry valley, with very little shelter around the colonies or the crop. This meant the site was prone to winds and high temperatures during the day, and sub-zero temperatures during the night. The lowest temperature recorded on site during the experiment was -3°C, while the highest was 43°C (recorded by the HiveMind monitors).

Figure 2.2: Treatment site (Marshalls Cottage), 15th November with young *Phacelia* starting to sprout.

2.1.4 Secondary control site

If funding and resources were no issue the initial control site would have been identical to the treatment site. However, funding limitations and water restrictions in Central Otago meant the sowing of a second (control) crop was not an option. The original control site was chosen due to the levels of food availability (predominantly thyme).

However by the 14th of January a large number of the thyme plants were dying. This may have left the control colonies with inadequate forage crop to last through the experimental period. For this reason a secondary control site was necessary to continue monitoring a site without pesticides. This secondary site was 18km away from the original control site on Booth Road, Otago (45°06'02.66S – 169° 37'53.34E). This site was chosen due to an abundance of forage, and a guarantee of no chlorpyrifos being sprayed within 2 kilometres of the site itself. The floral availability at this site was less abundant than at the treatment site, but was more diverse than the *Phacelia* crop on offer at the treatment site. The main foraging resources included lavender and clover.

2.2 Flower crop

A 1.26ha field of *Phacelia* (*Phacelia tanacetifolia*) was sown at the treatment site on the 1st of October 2016 (Figure 2.2 & 2.3). *Phacelia* was chosen as it was determined to be ideal for the arid conditions in Central Otago. *Phacelia* is a Californian desert plant, able to survive long periods of drought, hot days and cold nights. A site was chosen in the Ida Valley, Central Otago, where conditions are similar to the native conditions of the crop. The conditions were hot, dry and windy, with the monthly average rainfall for the area providing only 38mm. Initially 7kg of seed was to be surface dropped onto a field that had been prepared using a harrow, but due to an error when the seed was sown, thirty-five kilograms of seed was sown instead. *Phacelia* is a highly competitive plant species with a flowering period of 48-52 days (Stevenson, 1991). The *Phacelia* flowers began reaching bud burst by the 20th of December and continued flowering until mid-February. Honey bees, bumblebees and hover flies were observed foraging on the crop throughout the entire duration of the experiment. By the 15th of January, an estimated 50% of the crop had finished flowering, and about 80% had died off by January 24th. However due to the high density of the plants, large numbers of flowers were available for foraging throughout the majority of the experiment. By the 25th of February, all of the *Phacelia* crop was dead.

Figure 2.3: Treatment site on the 20th of December, the beginning of *Phacelia* bud burst.

Figure 2.4: *Phacelia* crop in full bloom, 30th of December. Photo provided by Alison Mercer

2.3 HiveMind monitors

Colony strength monitors were purchased from the Christchurch based company HiveMind. Each monitor reports in-colony temperature (°C), external temperature (°C) directly outside of the colony entrance, and the number of bees entering or exiting the colony (as a single count). The monitors do not distinguish whether bees are entering or exiting the hive. The monitors send out satellite reports once every three hours. Activity counts were recorded over a 90 minute period. This gave two readouts every three hours of the accumulated counts across each 90 minute period. This gave approximately 16 activity readouts within a 24 hour period. The readouts come in the form of a count on a logarithmic scale, with smaller measurements providing greater accuracy. Due to the way the data are sent, as the counts get higher, rounding of numbers occur, such as above 4000 bees, there are jumps of 256 for each count e.g. 4096,4352... etc. This was inherently built into the monitors for data transfer. Internal and external temperature were sampled every three hours, providing the temperature at the time the readout was taken. The monitors themselves are 150 mm x 100 mm, with an operating temperature of -20°C to 45°C. They attach directly above the colony entrance, slotting in between the bottom deep super and the second deep super (Figure 2.5).

Figure 2.5: Hive Monitor attached the front of a colony

A cable extending into the colony and sitting atop frames containing brood (Figure 2.6) enabled in-colony temperatures to be monitored. Each monitor responded to a satellite hub, placed within 50 m of each of the monitors. Two satellite hubs were used, one at the control site and one at the treatment site. Each satellite hub sat atop a central colony, firmly strapped under a ratchet colony strap. Data were transmitted via satellite to a data server, accessible through the HiveMind website in a raw form .csv file.

Figure 2.6: HiveMind colony strength monitor extending inside a hive. In the current experiment, the metal sheet extending into the hive was replaced by a cable with a temperature sensor at the end, extending to the same point in the hive. Image was sourced from https://hivemind.co.nz/.

Monitors were installed on the morning of the 19th of December 2016 on 16 of the 30 colonies at the control site, treatment groups are shown in Table 2.1. Monitors were also installed on three out of the five colonies on the treatment site on the morning of the 20th of December.

2.4 Baseline measurements

Prior to the spray event, all treatment groups were sampled for baseline levels of chlorpyrifos. Treatment group 1 was sampled on the treatment site prior to spraying. Each of the remaining sets of colonies (treatments 2, 3, 4 and a set of control colonies) were sampled while they were still at the initial control site at Golden Road. Foragers were collected in glass vials from the entrance of each of the colonies. Only exiting foragers were collected so as to reduce contamination from pollen sources. Nurse bees were also collected from each colony. Workers were determined to be nurses if they were found on a frame containing pupae and larvae, and were seen tending to brood. Larvae and pupae were collected from brood combs from the second deep super (so as not to disturb the colony monitors). These measurements were taken for the PhD of Sue Heath (Heath, in prep).

2.5 Spray event & treatment groups

At 8:45am on a still day in early January (January 8th), the *Phacelia* crop was sprayed with Lorsban 50EC, which contains the insecticide chlorpyrifos. Lorsban 50EC (400 ml diluted in 150 L of water) was applied by a professional spray contractor using a tractor with a 24 m wide boom. The boom covered the entire crop with spray during a single run up and down either side of the crop.

It is stated in the SDS for Lorsban 50EC that no pollinator should be present while the crop is being sprayed. It is also stated for kiwifruit crops, that spraying of the crop should occur 7 days prior to introduction of any pollinators. Colonies belonging to treatment group 1 were established at the treatment site three weeks prior to spraying to investigate effects of exposing bees to the pesticide immediately after the initial spray event.

A second group of colonies (treatment group 2) was introduced to the treatment site 48 hours after spraying (10th Jan). The third and fourth groups (Treatments 3 and 4) were introduced 96 hours (12th Jan) and 144 hours (14th Jan) after spraying, respectively. Hence, all colonies were introduced to the spray site within a 7 day period, which is all within the danger period for pollinator introduction suggested on the SDS. However, instructions also state spraying should occur before bud-burst to avoid contact with visiting pollinators. At the treatment site, spraying was delayed until *Phacelia* bud burst had already occurred and pollinators were present at the site, presenting a worst case scenario for pollinators. A summary of the number of colonies in each treatment, and when each treatment group was introduced to the spray site is provided in Table 2.1.

All colonies were transported at 7am in the morning to reduce the numbers of bees lost in transit and to ensure bees would re-orientate to their new location when they began foraging for the day. Colonies in treatment 2 were placed in two parallel rows, facing northwest (Figure 2.7). Colonies in treatment 3 were placed in two parallel rows, adjacent to the colonies belonging to the second treatment. Colonies belonging to treatment 4 were placed in a line adjacent to the third treatment.

Table 2.1: Description of each treatment group, its location during the spray event (initial site), and date of transfer to the spray site. Note: As food availability at the control site 1 declined, the control group was shifted from control site 1 to control site 2 on the 14^{th} of January.

Treatment group	Number of colonies	Number of colonies with monitor	Initial site	Transfer site	Date of introduction	Monitors installed	Monitors removed
Control	5	3	Control 1	Control 2	14^{th} January	19th December	1st May
Treatment 1	4	3	Treatment	None	10^{th} December	20th December	7th April
Treatment 2	10	5	Control 1	Treatment	10^{th} January	19th December	7th April
Treatment 3	10	5	Control 1	Treatment	12^{th} January	19th December	7th April
Treatment 4	5	3	Control 1	Treatment	14^{th} January	19th December	7th April

Figure 2.7: Treatment group 2 colonies in two parallel lines on the treatment site.

Figure 2.8: Colonies from treatment groups 1 and 2 on the treatment site. In the foreground is 3 of the 10 colonies belonging to treatment group 2. In the background all five colonies belonging to treatment group 1.

Figure 2.9: Wide angle shot of treatment groups 2, 3 and 4. Far left is treatment group 2 (red tags), middle is treatment group 3 (green tags), and far right is treatment group 4 (blue tags). Photo is sourced from Lenovo Thinkpad promotional video http://blog.lenovo.com/en/blog/harmony - in - the - hive - using - think - technology - for - beehive - monitoring/ .

2.6 Data collection and measurements

Information from colonies at Golden Road (control site 1) began streaming from the 19th of December 2016, and from the 20th of December from the Marshall's Cottage (treatment) site. A satellite hub was shifted from the Golden Road site to the Booth Road site (control site 2) on the 14th of January. Data collection ended at the treatment site on the 7th of April, 111 days after the start of the experiment. Data collection from the colonies at the secondary control site ended on the 1st of May, 134 days after the start of the experiment. However the data from the control site was only analysed up until the 7th of April for consistency.

Data from the satellite hub was lost on multiple occasions at both the control and treatment sites. On the 1st of January, the satellite hub at the initial control site stopped functioning. This was unable to be fixed until the 7th of January, where data began to stream again. Over this period all data measurements were lost. On the 3rd of March *Varroa* treatments were administered to the hives, which disconnected the internal temperature sensor of three colonies. This occurred due to the sensor itself

being attached to the second deep super through propolis. The subsequent shifting of the second deep super during application of a miticide accidentally disconnected the temperature sensors in these colonies. One colony each from treatment groups 1, 2 and 4 were affected by this.

Bees from the control colonies and colonies from each treatment group were repeatedly sampled for measurements of chlorpyrifos after the spray event. For each treatment group introduced to the spray site, samples were taken the first three days (treatment group 1 was sampled the day after spraying, rather than on the day of the spray event) that they were on site. After the first three days of sampling for each group, samples were taken every other day for the next six days. After this period, samples were taken every third day for the next 12 days. Within the first 21 days, all treatment groups were sampled a total of 10 times each.

2.7 Statistical analyses

All statistical analyses were carried out in the R environment (R Core Team, 2017). Initial tidying of the data utilised the lubridate package (Grolemund and Wickham, 2011). The lubridate package allowed for rounding of times used in analyses regarding circadian rhythms, and to transform the raw data's date-time set up into a specific date-time formats which could be processed by r for the GLMMs and LMMs analyses (Grolemund and Wickham, 2011). Both the GLMMs and LMMs were created and analysed with the R packages lme4 (Bates et al., 2015) and plyr (Wickham, 2011). For the LMMs, p-values were obtained through the package lmerTest (Kuznetsova et al., 2017). Graphs were created with base R and the plotrix package (Lemon, 2006).

2.7.1 Actograms

Actograms were constructed to determine whether activity rhythms could be detected and analysed. For detailed analysis, actograms require frequent measurements taken at regular time intervals. Bins of equal duration are required to plot changes in activity over time. Signals transmitted from the HiveMind monitors were not continuous, nor were they received at the same time every day. For this reason, activity measurements

were rounded into 16 equal length bins. The bin lengths were an hour and a half, the same length of time between measurements sent from the monitors. This gave 16 measurements every 24 hours, split into hour and a half bins. Times were either rounded up or down dependent on how close the data readout came to a later or earlier time bin (i.e. if a readout came in at 11:45, it would be rounded up to 12:00, however if it came in at 11:04, it would be rounded down to 10:30). This brought slight coercion into the data set, so the actograms provided purely descriptive statistics. As the resolution of the data set was not able to provide accurate measures of activity rhythms, daily measurements of maximum activity levels were used as an indicator of honey bee activity. Maximum daily activity levels were obtained once a day for each hive, taken from the time bin with the highest activity count each day.

2.7.2 Generalized linear mixed-model analyses (GLMMs)

Generalized linear mixed-effects models were used to investigate the effects of site, external temperature, spray exposure and crop availability on maximum activity levels. Any dates that had missing data due to technological failure (HiveMind failure, battery failure) or from large anthropogenic disturbances such as during miticide application, were omitted from the dataset. The data collected were tested for normality through the Shapiro-Wilk test and Q-Q plots, and were found to not be normally distributed. Log-transformations were unable to normalise the data. Due to the non-normality of the data, and the presence of repeated measures, a generalized linear model was indicated. Explanatory variables, such as external temperature, site, treatment level, spray event and crop death were included in the model as fixed effects. Spray event was defined as any activity measurement taken from a colony on the treatment site after the 8^{th} of January. Crop death was defined as any activity measurement taken after the 25th of February when complete death of the *Phacelia* crop occurred on the treatment site. As the experiment was carried out over a long period of time, there was possibly a seasonal effect to account for. For this reason a quadratic and cubic function was added to the analysis. No interpretation of the quadratic and cubic functions were carried out, they were purely for the visualisation of the data. To deal with repeated measures from the colonies, each colony was treated as a random factor ("colony" = (1|colony) (Baayen et al 2008, Bolker et al., 2009). Due to working with count data, this indicated the use

of a Poisson model. When graphing the relationships between treatments, regression lines were modelled independently for each control and treatment group, with no added interaction terms. With the above considered, the final equation for the GLMM in R (or a variant of it) is described below:

GLM<-glmer(Activity ~ Treatment + Site + Date + $Date^2$ + $Date^3$ + ExternalTemp + HiveTemp + Spray Event + Crop Death + (1|Colony.ID), family="Poisson", data=data)

Where activity is a single data point a day, the highest daily activity count (single data point per day). Treatment is split into control + four individual treatments. Site is split into three levels: initial control site, secondary control site and treatment site. Date is the specific day of any one recording. External temperature is the outside temperature readout correlated with the time of a daily maximum activity readout (single data point per day). Hive temperature is the internal temperature readout correlated with the time of a daily maximum activity readout (single data point per day). Data for spray event is split into date prior to the 8th of January (no spray = 0), and all date past the 8th of January till the end of the experiment (spray = 1), providing a binary factor. Data for crop death was is split into dates prior to the 25th February (prior to crop death = 0), and dates after the 25th February (crop death = 1), providing a binary factor, and finally colony as a random effect.

2.7.3 Linear mixed-model analyses (LMMs)

To analyse the effects of the pesticide on internal colony thermoregulation, linear models were utilised. For temperature data, residuals were approximately normally distributed, so a general linear mixed-model was not required for temperature analyses, and a linear model provided a better fit. However it was still necessary to include a random factor to deal with the repeated-measures, so a mixed effect model was chosen. Identical to the GLMMs, a random factor of the subject units (colonies) themselves was utilised. When graphing the relationships between treatments, regression lines were modelled independently for each control and treatment group, with no added interaction terms. The final equation for the LMM in R (or some variant of it) is described below:

LM <- lmer(InternalTemp ~ Treatment + Site + Date + ExternalTemp + Activity + (1|colony.ID), data = data)

Where internal temperature is the raw data for internal temperature throughout the entire experimental period, as opposed to the GLMMs where internal temperature was taken at the time of maximum activity. Treatment is split into control + four individual treatments. Site is split into three levels: initial control site, secondary control site and treatment site. Date is the specific day of any one recording. Outside temp is the temperature taken at the time of an internal temperature readout, activity is a simple count at the time of internal temperature readouts and finally colony as a random effect.

A further point to note prior to reading both the GLMMs and LMMs, is that the intercept of all tables is the control colonies. This means all treatment group colonies are compared directly to the control colonies, unless it is stated otherwise.

2.8 Breakdown of the data

Throughout the experimental period, there were two key events which prompted dividing up the data to obtain the most information possible, giving three data periods of analysis. These three time periods were:

1. Prior to the spray event for the treatment colonies (19th December 2016 - 7th January 2017), prior to the move event for the control colonies (19th December 2016 - 14th January 2017).

2. After the spray event for the treatment colonies (8th January 2017 - 25th February 2017), post-move event for the control colonies (14th January 2017 - 25th February 2017).

3. Post-crop death on the treatment site (25th February 2017 - 7th April 2017).

Further breakdown of the post-crop death data occurred when analysing internal temperature. The post-crop death measurements spanned exactly 6 weeks. Therefore the data was further broken into three two week periods:

1. The two weeks following crop death (25th February - 10th March)

2. Two weeks after crop death (11th March - 24th March)

3. Four weeks after crop death (25th March - 7th April)

Chapter 3

Results

3.1 Preliminary analysis of activity measurements

To begin, the entire dataset was examined to determine whether and how activity levels changed over the experimental period. Figure 3.1 a is a scatterplot showing maximum activity levels of all colonies across the entire experimental period. In this figure only linear functions are applied, however Figure 3.1 b is modelled with a quadratic and a cubic function applied to the date component. These functions were included to assess whether they better captured the seasonality of the data. All graphs pertaining to maximum activity levels and internal temperature readouts were graphed with and without the addition of these functions. After careful consideration, it was decided the quadratic and cubic functions improved the fit of the regression lines to the data. It should be noted however that the addition of these functions did not change the outcome or final interpretations of the data, rather they are believed to provide a better representation of changes in maximum activity and internal temperature over time, compared to the simple linear regression lines. As the quadratic and cubic functions improved the fit of the regression lines to the data, these functions were applied throughout the results.

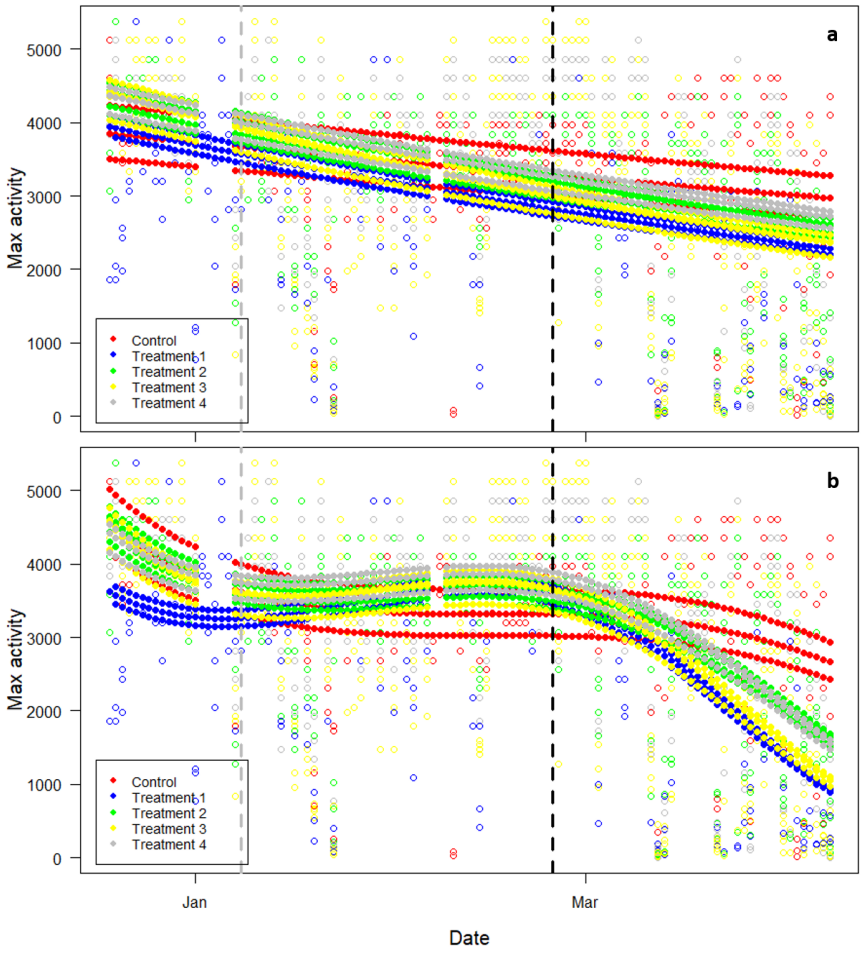

Figure 3.1: Scatterplot showing maximum activity counts over the experimental period between all colonies from the control and treatment groups. (a) is plotted with only linear functions. (b) is plotted with both a quadratic and cubic function applied to the date component to improve fitting of the regression lines to the data points over time. Regression lines are plotted in their respective colours. Grey dotted line indicates the day of the spray event (January 8th). Black dotted line indicates the day of complete crop death (25th February).

First, the data were examined to determine whether measurements of maximum activity provided evidence of (a) circadian rhythmicity and (b) temperature dependent foraging activity, both of which have been described extensively in the literature (see Introduction).

3.1.1 Activity Rhythms

Initially, activity levels were examined to look for evidence of circadian rhythmicity. One of the control colonies was used for this purpose (control colony 2). Figure 3.2 is an actogram showing the activity of control colony 2 recorded over the first 9 days of the experiment. As activity measurements were collected every one and a half hours, each 24 hour period is made up of 16 time bins (See M&M Section 2.7.1). At the onset of the experiment, sunrise occurred at 5:50am and sunset at 9:29pm. On average, foraging activity began approximately 2 hours after sunrise, some time between 7:30am and 9am (Figure 3.2). It was difficult to measure foraging onset more precisely because the resolution of the data was relatively low. A clear decline in activity is apparent around 7:30pm each night, providing evidence of activity rhythms occurring within the colony. The maximum daily activity observed throughout the 9 day period was 4352 bees for this colony, on days 2 and 9 (Figure 3.2). However the maximum activity measured over the entire experimental period for this colony was 5376 bees within a single time bin (Appendix A Table A.1). The average maximum activity across the entire experimental period for control colony 2 was 3717±883. Comparing all colonies in the experiment, the average maximum activity was 3278± 1297.

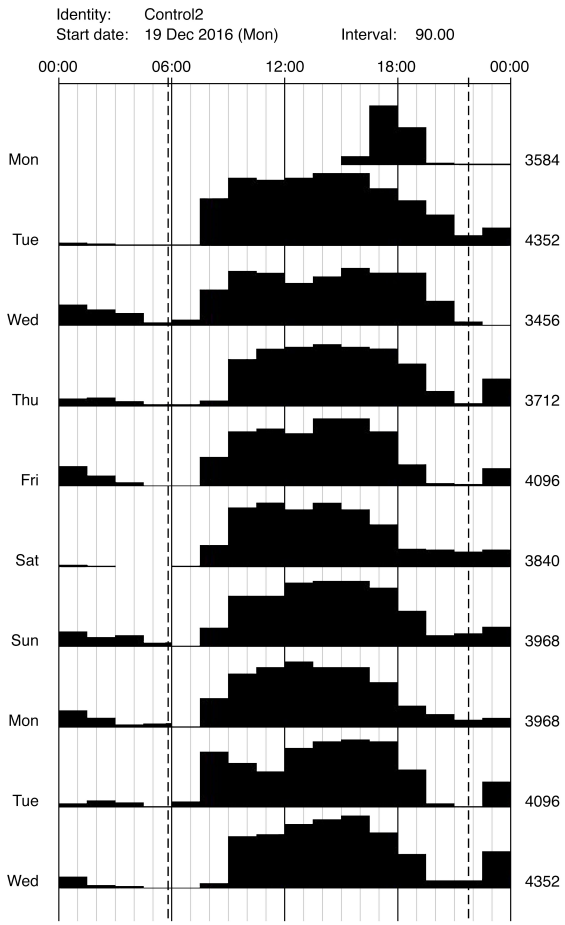

Figure 3.2: Actogram showing 9 days of activity counts from colony 2 at the control site. Time is displayed above the graph (24h time), each vertical grey line represents 1 hour. Left hand axis displays day of the week. Right hand axis displays the maximum activity recorded each day respectively. Measurement bins of 90 minutes are displayed in the graph as solid black bars. Collected data were rounded to obtain bin sizes of equal length. Dotted black lines indicate times of sunrise (5:50am) and sunset (9:29pm).

When comparing all colonies within the experiment, the average time maximum activity was achieved was around 3pm, with maximum activity rarely occurring before midday. By sunset, foraging activity was lower than during the daytime in all colonies, however it had not ceased. Over the night period (between 10:30pm and 6:00am), the mean activity in each bin was 279 ± 159 active bees averaged throughout all colonies. However

some colonies showed a tendency to be more active than other colonies across both the day and the night.

The actogram (Figure 3.2) provides evidence of circadian rhythmicity, but because the resolution of the data was relatively poor, activity patterns were not examined further, instead it was decided that maximum activity counts would be analysed, as they were the simplest measure of activity. To confirm that measurements of maximum activity levels provided an effective measure of shifts in behaviour related to environmental variation, predicted temperature-dependent changes in activity levels were evaluated.

3.1.2 Relationship between temperature and maximum activity levels

A strong correlation was identified between temperature and maximum activity levels. As temperature increased, activity also increased (Figure 3.3, Appendix B Table B.1).

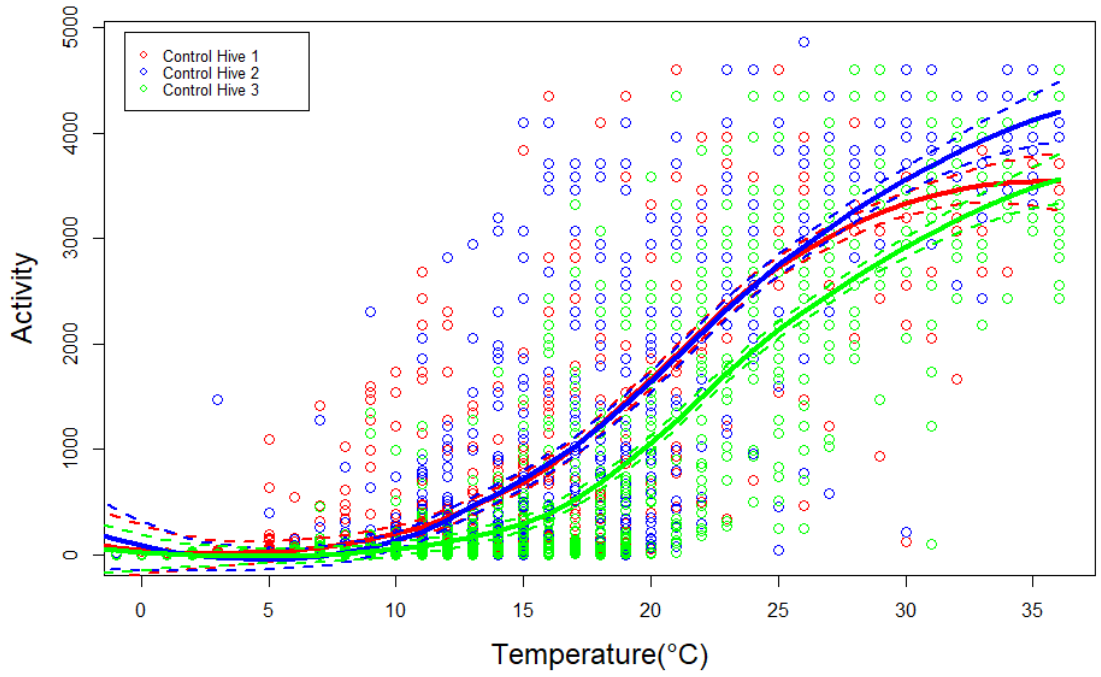

Figure 3.3: Scatterplot of activity versus temperature for all three colonies in the control group from 19th December - 7th April. Colour coded raw data points are used within the plot. Loess regression curves were fit using the loess function in R with a smoothing parameter of $\alpha = 0.75$. Confidence intervals (dashed lines) are plotted for each regression line.

While Figure 3.3 provides a purely descriptive statistic, the confidence bands do not overlap between the lower temperatures (e.g. 0°C-10°C) and the higher temperatures (e.g. 25°C-35°C) for all colonies, which provides evidence of activity increase at higher temperatures. This is consistent with the strong correlations between external temperature and activity levels, identified using GLMMs (Est. = 0.036, Z = 458.8, p <0.001; Appendix B Table B.1). When comparing between the control colonies, similar activity profiles are observed between colonies 1 and 2 for the majority of the temperatures, however colony 3 seems to have lower activity between about 11°C and 35°C when compared to the other two colonies. This highlights the variability between colonies within each treatment group. Effects of temperature will be examined further at the end of the results in section 3.4.

3.2 Activity levels in the control colonies

To understand how maximum activity levels of the colonies were changing over time (Figure 3.1), it was necessary to break the data down into three key time periods: 1) before the spray event, 2) immediately after the spray event on the treatment site (or immediately after translocation for control colonies), and 3) after crop death on the treatment site (Methods section 2.8).

First, activity levels of the control colonies were examined to determine whether they were affected by transportation during the shift from the site at Golden road, to the new site at Booth Road (see M&M section 2.1). This move was undertaken to improve resource availability for the control colonies as the thyme crop was seen to be dying off at the Golden Road site. Consistent with this, maximum activity counts in the controls showed a significant decline over the pre-move period (Figure 3.4a; Est. = -0.029, Z = -5.9, p <0.001, Appendix C Table C.1). Once the control colonies were moved to the secondary control site (Booth Road; 14th January), activity began increasing over time (Figure 3.4b; Est. = 0.023, Z = 2.72, p <0.001, Appendix Table C.2). This increase in activity was significant but overall, the average maximum activity count at the new site remained below the overall levels of activity recorded at the initial control site (Est. = -0.030, Z = -3.84, p <0.001; Appendix C Table C.2). Activity levels in the control colonies remained relatively stable even after crop death had occurred on the treatment site (Figure 3.4c). A decline in activity was apparent as the season progressed (Est. = -0.08, Z = -27.7, p <0.001; Appendix C Table C.3), however the overall average maximum activity during this period was higher in the post-crop death period than the period between spray and crop death (Est = 0.10, Z = 16.0, p <0.001; Appendix Table C.3). It should be noted that differences in maximum activity were apparent between individual colonies within the control group. These differences were apparent throughout the entire experimental period (Appendix C Table C.1).

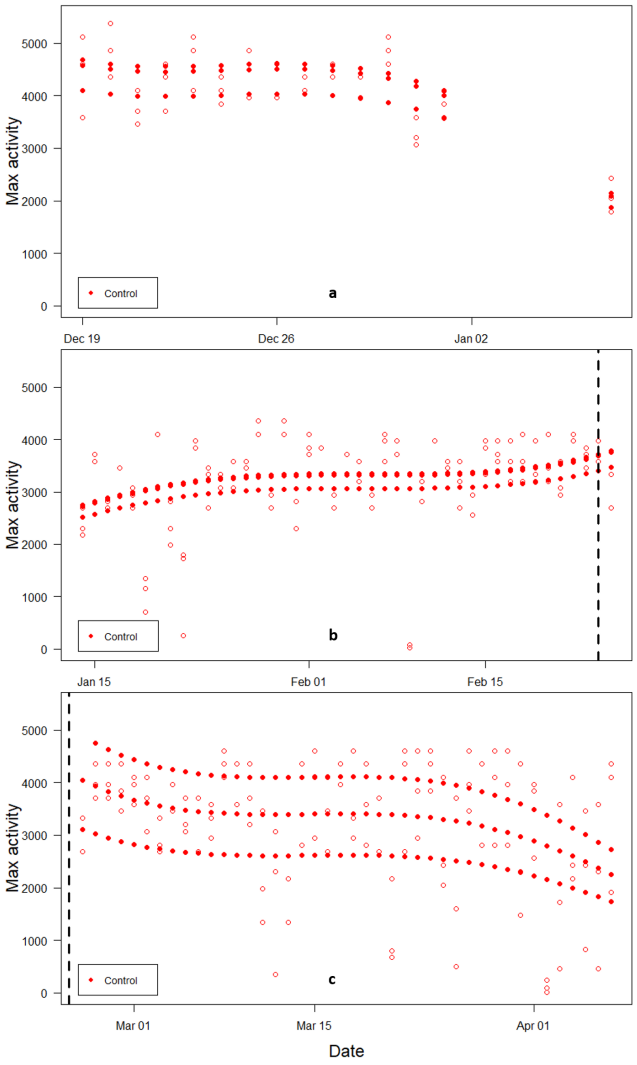

Figure 3.4: Scatterplots showing maximum activity levels in control colonies. (a) Before the move to Booth Road (December 19th - 14th January). (b) After the move to Booth Road, but prior to crop death on the treatment site (14th January - 25th February). (c) After crop death at the treatment site (February 25th - April 7th). Regression lines are plotted over the top as solid red circles. Black dotted line indicated time of complete *Phacelia* crop death on the treatment site. Data from January 2nd - 7th was lost due to satellite hub malfunction.

3.3 Activity in the treatment colonies

3.3.1 Activity prior to the spray event

Treatment group 1 was on the experimental site prior to and during the spray event, providing background activity levels for foragers at the treatment site itself. Prior to the spray event, maximum activity was increasing significantly over time for all three colonies in treatment group 1 (Figure 3.5a; Est. = 0.11, Z = 28.6, p <0.001, Appendix D Table D.1). The activity of control colonies (Figure 3.4a) are included within Figure 3.5a & b for comparison. Although the changes in activity over time seem to be different between the control and treatment group 1 colonies, there is no significant difference in their maximum activity levels in the pre-spray period (Figure 3.5, treatment group 1: Est. = -0.032, Z =-0.5, p =0.616, Appendix D Table D.2). Maximum activity levels of colonies in treatment groups 2-4 were then analysed. This was to assess whether the changes in activity over time in these colonies was similar to changes observed in the control colonies, or whether their activity levels were increasing over time as shown for the colonies belonging to treatment group 1. As shown in Figure 3.5b the activity levels of treatment groups 2-4 declined over time in a manner similar to what was observed in the control colonies. Overall there was no significant difference in maximum activity levels between treatment groups 2-4 and the control colonies (Figure 3.5, Appendix D Table D.2). In all colonies outside temperature was correlated with maximum activity over the period prior to the spray event (Est. = 0.018, Z = 66.1, p <0.001, Appendix D Table D.2). As in the control colonies, each of the 3 colonies from treatment group 1 showed significant variation in activity levels (Appendix D Table D.1), as did all colonies from treatment groups 2-4 (data not shown).

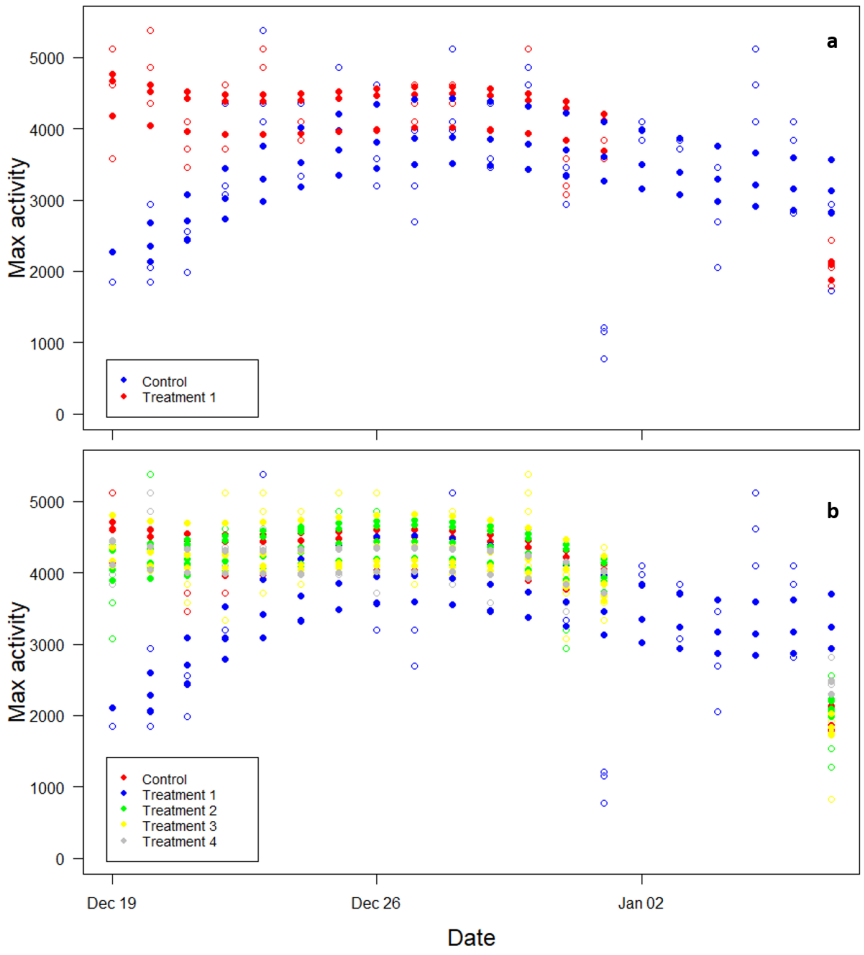

Figure 3.5: Scatterplot of maximum activity counts over the pre-spray period on the treatment site from 20th December - 7th January, data from the control colonies (Figure 3.4 a) are included for comparison. (a) Maximum activity counts recorded for treatment group 1 at the experimental (spray) site. (b) Activity of colonies in all treatment groups during the pre-spray period. Colonies in treatment groups 2-4 were located at the control site during this period. Regression lines are plotted in their respective colours

3.3.2 Activity levels following the spray event

After the spray event, but prior to crop death, maximum activity levels of all treatment group colonies increased significantly over time (Figure 3.6a & b; Est. = 0.016, Z = 15.5, p <0.001, Appendix E Table E.1). Treatment group 1 was subjected to the longest period of chlorpyrifos exposure, but showed no significant difference in overall activity

compared to colonies at the control site (Figure 3.6 A; Est. = 0.034, Z = 0.80, p = 0.42, Appendix E Table E.2). When comparing activity of treatment groups 2-4 to the control colonies, treatment group 2 was the only set of colonies that showed slightly higher activity levels than the controls, however the difference was not statistically significant (Est. = 0.074, Z = 1.90, p = 0.057; Appendix E Table E.2). Treatment groups 3 and 4 showed no significant difference to the controls over this time period. Between the spray event and prior to crop death, maximum activity was increasing over this time period for all colonies (Figure 3.6, Est. = 0.0061, Z = 6.26, $p<0.01$; Appendix E Table E.2). In all colonies maximum activity was significantly correlated with external temperature over this time period (Est. = 0.032, Z= 263, p-value <0.001, Appendix E Table E.2), and a decrease in internal hive temperature was correlated with a decrease in activity (Est. = -0.012, Z = -8.38, $p <0.001$; Appendix E Table E.2.

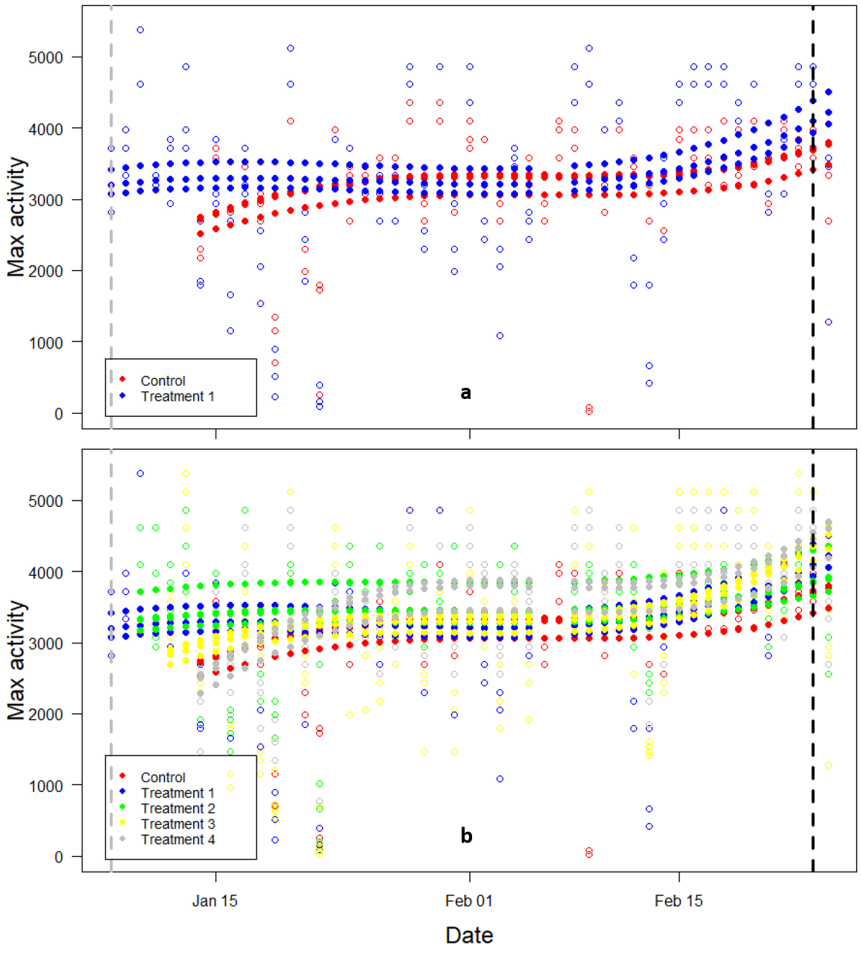

Figure 3.6: Scatterplot of maximum activity counts following the spray event and prior to crop death (8th January - 25th February), data from the control colonies (Figure 3.4 b) are included for comparison. (a) Data from treatment group 1. (b) Activity counts for all colonies over this period. For treatment groups 1-4 only data obtained from the treatment site are shown. Note that treatment groups 2, 3 and 4 were introduced to the site 2, 4 and 6 days after the spray event, respectively. Regression lines are plotted in their respective colours. Grey dotted line indicates spray event on the 8th January. Black dotted line indicates crop death on the 25th February.

3.3.3 Activity levels following crop death on the treatment site

After the death of the *Phacelia* crop on the treatment site a decline in maximum activity was observed in all colonies on this site (Figure 3.7; Est. = -0.16, Z= -100, p <0.001, Appendix F Table F.1). Treatment group 1 showed a significant reduction in activity

compared to the control colonies post-crop death (Est. = -0.25, Z = -2.1, p = 0.0385; Appendix F Table F.2). Maximum activity levels of colonies belonging to treatment groups 2-4 were also declining over this period (Figure 3.7b). Overall however, the activity decline in these colonies was not significantly different from that observed in the control colonies (Appendix F Table F.2). However treatment group 4 did exhibit significantly higher activity than treatment group 1 over this period (Est. = 0.39, Z = 2.9, p = 0.0040; Appendix F Table F.1). External temperature was also correlated with maximum activity over this time period (Est. = -0.048, Z = 347, p <0.001, Appendix F Table F.2)

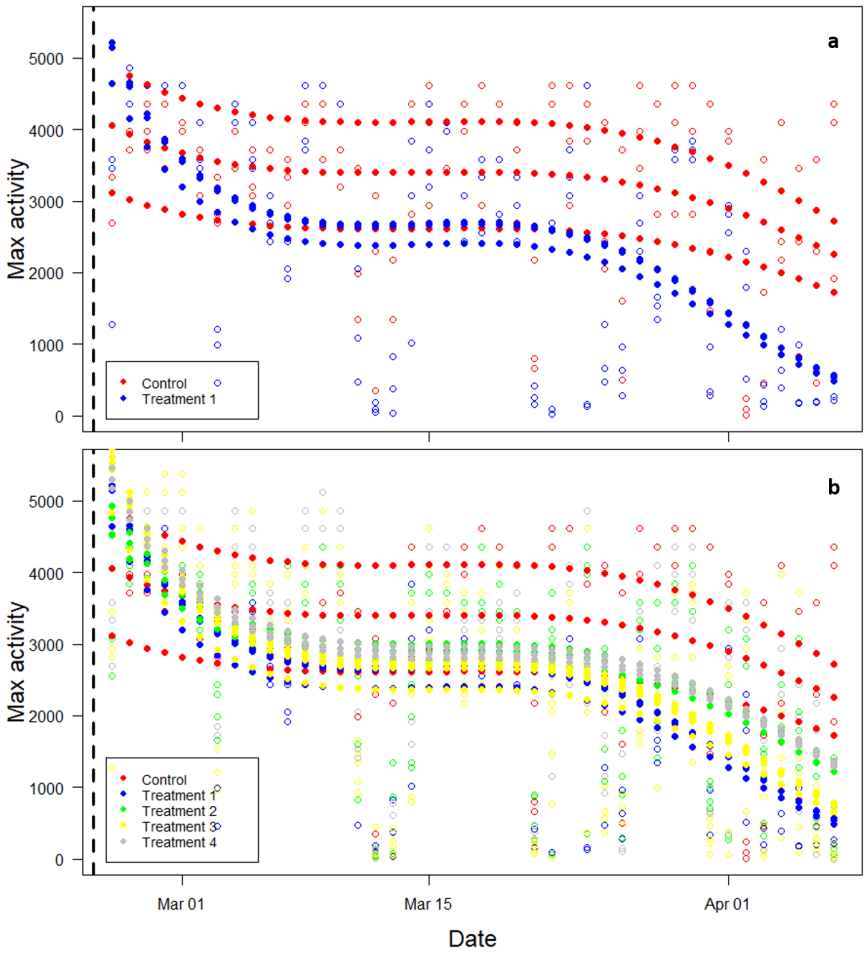

Figure 3.7: Scatterplot of maximum activity counts post-crop (25th February-7th April), data from the control colonies (Figure 3.4 c) are included for comparison. (a) Data from treatment group 1. (b) Activity counts of all colonies during this period. Regression lines are plotted in their respective colours. Black dotted line indicates time of complete *Phacelia* crop death (25th February).

3.4 Responses to changes in external temperature

Over the final month of the experiment there was an observed decline in maximum activity levels that appeared to be more prominent in the treatment colonies than in the controls. To evaluate this pattern more closely, further investigation of the effects of external temperature was carried out. Figure 3.8 shows the average external

temperature on both the control site and treatment site over the post-crop death period. Over time external temperature is decreasing on both sites, dropping from an average of 20°C, to close to 10°C by the final week (Figure 3.8).

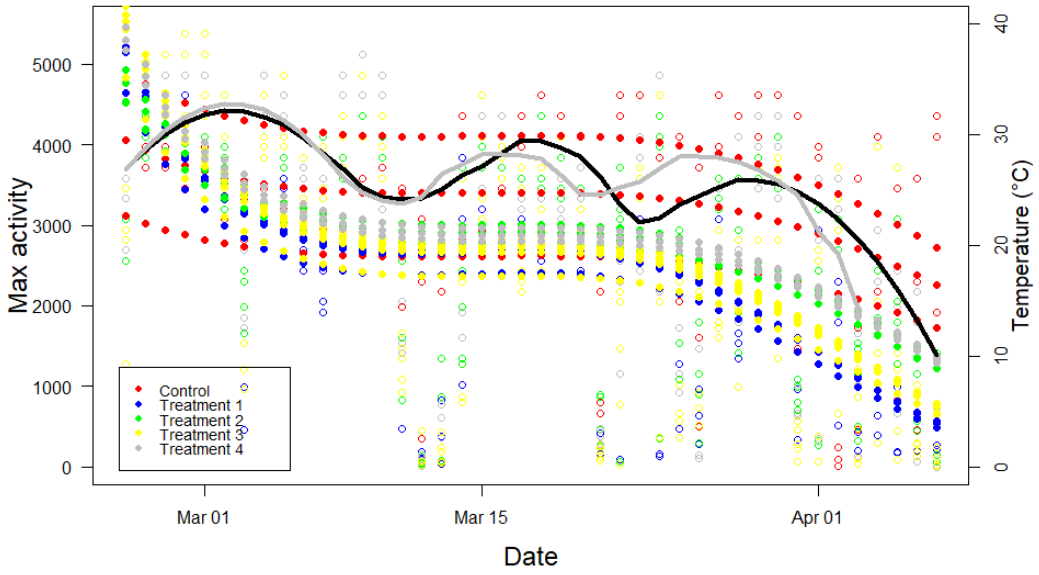

Figure 3.8: Scatterplot of maximum activity post-crop death for all colonies on the control and treatment sites, the same data as seen in Figure 3.7b (25th February - 7th April). Loess regression lines of average temperatures at the time of maximum activity are overlaid on the graph as solid lines. The grey line is the average temperature at the control site, and the black line is the average temperature at the treatment site.

As observed earlier in the results (Figure 3.3, section 3.1.2), external temperature is correlated with the levels of activity observed for a colony. However it is possible the spray event could have an effect on activity alongside temperature. To determine whether this was the case, the activity levels of the colonies in treatment group 1 were compared before and after the spray event.

3.4.1 Response to external temperature before and after the spray event

Activity data from treatment group 1 colonies pre- and post-spray event were compared to determine whether the spray event had an effect on the relationship between temperature dependent changes in activity (Figure 3.9 a & b respectively). In the pre-spray period, activity began to increase around the 14-15°C temperature range (Figure 3.9 a). Differences were apparent between treatment 1 colony 1 and the other two colonies between the temperature ranges of 20-26°C. Treatment 1 colony 1 displayed lower average maximum activity over the 20-26°C temperature range compared to the other two hives, as the confidence bands do not overlap between hives. In the post-spray period, activity began to increase around 15-17°C (Figure 3.9 b), slightly higher than prior to the spray event. However, both before and after the spray event, the highest activity counts for all colonies were observed above 30°C. Between the pre- and post- spray period average activity seemed similar, although there was variability within the colonies.

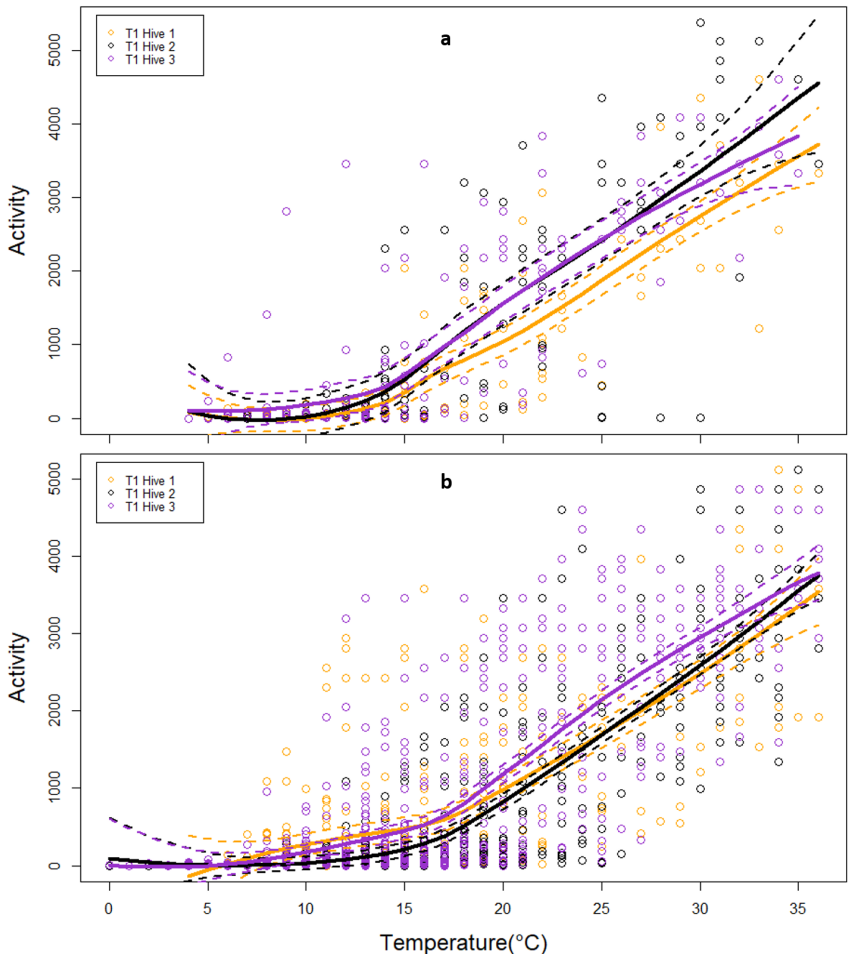

Figure 3.9: Scatterplot of maximum activity counts for all temperatures between 0°C and 36°C. (a) Data from the pre-spray period for treatment group 1 colonies. (b) Data from the post-spray period until the end of the experiment for treatment group 1 colonies. Loess regression curves were fit using the loess function in R with a smoothing parameter of $\alpha = 0.75$. Confidence intervals (dashed lines) are plotted for each regression line.

Next comparisons between the colonies on the control sites was carried out, comparing pre- and post- move activity levels (Figure 3.10 a & b). Prior to the move event, activity began to increase to above night time activity levels somewhere between 9-15°C (Figure 3.10 a). All three colonies showed very similar activity profiles across this period, with the confidence bands overlapping for the majority of temperatures for all three hives. In the post-move period, activity began to increase above the average night time activity levels between 11-17°C (Figure 3.10 b). However this was also different between colonies,

namely control hive 3 on average had lower activity than the other two colonies between the external temperature ranges of 13-33°C (Figure 3.10 b).

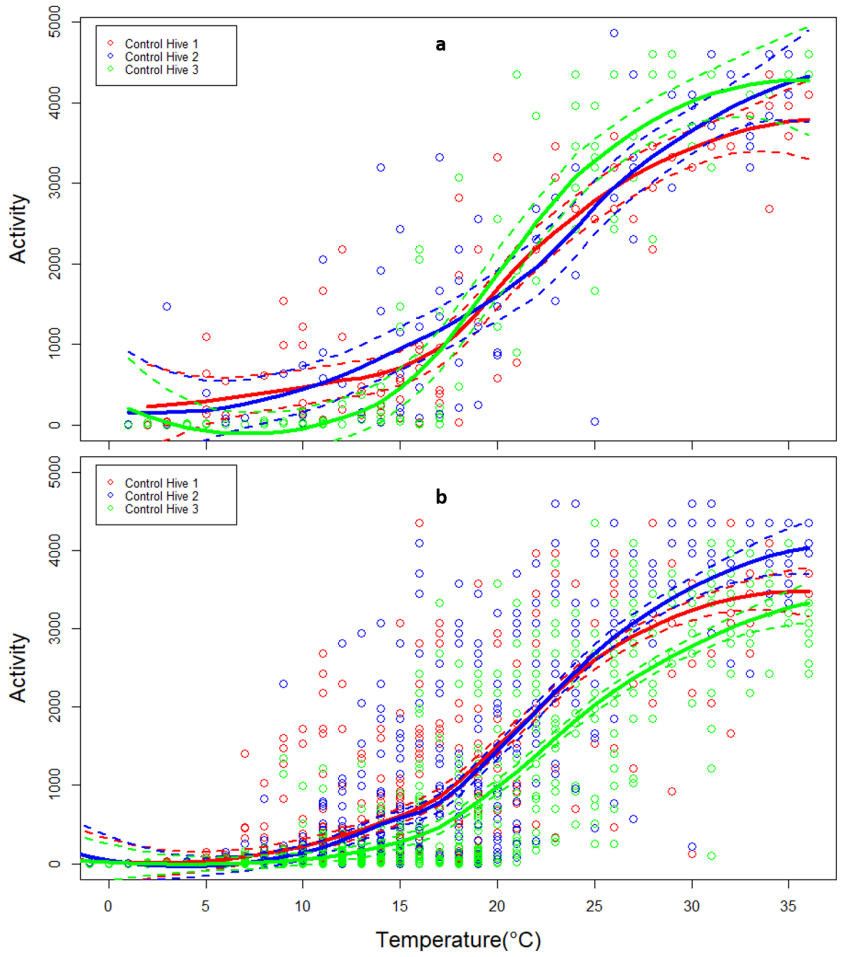

Figure 3.10: Scatterplot of maximum activity counts for all temperatures between -3°C and 36°C. (a) Data from the pre-move period for the control colonies. (b) Data from the post-move period until the end of the experiment for control colonies. Loess regression curves were fit using the loess function in R with a smoothing parameter of $\alpha = 0.75$. Confidence intervals (dashed lines) are plotted for each regression line.

Finally the post-spray (treatment colonies) and post-move (control colonies) periods were compared to assess whether any major differences in activity were observed between the control and treatment colonies, and whether these could be attributed to the spray event (Figure 3.11). There were individual colony differences in regards to the temperature at which activity exceeded average night time temperature in both the

control and treatment colonies. Control hive 1 had significantly higher activity than all of the treatment group 1 colonies between the temperature ranges of 20-30°C. Control hive 2 also had significantly higher activity than any treatment group 1 colony, between the temperature ranges of 20-33°C. However control hive 3 showed similar activity profiles to all three treatment group hives over these temperature ranges (Figure 3.11).

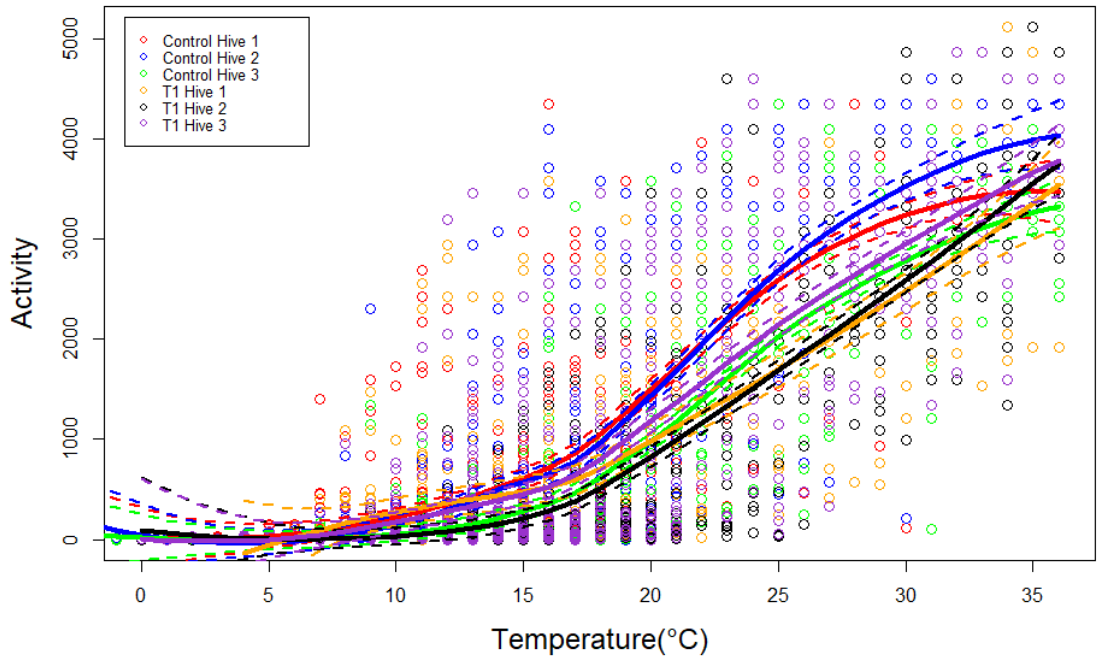

Figure 3.11: Scatterplot of maximum activity counts for all temperatures between -3°C and 36°C, comparing control and treatment colonies post-move and post-spray event, until the end of the experiment. Loess regression curves were fit using the loess function in R with a smoothing parameter of $\alpha = 0.75$. Confidence intervals (dashed lines) are plotted for each regression line.

3.5 Internal temperature as an indicator of colony activity

Measurements of internal hive temperature were used as an indicator of conditions within the colonies. Previous literature has described a relationship between internal colony temperature and brood count, with high internal temperatures (32°C-34°C) necessary for optimal brood growth (Stabentheiner et al., 2010). Over the entire experimental period internal temperature declined in all colonies (Figure G.1; Est. = -1.25, t = -33.9, p<0.001, Appendix G Table G.1).

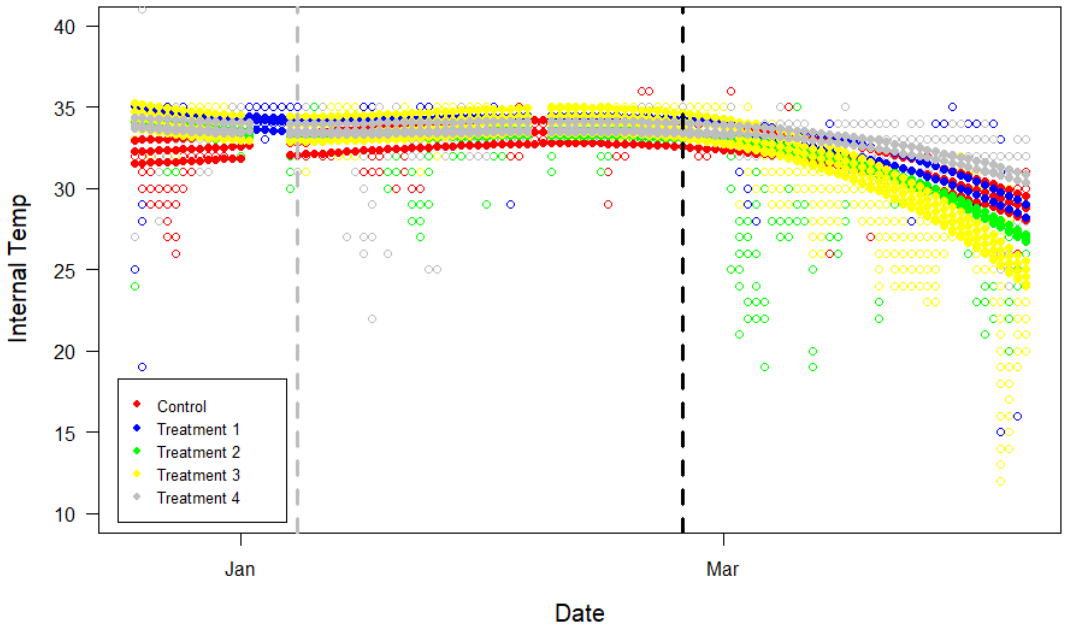

Figure 3.12: Scatterplot of internal temperatures (°C) across the entire experimental period (19th December - 7th April). The control group and all treatment groups are shown on the graph. Grey dotted line indicates spray event (January 8th). Black dotted line indicates complete *Phacelia* crop death (25th February).

To further understand how internal temperature was changing over time in the control versus treatment colonies, the data was split up into the same periods as seen in section

3.2, (a) prior to the spray event, (b) after the spray event but prior to crop death and (c) after complete *Phacelia* crop death.

3.5.1 Internal temperature during the pre-spray period

Over the pre-spray period internal temperature of all colonies was between 32-35°C (Figure 3.13 a & b). Internal temperature was decreasing significantly over this time period, however the decrease was small (-0.15°C, Est. = -0.15, t = 7.23, p <0.001; Appendix G Table G.2). Internal temperature in all treatment groups was significantly higher than in the control colonies, between 1.2-1.7°C higher (T1 = 1.7°C, T2 = 1.2°C, T3 = 1.4°C, T4=1.2°C: Appendix G Table G.2).

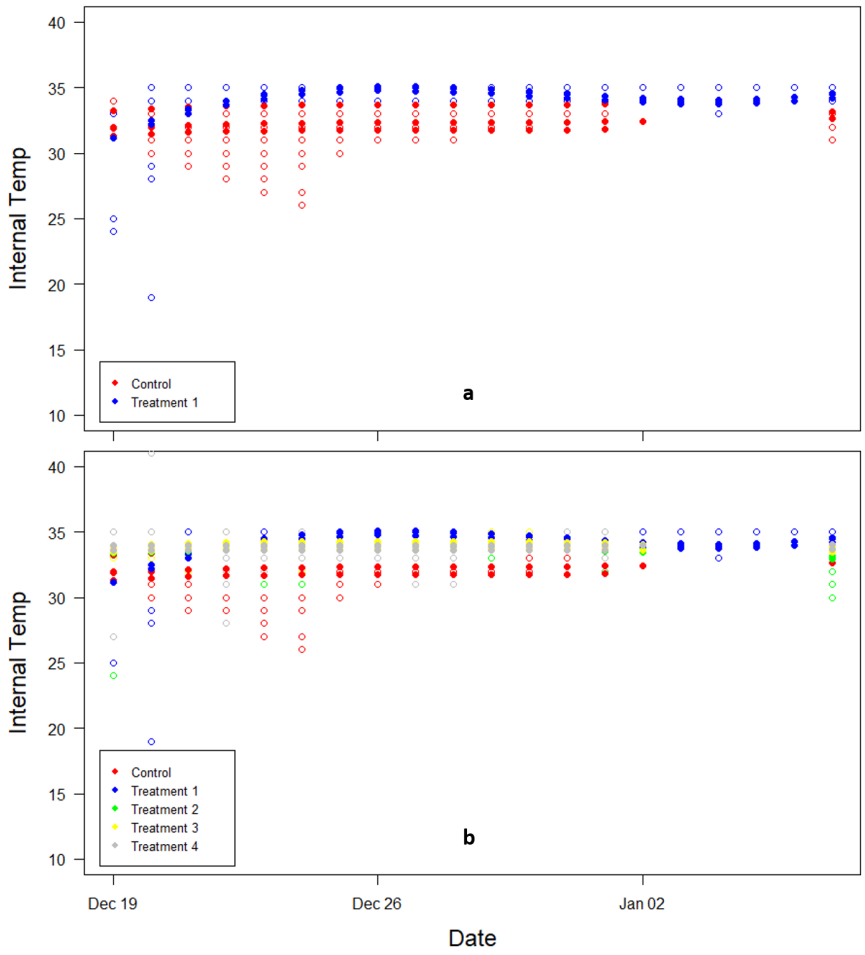

Figure 3.13: Scatterplot of internal temperatures (°C) over the pre-spray period (19th December - 7th January). (a) Data points from treatment group 1 and the control colonies. (b) Data from all colonies across this period. Regression lines are plotted in their respective colours.

3.5.2 Internal temperature during the post-spray period

Over the post spray period (but prior to crop death) internal temperatures in all colonies remained between 32-35°C (Figure 3.14 a & b). Internal temperatures were increasing significantly over this time period, however the magnitude of the increase was relatively small (0.095°C, Est. = 0.095, t = 11.2, p <0.001, Appendix G Table G.3). Over this period on average, only colonies in treatment groups 1 and 3 had significantly higher

internal temperatures than the control colonies (T1 = 0.82°C, T3 = 0.57°C; Appendix G Table G.3).

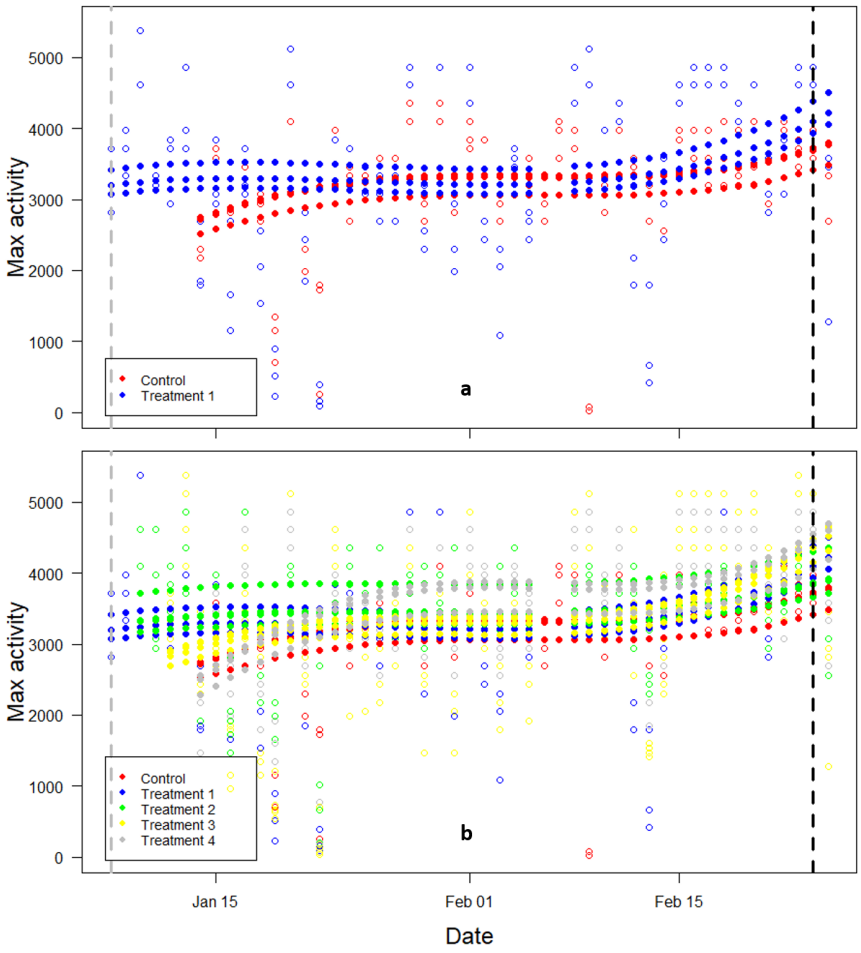

Figure 3.14: Scatterplot of internal temperatures (°C) following the spray event and prior to crop death (8th January - 25th February). (a) Data points from treatment group 1 and the control colonies. (b) Data from all colonies across this period. Regression lines are plotted in their respective colours.

3.5.3 Effects of crop death on internal temperature

In the period following the death of the crop on the treatment site, internal temperature was declining significantly in all colonies (Figure 3.15, Est. = -1.6, t = -48.4, p <0.001, Appendix G Table G.4). Over the entire post-crop death period there was no significant

difference between internal temperatures of colonies in treatments groups and control colonies (Appendix G Table G.4). The average internal temperature of the control colonies during this period was 30.7°C (Est. = 30.7, t = 45.2, p <0.001; Appendix G Table G.4). In comparison, internal temperature averaged across all of the treatment colonies was 30.3°C (Appendix G Table G.4). Comparing average internal temperature of the treatment groups between the pre-spray period- and the post-crop death period, a decline in internal temperature was observed in all treatment groups, anywhere between 2.8-4.5°C, where the controls dropped 2°C (Observed declines: T1=-3.6°C, T2=-3.9°C, T3 =-4.5°C, T4=-2.8°C, Figures 3.13b & 3.15b, Appendix G Tables G.2 & G.4).

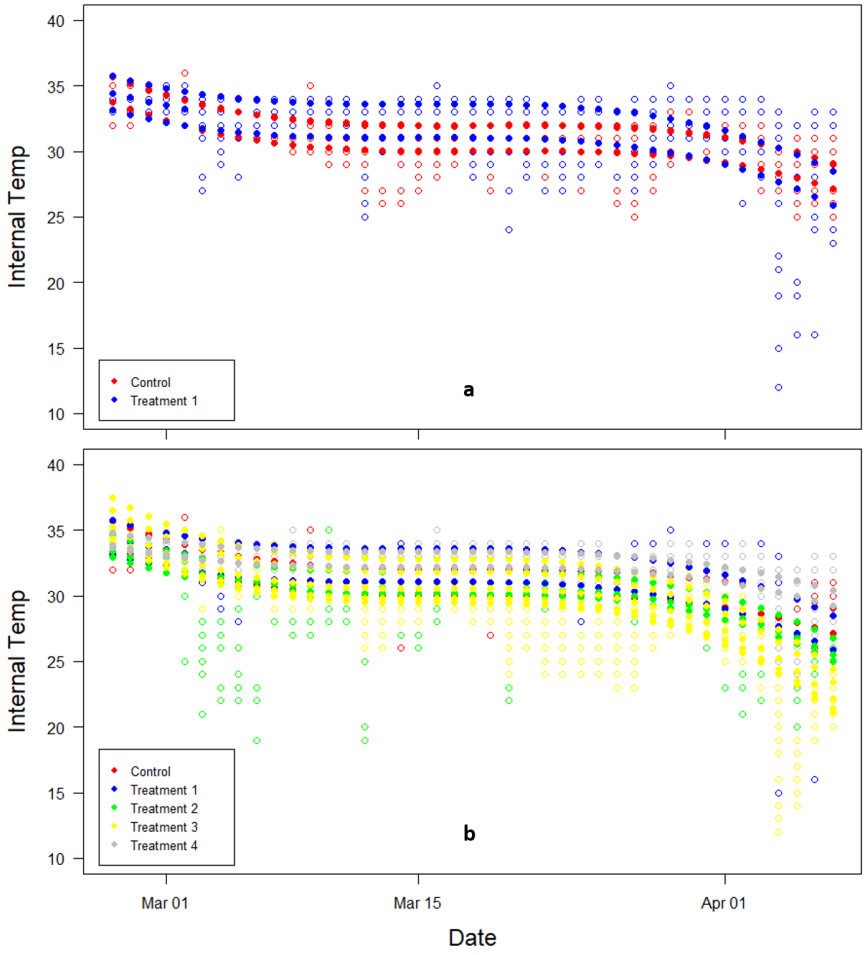

Figure 3.15: Scatterplot of internal temperatures (°C) following crop death on the treatment site (25th February - 7th April). (a) Data points from treatment group 1 and the control colonies. (b) Data from all colonies across this period. Regression lines are plotted in their respective colours.

3.5.4 Further breakdown of the period following crop death

To further investigate the major decline in internal hive temperature observed post-crop death, the data from this period was broken down into three 2 week time periods (Figure 3.16, Method section 2.8). The three periods spanned between the 25th February - 10th March, 11th March - 24th March and 25th March - 7th April, respectively.

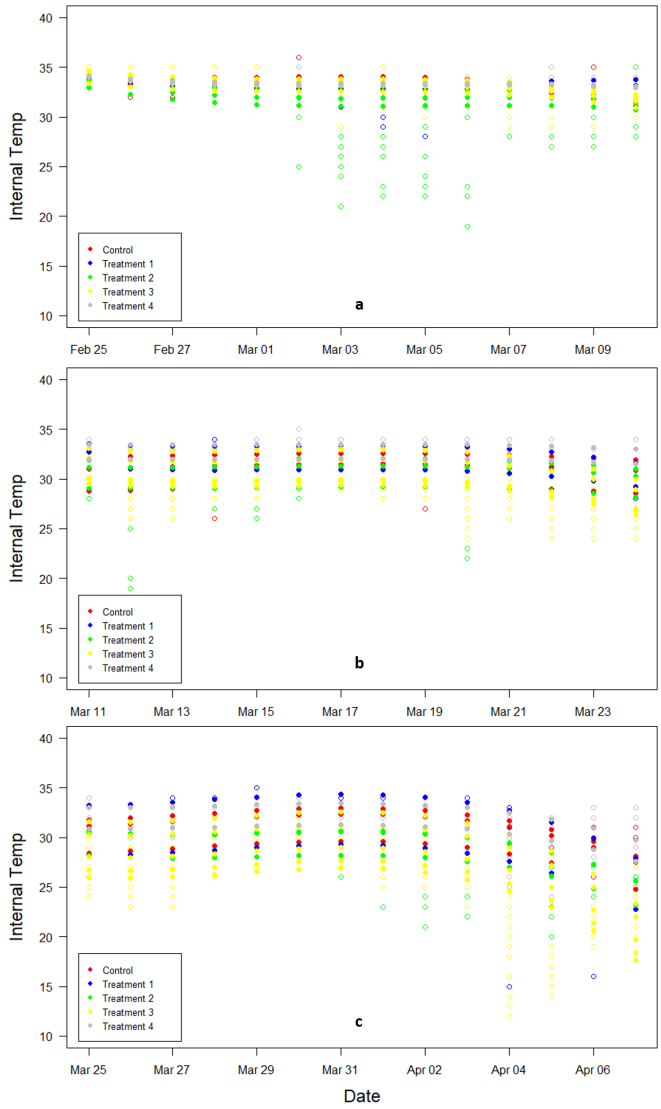

Figure 3.16: Scatterplot of internal temperatures(°C) over time post-crop death of all colonies in the control and treatment groups: (a) Initial 2 weeks after crop death (25th February - 10th March), (b) Two weeks after crop death (11th March - 24th March) and (c) Four weeks after crop death (25th March - 7th April). Regression lines are plotted in their respective colours.

Over the first 2 week period (Figure 3.16 a, 25th February - 10th March) the control colonies showed an average internal temperature of 33°C (Appendix H Table H.1), similar to the internal temperature observed in the pre-spray and post-spray periods. (Appendix G Tables G.2 & G.3). The combined average internal temperature of all

treatment colonies over this period was 33.06°C. Colonies in treatment group 2 showed significantly lower internal hive temperature compared to the control colonies over this first 2 week period (Est. = -0.90, t = -2.3, p=0.033, Appendix H Table H.1). Internal temperature of all other treatment groups was similar to that recorded for the control colonies.

Over the period from the 11th March - 24th March (Figure 3.16b) average internal temperature of the control colonies had declined to 30.7°C (Appendix H Table H.2). The combined average internal temperature of all treatment colonies over this period was 31.2°C (Averaged data from Appendix H Table H.2). None of the treatment groups showed any significant difference in internal temperature compared to the colonies at the control site over this period, and internal temperature was reducing over time for all colonies throughout this period (Est. = -0.35, t = -9.26, p <0.001, Appendix H Table H.2).

Over the final two weeks of the experimental period (Figure 3.16c, 11th March - 24th March) the colonies on the control site had an average internal temperature of 28.6°C (Appendix H Table H.3). The combined average internal temperature of all treatment colonies over this period was 27.6°C. However none of the colonies on the treatment site showed any significant difference to the control colonies during this period (Appendix H Table H.3). Treatment group 3 displayed the lowest average temperature over this period (25.66°C), but it was not a significantly different from the control colonies (Est.= -2.9, t = -1.89, p = 0.085 (Appendix H Table H.3). Internal temperature was declining significantly for all colonies over this period (Est.= -1.2, t = -18.3, p<0.001, Appendix H Table H.3). Within these last 6 weeks of the experiment, all colonies showed a large decrease in internal hive temperature, anywhere from a 3.5-5°C drop in internal temperature.

Chapter 4

Discussion

4.1 The success of the HiveMind monitors

One key aim of this study was to assess whether a satellite based monitoring system HiveMind could provide measurements of foraging activity and of the internal temperature of *Apis mellifera* colonies. Overall the monitoring system was a success, providing measurements over a four month period of activity at the entrance of 19 separate colonies, as well as their internal temperature. The information provided by these monitors would be difficult to obtain through other measurement systems, giving a unique look into the overall foraging activity of honey bee colonies. Despite the issues of resolution of the data, the information collected by the monitors still enabled investigation into changes of maximum activity over the entire season, the effects of temperature on activity, deviations in internal hive temperature and the effect of a spray event on maximum daily activity.

The use of the HiveMind monitors for beekeepers may be invaluable, allowing for long range monitoring of hives within remote regions, or just keeping check on hives without physically disturbing them. The monitors can provide a measure of activity levels, internal temperature, colony weight and internal humidity. Colony weight and humidity sensors were not used in the current experiment due to funding restrictions. However, between these four measures, one can gauge when a colony is thriving, and also when an intervening hand is necessary (e.g. addition of sugar solution as food stores). The

measurements allow the health of colonies hundreds of kilometres away to be measured, and they give insight into how the current environment is affecting the health of a colony. The initial design of these monitors was not for scientific measurement. They were initially designed as a long range monitoring system for apiaries in remote locations, so bee keepers did not have to physically check the colonies.

As far as this study is concerned, the monitors worked effectively to gather enough information to answer some specific questions regarding foraging activity and internal thermoregulation. However there are improvements that could be made in order to fully utilise this technology, and allow for more rigorous scientific studies in the future. Rather than relying on satellite based technology, utilising WiFi based hubs would be more suitable, allowing the data to be downloaded from the central hub when in range. This would minimise data loss when data signals are not transmitted to a satellite, which in the present study resulted in periodic loss of data. There were multiple data loss events throughout the experiment due to information not reaching the satellite, or not being sent to the satellite hub itself. With the use of WiFi, information would not get lost between the hub and the satellite, with data stored on site instead. However this would not solve the issue of data loss between the monitors and the satellite hub itself. There were also issues with battery life of the satellite hubs, which caused up to one week of data loss from the initial control site prior to the spray event. The HiveMind monitor technology had not previously been tested when connected to such a large number of HiveMonitors at a single time or over such a long period of time.

A secondary improvement would be reducing the time between reports from the monitors themselves. Instead of reporting once every hour and a half, reports could be taken at more regular time intervals, allowing for more specific questions to be asked of the data set related to circadian rhythmicity. For example, onset and offset of foraging could be measured more accurately. The current data recordings sent by the HiveMind monitors are adequate for beekeepers to understand whether their colonies are surviving and producing honey. However if the technology is to be used to pursue further scientific ventures, the issues outlined above will need to be addressed.

4.2 Temperature effects on honey bee foraging activity

Over the experimental period, foraging activity was shown to be highly correlated with external temperature. The effects of temperature on activity levels have been described within previous literature, such an activity increase as temperature rises (Heinrich, 1979; Blažytė-Čereškienė et al., 2010; Joshi and Joshi, 2010; Abou-Shaara et al., 2012; Tan et al., 2012). Joshi and Joshi (2010) studied the foraging behaviour of *Apis mellifera*, observing a temperature threshold for the onset of foraging between 12-15°C, with little to no activity occurring below 10°C. They also observed activity increases as temperature rose, with a large increase between 15°C and 20°C, after which the activity of a colony remained high (Joshi and Joshi, 2010). In the current investigation there also seemed to be an activity threshold which was dependent on temperature, between the temperature ranges of 9-17°C. However the temperature at which activity began to rise was colony dependent, and also changed throughout the experimental period (i.e. with the seasonal change). Time also seemed to play a role in onset, some time between 7:30-9:00am in the current study, which is later than the times found by Joshi and Joshi (2010) (6:17am). However, unlike Joshi and Joshi (2010), the measure in the current study was not the first forager to emerge, rather the time at which average night activity was surpassed. All colonies within this investigation displayed high levels of activity above 20°C, which is consistent with earlier studies (Joshi and Joshi, 2010). A point of interest from the current study was activity levels of foragers above 40°C, which were found to be as high as activity between 20-30°C (data not shown). This is in contrast with a study by Blažytė-Čereškienė et al. (2010), which found foraging activity above 43°C+ temperatures to be very low. However their measure of foraging activity was visitation rates on their measured crop (oilseed rape), whereas in the current study it was activity recorded at the entrance of the hive. The high levels of activity in the current study observed above 40°C could be due to fanning behaviour of the colonies around the entrance of the hive, rather than a measure of foraging activity. The trend of high foraging activity at high temperatures may be regionally dependent, or dependent on available forage, as other observations of this kind have not been carried out in New Zealand or with *Phacelia*.

For insects to fly, they must attain a minimum thoracic temperature in order to effectively beat their wings. This minimum temperature is dependent on the temperature of the muscles involved in flight (Heinrich, 1974). Honey bees have been shown to warm up their thoracic temperature to between 36-38°C before departing the hive, by shivering their wing muscles (Heinrich, 1979). Honey bees are also able to maintain their thoracic temperatures above 30°C while in flight, but this is dependent on the external temperature (Heinrich, 1974; Dudley, 2002). Stationary bumblebees are able to keep their body temperature around 36-38°C for hours or even days at ambient temperatures as low as 2°C, but this is metabolically expensive (Heinrich and Raven, 1972). At low ambient external temperatures (0°C), convective heat loss is too much for honey bee foragers to continuously fly, so they must warm their thoracic muscles up during the intermittent stops on flowers during foraging (Heinrich, 1974). This may provide evidence for the observed low levels of activity below certain temperature thresholds, due to the high energetic cost of maintaining flight muscles at optimal temperatures to carry out successful foraging flights. At high ambient temperatures, without active cooling mechanisms there is a chance of overheating due to the combination of the high metabolic rate during flight and insulation (Heinrich, 1974). However honey bees are able to thermoregulate during flight by regurgitating contents from their honey stomach (Cooper et al., 1985), enabling evaporative cooling of the thorax and head (Cooper et al., 1985; Roberts and Harrison, 1999). The relationship between temperature and activity levels within the current investigation is supported by findings in previous literature, with activity showing a strong temperature dependence between the temperatures of 9°C and 36°C.

4.3 Activity levels are affected by forage availability

Forage availability, while not monitored directly, appeared to be a major factor influencing foraging activity. Three major examples of forage affecting activity levels within the experiment were the increase in activity levels associated with the bloom of the *Phacelia* and the decline in activity levels associated with the death of the *Phacelia* and thyme crops. The initial bloom of the *Phacelia* crop at the onset of the experiment was correlated with an increase in activity observed in the treatment group 1 colonies.

When the HiveMind monitors were first installed on the treatment group 1 colonies at the treatment site, the maximum activity levels recorded over the first few days hovered between 2000-3000 bees. However 3-4 days after the installation of the monitors the *Phacelia* flowers began to bloom, which coincided with an increase in maximum activity, peaking at a single day maximum activity count of 5376 individual foragers. The *Phacelia* crop provided the majority of available forage around the treatment site, which may explain the rise in activity once the flowers began blooming. On the initial control site, the death of thyme coincided with declining activity in the control colonies and treatment groups 2-4. The disappearance of thyme was occurring at the same time as the bloom of *Phacelia*, which may explain the increase in activity of the T1 colonies which were located at the treatment site throughout the experiment, but the decline in the controls and T2-4 colonies which at the time were located at the initial control site. The death of the *Phacelia* crop was also correlated with a reduction in activity for all treatment group colonies, but not for the control colonies which were not exposed to a reduction in forage availability over this period.

The availability of forage has been identified previously as a major factor influencing foraging activity, such as attracting a greater number of pollinators, and a greater diversity of pollinators (Ghazoul, 2006; Klein et al., 2007; Kremen et al., 2007). As mentioned in the introduction, Van Nest and Moore (2012) described scout bees which gather information to pass to reticent bees which wait in the hive. When forage availability is high, this may increase the number of reticent bees foraging at any one time, due to the high chance of a scout bee passing on information regarding good forage. Within the current investigation this may suggest that the increase in activity observed in T1 colonies during the onset of blooming of *Phacelia* was due to increased recruitment of reticent bees. This may also suggest that the decrease in activity could be related to a decrease in recruitment levels within the colonies.

Activity measurements at the initial control site spanned over three weeks. During this period, the control colonies displayed their highest recorded single day maximum activity over the entire experimental period (5376 bees). This may be due to a number of factors, such as forage availability (thyme in particular), high temperatures or seasonal timings (i.e. the height of Austral summer). All of the treatment groups present on the initial control site also displayed their highest, or equal highest recorded single day

maximum activity (5376 bees). This is not surprising because of the forage availability on the initial control site. The initial control site had abundant thyme plants, which provided large amounts of forage prior to transportation of the control colonies and colonies belonging to treatment groups 2-4. However because the thyme plants were beginning to die, the control colonies were moved to a new site to ensure foraging activity would be retained at a high level. Under ideal conditions the initial control site would have been identical to the treatment site, however water limitations made this impossible. In the Ida Valley the available water is sub-divided into shares for all farmers to take from the multiple races that run through the hills. However shares were not able to be purchased to provide irrigation for a secondary plot, or for the treatment site itself. In terms of forage the secondary control site was not identical, and provided a different range of floral resources such as lavender and clover. This was not as realistic as desired, however the death of the thyme crop forced a move of the colonies and the most appropriate available site was the site at Booth Road (secondary control site).

After the control colonies were moved to the secondary control site, the average maximum activity count was lower on the new site compared to prior to the move. While the relocation of the hives could have had an effect on activity levels, e.g. transportation damage, it was unlikely these effects were the main cause of activity reduction. A study by RiddellPearce et al. (2013) looked at the effects of relocation on *Apis mellifera* colonies, finding no consistent differences in foraging activity between resident and relocated hives. Furthermore, the authors suggest any differences between foraging activity at different locations was likely due to climatic conditions and floral availability on their chosen sites. In the current study the change in forage availability is the likely candidate for the activity difference between the two control sites.

Activity levels of the control colonies were steadily increasing after the move event up until mid-March. It is important to note however average activity levels never reached levels observed prior to the move event. This could be due to the diversity of forage available or new sources of forage appearing over time on this site, however this was not measured. The large reduction in foraging activity observed once crop death had occurred on the treatment site supports the conclusion that foraging activity levels are strongly affected by forage availability. At the control site the decline in foraging was less dramatic, suggesting the strong link between available forage and activity levels.

Since this trend was not apparent on the control site, it is most likely caused by a factor that is present on the treatment site, but absent from the control site. While maximum activity was declining on the secondary control site throughout this period, the reduction is more likely what would be expected from the decrease in temperature associated with the shift between seasons (Austral Summer into Austral Autumn).

The early death of the *Phacelia* crop on the treatment site was believed to be caused by two major factors. The first was the amount of seed sown on the treatment site. A study looking at multiple factors affecting growth of *Phacelia tanacetifolia* in New Zealand was carried out by Stevenson (1991). The study asked four companies in Canterbury, New Zealand to grow *Phacelia* on four different sets of advice from European seed growers. They found that between 4-10kg/ha of seed was appropriate for a green manure crop. However they also state that *Phacelia* is an indeterminate plant, which means it will not terminate growth when a certain size is reached (Stevenson, 1991). Rather it will continue competing for resources and grow until its death is caused by some other factors (e.g. frosts). Therefore increasing sowing rate will increase inter-plant competition, sharing available nutrients between more plants, resulting in a shortening of the flowering period. The amount sown in the current experiment was 35kg/ha, between 3-7x more than the recommended amount for the field size. This may have shortened the flowering period, leading to a reduction in available foraging resources earlier than intended. The second factor that may have played a part in crop death was the lack of a major source of irrigation throughout the experimental period. Despite being known as a drought resistant plant, it is suggested by Stevenson (1991) that *Phacelia* may need a modest amount of moisture to thrive. In Central Otago water availability is scarce, with temperatures reaching above 30°C on most days in summer, causing high levels of evaporation.

An important measure that was overlooked in the current study was the amount of available forage crop. In hindsight this would have been very helpful to measure accurately as it could give a much stronger indication of the effect of forage, or lack thereof, on forager activity levels. For future field studies regarding foraging activity or behaviour, it is recommended that forage availability is monitored closely, allowing for comparisons between activity and increasing/decreasing levels of forage. It is suspected that maximum activity was affected by forage availability in this experiment during the

floral bloom in December 2016, and the death of the crop in February 2017. Alternative explanations to large deviations in activity could be due to a change in population size or temperature deviations. Foraging behaviour has been observed to differ between small and large colonies (Eckert et al., 1994). An increase in activity counts should be correlated with an increasing colony size as the number of viable foragers at any one time would be higher in a larger colony compared to a small colony. As for temperature deviations, temperature has been shown to be an indicator for the levels of activity observed, both within this investigation and previous literature, for example, that foraging is dependent on temperature (Abou-Shaara, 2014). However temperature differences are unlikely to be the cause of activity differences between the sites, as both sites experienced similar external temperatures. Therefore if an activity increase was observed at the treatment site and not at the control site, it was not likely to be related to a change in external temperature, rather some other difference that was unique to the sites themselves.

4.4 Activity levels of all colonies were increasing in the post-spray period

Treatment group 1 was onsite during the spray event, so it is likely that these colonies would have been exposed to the highest concentration of pesticide. Therefore if the pesticide was having an effect, the largest effect was predicted to be observed in these colonies. However maximum activity levels in colonies belonging to treatment group 1 showed no significant difference from the levels recorded in the control colonies, or in any other colonies on the treatment site in the period post-spray. All colonies on the treatment site exhibited an increase in activity in the month following the spray event (i.e. prior to crop death). This was most likely due to the warm temperatures and abundant food supply present on the treatment site. The same trend was also apparent at the secondary control site, with activity increase occurring over the same time period. Hence an activity increase was occurring both on sites with pesticide present, and also without pesticide exposure.

There are two major types of exposure when regarding pesticides. The first is acute exposure, which refers to a bee coming into contact with a pesticide over a short

amount of time, possibly leading to adverse effects. If acute exposure occurred, it may lead to a change in behaviour immediately, or within the first few hours of both contact or ingestion of chlorpyrifos. Examples of the effects of acute exposure to pesticides are increased mortality rates such as reported by Lunden et al. (1986) regarding chlorpyrifos, decreased short term memory and impairment of associative learning as reported by Herbert et al. (2014) with glyphosate, difficulty in navigation such as finding floral resources or difficulty in returning to the hive as reported by Balbuena et al. (2015) with glyphosate, and hyper-activity and hyper-responsiveness, followed by hypo-activity and ataxia reported by Suchail et al. (2001) with imidacloprid. The current experiment observed an increase in activity after the spray event, which could indicate a form of hyperactivity. However acute poisoning induced hyperactivity of invertebrates usually disrupts motor functions within the insect (Williamson et al., 2013). This can lead to attempts at foraging flights, but overall unsuccessful trips or death outside of the hive. This was not observed within this study as direct observations of the colonies and bees on the day of the spray was not carried out due to unsafe levels of chlorpyrifos present for humans. However when the colonies were first examined post-spray (2 days after), there were no signs of dead bees in front of any colonies. For the current study to address questions such as affected foraging flights, foragers would need to be tracked using RFID (radio-frequency identification) (Balbuena et al., 2015), and colony weights would need to be measured to investigate whether the rate of colony-weight increase is affected after a spray event. A slower weight gain might signal unsuccessful foraging trips and/or death of workers outside the colony.

The second form of exposure is chronic exposure, with a build-up of chlorpyrifos over time due to repeated exposures, leading to long term effects (Desneux et al., 2007). Field studies regarding chronic exposure to pesticides in honey bees are scarce, with the majority carried out in-vitro. Suchail et al. (2001) tested chronic exposure of caged honey bees to imidacloprid over 10 days, using concentrations similar to what is found during spray events on plant material. They observed an increase in mortality after 3 days. Moncharmont et al. (2003) carried out a similar study testing imidacloprid on caged forager bees. They found after chronic pesticide exposure using two concentrations (4μg/L & 8μg/L), mortality rates increased. A similarity between the studies by Suchail et al. (2001) and Moncharmont et al. (2003) is that the lower concentrations of pesticides tested caused a faster increase in mortality than the higher

concentrations. The authors suggest age-related sensitivity as a major factor of the mortality increase, as they believe sensitivity increases with age, however this is purely speculation on the author's part, with further investigation necessary (Moncharmont et al., 2003). In the current experiment, mortality rates were not assessed, however observations were made outside the entrances of the hives. A small percentage of honey bees within a colony display necrophoric behaviour, meaning they regularly clear out corpses from a hive (Visscher, 1983). This behaviour leads to corpse removal through the front of the hive, usually resulting in pooling of corpses outside the entrance. No large pools of dead bees were found at the entrance of any of the hives over the experimental period, so it is unlikely a large change in mortality occurred within the hives. However if the bees had problems homing, such as reported in Balbuena et al. (2015) with the use of glyphosate, they may have died away from the colonies themselves. Further studies into whether chlorpyrifos affects homing behaviour need to be carried out with RFID, as described in the study carried out by Balbuena et al. (2015).

If acute pesticide poisoning occurred on the treatment site, it is predicted treatment group 1 would show the greatest response. At the very least the effects on treatment group 1 would be expected to occur before effects on treatment groups exposed at a later date. Activity levels of treatment group 1 were increasing over time after the spray event, with one of the colonies exhibiting their highest maximum activity readout on the 9th of January (5376 bees), the day following the spray event. This may suggest hyperactivity of the colonies, which has been observed with acute poisoning. However the hyperactivity observed in previous literature was at levels at or above the LD50. So while hyperactivity was observed in these studies, this meant a level of acute poisoning had occurred within the bee, which then lead to hypoactivity shortly after, followed by death (Suchail et al., 2001). In the aforementioned study, hyperactivity seems to be a precursor to a level of poisoning that leads to the death of the bee. In the current study, it is unlikely that acute poisoning occurred as no large pools of bees were found in front of exposed hives. While it seems unlikely hyperactivity was induced by chlorpyrifos in this study, this possibility cannot be ruled out.

The other treatment groups, once transferred to the treatment site also showed an increase in activity over the month following the spray event, including colonies introduced 6 days after the spray event. This suggests the activity changes were being driven by

forage availability rather than by effects of pesticide exposure, especially as activity also increased during this period in hives on the control site without the presence of pesticides.

4.5 Activity levels of all colonies were decreasing in the post-crop death period

After the death of the crop on the treatment site, activity began to plummet within all of the treatment group colonies. The treatment group 1 colonies displayed the largest activity drop, showing significantly different activity compared to the control colonies. This could suggest that the spray may have had long term effects on colony health, disrupting the colonies' long term viability. This would be expected to the greatest extent in treatment group 1 colonies, as they likely were exposed to the highest concentrations of chlorpyrifos. However as activity levels in all of the other treatment groups were declining in a similar fashion, it is difficuly to determine whether chlorpyrifos played a role in the observed results. At the same time however, activity in the colonies on the control site was also declining, although not to the same magnitude as the treatment colonies. This suggest that one of the factors on the treatment site, whether it be the presence of a pesticide, or reduction in forage availability, was the likely cause of the decline. There is no strong evidence to suggest that the pesticide had an effect on foraging activity within this study. Evidence seems to suggest forage availability was the driving factor behind activity reduction of the treatment colonies. There have been many studies suggesting that landscape type (i.e. site of apiary) can have a large factor in foraging success of honey bees (Steffan-Dewenter et al., 2002; Klein et al., 2007; Alburaki et al., 2017). The differences in site can range from weather conditions, forage availability, floral diversity, urbanisation and pesticide exposure. In the current experiment the main differences between the two sites were the presence of a spray event, forage availability and forage diversity.

The second measure of colony health within this study was colony thermoregulation, which may be affected by pesticides brought back to the colony through contaminated pollen and nectar. If chlorpyrifos was having an adverse effect, it was predicted to affect honey bee workers' ability to thermoregulate, therefore increasing the variability

of temperatures present within a hive. This would be due to pesticides effects on motor function such as muscular excitation causing temperature increase (Tosi et al., 2016), or impaired cognitive ability, leading to the inability to achieve the muscle shivering necessary to warm themselves (Esch, 1976; Vandame et al., 1995), .

4.6 Chlorpyrifos has no discernable effect on colony thermoregulation

Honey bees rely on rapid brood production to gather enough workers to build food stores for overwintering (Winston, 1991). If the stores are not adequate for the winter months, the colony will perish. During the summer period, the number of worker bees inside a colony is at its highest, sometimes averaging over 60 000 individual bees (Winston, 1991). With this number of workers foraging and maintaining the hive, large amounts of brood are able to be produced. During this period, internal temperatures are maintained between 33-36°C to ensure optimal brood growth. Brood developmental time is reduced when a colony is regulated within 33-36°C, with lower temperatures greatly increasing incubation time, in part through decreased larval respiration at low temperatures (Petz et al., 2004). Reduced temperature during development can also affect short term learning and memory within adult workers (Jones et al., 2005). It is therefore essential that brood production be as efficient as possible, which is achieved by worker bees working to maintain optimal hive temperatures. This is why internal temperature and its variability may provide a good indicator for levels of brood production, and therefore the chance of surviving overwinter (Meikle et al., 2017). Tosi et al. (2016) tested the neonicotinoid pesticide thiamethoxam on thermoregulation of individual harnessed bees, exposed to 22°C and 33°C environments, showing that sub-lethal doses of pesticides (between 0.2 - 2ng/bee) affected the ability to thermoregulate at both temperatures. They reason that as honey bees use their muscles to produce heat (Esch, 1976), the pesticide could affect activity of the thoracic muscles, not allowing for proper thermoregulation (Tosi et al., 2016).

Within the current investigation, internal temperature was stable in all colonies throughout the summer period of December through to February, hovering around 32-34°C for the colonies at the treatment and at both control sites. This provides evidence that the

colonies were successfully thermoregulating throughout the period immediately after the spray event, as hives remained at optimal temperatures for brood rearing (Petz et al., 2004). However a large reduction in internal hive temperatures occurred halfway through March, and continued to drop throughout the remainder of the experiment. This trend occurred in all treatment group colonies and the control colonies. This is at odds with observed maximum activity declines, where the treatment colonies displayed a much larger decrease in maximum activity. The colonies from treatment group 1 showed a faster decline in maximum activity in the post-crop death period compared to the control colonies. However there was no significant difference in internal temperature over the period between the control and treatment group 1 colonies. The decline in internal temperature was also similar for treatment groups 2-4. The uniformity of colony temperature decline between all colonies regardless of treatment suggests that neither the pesticide or activity reduction was the main driver behind the internal temperature reduction. The evidence for this conclusion is that the control colonies were not exposed to pesticides, and also did not show the same magnitude of activity decline shown by the treatment groups, yet the internal temperature decline was very similar to the treatment groups.

Pesticide have been shown to alter individual honey bees ability to thermoregulate (Vandame and Belzunces, 1998; Tosi et al., 2016; Schmaranzer et al., 1987). Vandame and Belzunces (1998) tested a commonly used fungicide deltamethrin on the thermoregulation of individual harnessed bees. They found that the concentrations of 2.5ng/bee and 4.5ng/bee administered orally caused severe hypothermia at 22°C, an hour after oral application, and for the following 24 hours. However deltamethrin is not known to cause problems with the muscles at the levels tested within their study (Vandame et al., 1995), rather they suggest action occurred at the level of the brain which is affecting the ability to thermoregulate. They suggest that at the concentrations tested, the bees would still be able to carry out foraging flights, but may not be able to undertake the necessary shivering required for thermoregulation due to possible neural issues (Vandame and Belzunces, 1998). In the current study, it is possible concentrations of chlorpyrifos were high enough to alter motor or neural function, however this area is very understudied in organophosphate pesticides. It is known however that inhibition of cholinesterase, caused by organophosphate pesticides can lead to hypothermia in honey bees (Schmaranzer et al., 1987). The acute effects mentioned above would be

especially prominent in treatment groups that were exposed on the initial day of spray (i.e. treatment group 1) versus treatments that were introduced at a later date (e.g. treatment group 4), as the concentrations within the environment would likely be higher. However this was not the observed trend, with treatment group 3 showing the greatest decline in internal temperature.

The HiveMind temperature sensors were located between the first brood super and the second brood super. These two supers are where all of the brood are stored, which means the temperature sensors were approximately at the centre of the highest concentration of brood within the hive. Any change in temperature that was picked up by these sensors would be directly affecting the brood. Due to all colonies decreasing in internal temperature regardless of site, this may suggest a correlation with a reduction in brood. If the colonies have produced their last set of brood for the overwinter months (Winston, 1991), there is no longer a need to maintain the internal temperature between 33-35°C. To maintain these temperatures is very energetically demanding (May, 1979), so once brood production has finished for the season, the workers will let the internal temperature of a hive decrease to save energy. Over winter, internal colony temperature averages around 21°C, but shows much greater fluctuations than in any other season, falling as low as 11°C and reaching as high as 33°C (Fahrenholz et al., 1989). The temperature declines observed within the current study could indicate the beginning of the normal variance seen within colonies across the winter periods. It seems unlikely therefore that the decrease in internal hive temperature observed within this study is due to chlorpyrifos, as internal temperature does not show the variability expected if chlorpyrifos was affecting thermoregulatory ability. There is limited research on the effects of pesticides on colony thermoregulation, but this could be a promising area of future research. Further research on whether a pesticide effects thermoregulatory ability overwinter and in the following season would be an important future study. Furthermore, certain pesticides have been found to have a hypothermic effect on honey bees at certain concentrations (Schmaranzer et al., 1987; Vandame and Belzunces, 1998), but has yet to be proven within a field realistic setting. Further investigations on whether sublethal levels of pesticides can cause thermoregulatory issues within honey bees needs to be carried out.

4.7 Effects of chlorpyrifos on pollinators

Multiple studies have investigated the effects of chlorpyrifos on pollinator species. Urlacher et al. (2016) assessed sub-lethal effects of chlorpyrifos on aversive learning, appetitive learning, and mortality rates through proboscis extension response (PER) and sting extension response (SER) tests in honey bees. The chlorpyrifos concentrations tested were field realistic doses found within the Otago region of New Zealand (less than 286pg\bee). In contrast to what was observed by Lunden et al. (1986) at higher concentrations of chlorpyrifos, mortality rates were not increased by the presence of pesticide exposure at these low levels. The PER test showed slower learning capabilities of bees exposed to 50pg of chlorpyrifos for both winter and summer bees. A reduction in appetitive learning could adversely affect foraging efficiency as foragers may find it difficult to associate reward with floral signals such as scent and colour. In contrast to the effects of chlorpyrifos on appetitive learning, Urlacher et al. (2016) found no significant difference in SER tests from chlorpyrifos which suggests aversive learning is not affected by chlorpyrifos. A study by Bakker and Calis (2003) tested foraging rates and mortality levels of bees treated with Dursban 75 WG , which contains chlorpyrifos. Their experiment was carried out on bees in wire mesh cages, with untreated *Phacelia* plants as forage. They applied Dursban 75 WG at a rate of 1000g a.i./ha in a water solution to fresh *Phacelia* plants, swapping them with the untreated *Phacelia* in the mesh cages. This was 5x the concentration of chlorpyrifos used within the current study. They observed an increase in mortality the day after treated plants were introduced, and reduced activity for 4 days post-spray.

Field studies involving chlorpyrifos have also been carried out to assess mortality and effects on honey bee foraging. Lunden et al. (1986) carried out multiple studies on a variety of crop types sprayed with different "over the counter" chlorpyrifos products. The concentrations tested were within the ranges suggested for applications by the manufacturer, however they were on the high end of the concentration ranges (see Introduction for application ranges). They tested Lorsban 4E applied by aircraft at 1.12kg a.i./ha, spraying in early evening between 19:00-21:00h onto alfalfa crops, over 4 times the applied concentration within the current investigation. Mortality rates of the honey bees on the treated plots were 5-8 fold higher than prior to treatment.

There was also a 56-67% reduction in nesting leafcutting bees, *Megachile rotundata*, next to the treated crops, with large reductions of floral visitation by these leafcutters. However a second test of Lorsban 4E applied to dandelions in multiple 0.004ha plots, at a concentration of 1.12kg a.i./ha caused no reductions in the number of foraging bees present within the plots, and no erratic behaviour was observed (Lunden et al., 1986). These authors further tested Lorsban 50 WP applied with a ground sprayer on raspberry plots in the evening (1.68kg a.i./ha). They observed erratic behaviour of honey bees on the forage crop 1 day after the treatment, with a 40% decrease in visitation rate compared to controls examined on the first day of the experiment. Foraging activity remained reduced for 7 days (Lunden et al., 1986). In the current experiment visitation rates were not assessed, however they are a very useful measure to assess behaviour while foraging, and would be recommended for any future studies regarding foraging behaviour. Finally Lunden et al. (1986) tested Lorsban 50 W, applied to a blooming carrot field (8.1ha; 1.12kg a.i./ha). They reported a 12-fold increase in dead bees and reduced foraging activity the day after the spray application, and three-fold increase of dead bees 2 days after spray. They conclude however that the mortality increase caused by the pesticide, and subsequent loss of bees from each hive (between 250-500), was not enough to affect the hives long term survival (Lunden et al., 1986). The studies of Bakker and Calis (2003) and Lunden et al. (1986) both indicate that foraging behaviour and mortality of honey bees and wild bees were heavily affected by the concentrations of chlorpyrifos applied. However these effects may be occurring due to the high concentrations of chlorpyrifos used within their experiments. In the current study, the spray event was carried out by a registered spray contractor, with 400/mL of active ingredient applied over 1.6/ha, which translates to 0.20kg a.i./ha. This is under a quarter of the concentrations used in the experiments by Bakker and Calis (2003) and Lunden et al. (1986), and what was recommended as an effective dose for crop treatment by the manufacturer. The higher concentrations used in the earlier experiments may induce side effects on foraging bees, however it is unlikely that managed honey bee colonies would normally be exposed to such high concentrations of pesticide. The guidelines for the use of chlorpyrifos state that at these higher concentrations, foragers should not be introduced until 7 days after spraying (Chlorpyrifos SDS)

One of the main routes of dissipation of chlorpyrifos is volatilisation, the process of evaporation from a liquid to a gas (Solomon et al., 2014). Chlorpyrifos has been found

large distances away from spray sites, up to 300km away from a source, suggesting short- (Muir et al., 2004) and long-range atmospheric transport (Mackay et al., 2014). In the present study, the temperatures on the day of the spray event (35°C maximum temperature) would likely have caused volatilisation of the chlorpyrifos, possibly aiding the spread of chlorpyrifos from the experimental field onto the surrounding environment. A parallel study was undertaken to determine the exact atmospheric spread of chlorpyrifos, and to what extent chlorpyrifos was carried away from the experimental field (Das, in prep). The results suggest chlorpyrifos could have drifted onto the colonies themselves, contaminated nearby water sources and other floral species around the experimental site. Mackay et al. (2014) suggest the persistence of chlorpyrifos residues in air samples is minimal, with maximum concentrations of 250ng m^{-3}detected in their study. When compared with the LD50 of between 70-80ng /bee (Chlorpyrifos SDS, Cutler 2014), this would pose minimal exposure risk to foragers. However if sublethal effects occur at exposure levels much lower than the LD50 of the bee, this exposure route may provide some risk (Decourtye et al., 2005; Desneux et al., 2007; Balbuena et al., 2015; Urlacher et al., 2016).

4.8 Multiple chemical stressors may play a role in global pollinator decline

In Poland, Kiljanek et al. (2017) found chlorpyrifos was one of the most common compounds found within the bodies of live and dead bees during a survey in 2014-2015. They collected live bees from 16 different provinces, sampling 343 apiaries over this two year period. Dead honeybees were taken from hives with symptoms of acute poisoning, whereas live bees were taken from a randomly selected colony within each sampled apiary. They also found that poisoned honey bees were more likely to have multiple pesticides within their body (85% of poisoned bees had two or more pesticides present); whereas only 24% of live honey bees showed evidence of multiple pesticides. In the present study only the presence of chlorpyrifos was tested for, however both thymol and formic acid were used in the treatment of the parasitic mite *Varroa destructor*. Due to prevalence of honey bee parasites and pathogens, miticides and fungicides are often applied within the colonies themselves (Sánchez-Bayo et al., 2016). Pettis et al. (2013)

investigated the effect of feeding bees with fungicide-spiked pollen to assess whether this affected pathogen prevalence. They found high loads of fungicides increased the probability of *Nosema* infection in the bees. Infections such as *Nosema* and *Varroa* can pose serious health risks for colonies if left untreated, possibly leading to colony failure (Cox-Foster et al., 2007; Higes et al., 2008). Furthermore, pesticides present within a colony can affect immune functions of honey bees, leading to an increase prevalence of pathogens within a colony.

Di Prisco et al. (2013) observed that the neonicotinoid pesticides, clothianidin and imidacloprid, negatively modulated the transcription factor NF-$_{K}$B, which affected the antiviral defence of the honey bees. This same trend did not occur following treatments with chlorpyrifos (Di Prisco et al., 2013). However, chlorpyrifos induced alterations in the expression of immune-related genes have been detected (Christen and Fent, 2017), which could potentially lead to viral, bacterial or fungal infections that affect the overall health of the colony (Pettis et al., 2013; Sánchez-Bayo et al., 2016). Christen and Fent (2017) also found expression of vitellogenin was altered in the presence of low levels of chlorpyrifos. Vitellogenin is thought to play a role in behavioural plasticity of workers in the colony (Peso et al., 2016). Changes in vitellogenin correlate with the shift from in-hive duties to foraging, with high levels of vitellogenin associated with in-hive tasks (Antonio et al., 2008). Silencing expression of the vitellogenin gene, therefore lowering the amounts of vitellogenin present, has been found to induce the onset of foraging behaviour 3-4 days earlier than normal forager onset (Antonio et al., 2008). Chlorpyrifos was found to upregulate the expression of vitellogenin transcripts by Christen and Fent (2017), which would be predicted to delay onset of foraging. However, in the present investigation, activity levels increased after the spray event, which suggests no delay of foraging occurred.

All colonies used in the present investigation were treated against *Varroa* prior to the spray period, and also again during the experimental period. Detection of chlorpyrifos within the bodies of the bees from the treatment colonies (Heath, in-prep), raises the possibility of synergistic effects with miticides. Multiple studies have looked at accumulation of chemical residues within hive matrices, noting there could be long-term effects on honey bee health from exposure to multiple chemicals simultaneously (Chauzat et al., 2006; Lambert et al., 2013). A study by Rinkevich et al. (2017) tested *Varroa*

management practices on insecticide sensitivity in honey bees. Significantly increased sensitivity was observed with the neonicotinoid pesticides, however the overall synergies between pesticide effects and miticide treatments were suggested to be minimal. Chauzat et al. (2009) sampled colonies for pesticide residues throughout France, sampling 120 colonies from 24 different apiaries. They found the neonicotinoid imidacloprid to be the most widespread pesticide, but many organophosphate pesticides such as chlorpyrifos were also present. Within each colony, they found between 0-9 residues, with the most common number of pesticide residues per hive being 2 (29.6%). After analyses of all colonies, they found no significant effect of pesticide residue on colony mortality. They also found no statistical relationship between pesticide residues and the abundance of brood or adults in a colony. Other studies have found high levels both of miticides and of organophosphates in the hive matrices and bodies of bees (Lambert et al., 2013; Kiljanek et al., 2017). However the effect of interactions between miticides and organophosphate pesticides needs to be further explored to fully understand any possible relationships on colony health.

The effects of formic acid and thymol miticides used in the current experiment are unknown. However on the day of formic acid treatment, a few hives showed erratic internal temperature profiles, deviating from the average internal temperature by upwards of 6°C. This could have been due to the opening of the hives themselves for extended periods of time rather than direct effects of the miticides themselves. A couple of the colonies that showed large deviations in temperatures did not return to the same temperature as prior to miticide treatment for up to a week. Further exploration into this trend would require a more controlled experimental set-up to assess possible miticide effects.

4.9 Future study directions

The current study successfully measured activity and internal temperature over the austral summer and autumn. While the data collected provided useful information on the activity of colonies exposed to chlorpyrifos over summer, it did not address questions related to the long term effects of chlorpyrifos. It would be useful to know whether colonies exposed to chlorpyrifos displayed high mortality levels over winter,

and whether colony productivity is affected by this pesticide in the following seasons. This is especially important as during the investigation, sublethal levels of chlorpyrifos were detected within the bodies of nurse bees and brood (Heath, in prep). Future work should also examine the effects of chlorpyrifos on other pollinators, such as bumblebees, native bees and flies (Velthuis and Van Doorn, 2006; Jauker and Wolters, 2008), to ensure not only honey bees, but other important pollinators are protected and valued as well.

Within the current study, the sample sizes of all treatment groups were relatively low. This may have impacted the ability to detect any sublethal effects on foraging activity and colony thermoregulation caused by chlorpyrifos. To detect any changes in activity or internal thermoregulation with small sample sizes, the changes would have to be very pronounced. Future field studies should aim for large sample sizes to minimise the effect of variation between colonies.

4.10 Overall conclusions and final remarks

The key aims of this study were to assess the effects of the pesticide chlorpyrifos on maximum foraging activity and hive internal thermoregulation through the use of HiveMind activity monitors. The use of the monitors was an overall success, providing valuable data in a field realistic setting over four months. Over this time, there was no significant evidence to suggest that chlorpyrifos affected foraging activity or thermoregulation of exposed honey bee colonies. The key driving factors behind observed daily and monthly variations in foraging activity appeared to be forage availability and external temperature. The reduction of internal temperature observed in the experiment was likely due to natural causes, and potentially associated with a reduction in brood rearing that normally occurs with the shift from summer to autumn. However this does not mean chlorpyrifos had no effect on the colonies foraging and internal thermoregulation. It is possible that any changes were too small to detect with the current experimental set-up and measurement tools. While this study focussed on changes over a long period of time, further research is required to determine the long-term effects of chlorpyrifos not only on foraging success, but choices made during foraging trips, and the homing abilities of foragers exposed to pesticides. Furthermore,

it is crucial to understand the effects of chlorpyrifos on nurse bees and brood due to the transport of the pesticide into the hive through contaminated pollen and transport through the hive matrix. Providing answers to these questions may contribute useful insight for further conservation of all managed *Apis mellifera* colonies.

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Appendix A

Appendix 1

Table A.1: Maximum recorded activity counts for each treatment group prior to spray event, post-spray event and post-crop death. Readouts taken as single highest activity readout over the described period, pre-spray (19th December - 7th January), post-spray (8th January- 25th Febraury) and post-crop death (25th February - 7th April) from any single colony within a treatment group.

Treatment Groups	Pre-Spray	Post-Spray	Post-Crop Death
Controls	5376	4352	4608
Treatment Group 1	5376	5120	4608
Treatment Group 2	5376	4864	4864
Treatment Group 3	5376	5376	5376
Treatment Group 4	5120	4864	4864

Appendix B

Appendix 2

Table B.1: Output of GLMM analyses conducted on the full dataset. Model is described in methods (X ~ Y1 + Y2 ... + 1|colony). The analyses carried out was a generalized linear mixed-effects model, all response and explanatory variables are stated, as well as fixed effects. The GLMM's contained one random effect which was the subject units, the colonies themselves. Intercept values are the control colonies, with p-values for each treatment group a comparison with the controls.

Model/response ~ Explanatory variables considered	Fixed effects	Estimate Value	Std. Error	Z-value	p-value
Activity ~ Treatment + Site + Date + Outside Temp + Hive Temp + Crop Death + Spray Event + $Date^2$ + $Date^3$+ (1|colony)	Intercept	5.5	0.029	186	<0.001***
	Treatment Group 1	-0.031	0.037	-0.8	0.40
	Treatment Group 2	0.075	0.033	2.2	0.026*
	Treatment Group 3	0.014	0.033	0.4	0.66
	Treatment Group 4	0.031	0.037	0.8	0.40
	Treatment Site	0.084	0.0039	21.4	<0.001***
	Control Site 2	0.13	0.0040	32.4	<0.001***
	Date	-0.0075	0.0014	-52.5	<0.001***
	Outside Temperature	0.036	0.000080	458.8	<0.001***
	Hive Temp	0.042	0.00042	101.2	<0.001***
	Crop Death	-0.0325	0.0039	-9.0	<0.001***
	Spray Event	-0.030	0.0022	-13.8	<0.001***
	$Date^2$	-0.088	0.0011	-78.7	<0.001***
	$Date^3$	-0.034	0.00089	-38.2	<0.001***

Appendix C

Appendix 3

Table C.1: Differences in maximum activity levels between the control colonies at the initial control site (before the move event). Model is described in the methods (X ~ Y1 + Y2...). All response and explanatory variables are stated, as well as fixed effects.

Model	Fixed effects	Estimate	Std. Error	Z-value	p-value
Activity ~ Colony ID + Date + Outside Temp + Hive Temp	Intercept	7.01	0.14	49.8	<0.001
	Hive1:Hive2	0.12	0.011	10.5	<0.001
	Hive1:Hive3	0.18	0.011	15.4	<0.001
	Hive2:Hive3	0.064	0.011	5.85	<0.001
	Date	-0.029	0.0050	-5.9	<0.001
	Outside Temp	0.017	0.0050	-5.9	<0.001
	Hive Temp	0.019	0.0044	4.33	<0.001

Table C.2: Interactions between pre-move maximum activity levels at control site 1 and post-move maximum activity levels at control site 2 for the control colonies (refer to table 2.1 for the move event).

Model/response ~ Explanatory variables considered	Fixed effects	Estimate	Std. Error	Z-value	p-value
Activity ~ Date * Move + Outside Temp + Hive Temp + (1|colony)	Intercept	7.34	0.067	108.2	<0.001***
	Date	-0.038	0.067	-5.61	<0.001***
	Move	0.023	0.0086	2.72	0.0064**
	Outside Temp	0.028	0.00025	108.7	<0.001***
	Hive Temp	-0.00063	0.0020	-0.31	0.75
	Date*Move	-0.030	0.008	-3.84	<0.001***

Table C.3: Interaction table of maximum activity levels within the control colonies prior- to and post- complete crop death on the treatment site. Note: The control colonies were on the secondary control site for this period

Model/response ~ Explanatory variables considered	Fixed Effects	Estimate	Std. Error	Z-value	p-value
Activity ~ Date * Crop Death + Outside Temp + Hive Temp + (1|colony)	Intercept	7.02	0.054	129.3	<0.001***
	Date	-0.08	0.0029	-27.7	<0.001***
	Crop Death	0.10	0.0067	16.00	<0.001***
	Outside Temp	0.024	0.00019	127	<0.001***
	Hive Temp	0.0010	0.0012	8.12	<0.001***
	Date*Crop Death	0.040	0.0075	5.33	<0.001***

Appendix D

Appendix 4

Table D.1: Differences in maximum activity levels between all three treatment group 1 colonies at the treatment site prior to the spray event

Model	Fixed effects	Estimate	Std. Error	Z-value	p-value
Activity ~ Colony ID + Date + Outside Temp + Hive Temp	Intercept	10.34	0.28	36.35	<0.001***
	Hive4:Hive5	0.26	0.0079	32.8	<0.001***
	Hive4:Hive6	0.12	0.0080	15.7	<0.001***
	Hive5:Hive6	-0.13	0.08	-15.7	<0.001***
	Date	0.11	0.00383	35.5	<0.001***
	Outside Temp	0.030	0.00071	42.5	<0.001***
	Hive Temp	-0.090	0.0085	-10.6	<0.001***

Table D.2: Comparisons of maximum activity levels of all treatment colonies prior to the spray event with the control colonies. The control colonies and treatment groups 2-4 were on the initial control site at this point. Treatment group 1 was on the treatment site.

Model/response ~ Explanatory variables considered	Fixed effects	Estimate	Std. Error	Z-value	p-value
Activity ~ Treatment + Date + Outside Temp + Hive Temp + (1|colony)	Intercept	7.75	0.096	80.1	<0.001***
	Treatment Group 1	-0.032	0.064	-0.5	0.616
	Treatment Group 2	0.066	0.057	1.15	0.252
	Treatment Group 3	0.028	0.057	0.49	0.621
	Treatment Group 4	-0.0019	0.064	-0.03	0.976
	Date	0.010	0.0016	6.45	<0.001***
	Outside Temp	0.018	0.00027	66.1	<0.001***
	Hive Temp	-0.00067	0.0026	-0.25	0.80

Appendix E

Appendix 5

Table E.1: Comparisons of maximum activity levels of all treatment colonies in the post-spray period, but prior to complete crop death on the treatment site (25th February). As the control control colonies are excluded from this analyses, the treatment group 1 colonies are now the intercept.

Model/response ~ Explanatory variables considered	Fixed effects	Estimate	Std. Error	Z-value	p-value
Activity ~ Treatment + Date + Outside Temp + Hive Temp (1|colony)	Intercept	7.52	0.68	109.76	<0.001***
	Treatment Group 2	0.042	0.040	1.03	0.30
	Treatment Group 3	-0.024	0.040	-0.59	0.55
	Treatment Group 4	0.0035	0.045	0.08	0.93
	Date	0.016	0.0010	15.5	<0.001***
	Outside Temp	0.031	0.0001	237	<0.001***
	Hive Temp	-0.0086	0.0018	-4.79	<0.001***

Table E.2: Comparisons of maximum activity levels in all treatment colonies post-spray period with the control colonies (post-move event). All data within this table is prior to complete crop death on the treatment site (25th February)

Model/response ~ Explanatory variables considered	Fixed effects	Estimate	SE	Z-value	p-value
Activity ~ Treatment + Date + Outside Temp + Hive Temp (1|colony)	Intercept	7.59	0.056	135.1	<0.001***
	Treatment Group 1	0.034	0.043	0.80	0.42
	Treatment Group 2	0.074	0.039	1.90	0.057
	Treatment Group 3	0.012	0.039	0.31	0.75
	Treatment Group 4	0.039	0.043	0.89	0.37
	Date	0.0061	0.00098	6.26	<0.001***
	Outside Temp	0.032	0.00012	263.3	<0.001***
	Hive Temp	-0.012	0.0014	-8.38	<0.001***

Appendix F

Appendix 6

Table F.1: Comparisons of maximum activity levels in all treatment colonies in the post-crop death period (25th February onwards). As the control colonies are excluded from this analyses, the treatment group 1 colonies are now the intercept.

Model/response ~ Explanatory variables considered	Fixed effects	Estimate	Std. Error	Z-value	p-value
Activity ~ Treatment + Date + Outside Temp + Hive Temp (1|colony)	Intercept	5.79	0.099	58.4	<0.001***
	Treatment Group 2	0.17	0.12	1.40	0.15
	Treatment Group 3	0.10	0.12	0.8	0.41
	Treatment Group 4	0.39	0.13	2.9	0.0040**
	Date	-0.016	0.0016	-100	<0.001***
	Outside Temp	0.054	0.0001	352	<0.001***
	Hive Temp	-0.012	0.0006	19.9	<0.001***

Table F.2: Comparisons of maximum activity levels in all treatment colonies post-crop death on the treatment site (February 25th) with the control colonies on the secondary control site.

Model/response ~ Explanatory variables considered	Fixed Effects	Estimate	SE	Z value	p-value
Activity ~ Treatment + Site + Date + Outside Temp + (1|colony)	Intercept	5.91	0.090	65.6	<0.001***
	Treatment Group 1	-0.25	0.12	-2.1	0.0385*
	Treatment Group 2	-0.094	0.11	-0.8	0.39
	Treatment Group 3	-0.16	0.11	-1.5	0.12
	Treatment Group 4	0.089	0.12	0.7	0.47
	Date	-0.11	0.0014	-83.4	<0.001***
	Outside Temp	0.048	0.00014	347	<0.001***
	Hive Temp	0.023	0.00059	39.7	<0.001***

Appendix G

Appendix 7

Table G.1: Comparisons of internal hive temperature between all treatment and control colonies throughout the entire experimental period. The analyses carried out was a linear mixed-effects model, all response and explanatory variables are stated, as well as fixed effects. The LMM's contained one random effect which was the subject units, the colonies themselves.

Model/response ~ Explanatory variables considered	Fixed effect	Estimate	SE	df	t-value	p-value
Hive Temp ~ Treatment + Date + Outside Temp + Activity + Site _ Spray + Crop Death + Date^2+ Date^3+ (1|colony)	Intercept	30.5	-0.31	18	95.7	<0.001***
	Treatment Group 1	1.5	0.43	15	3.4	0.0035**
	Treatment Group 2	0.74	0.38	15	1.90	0.074
	Treatment Group 3	0.81	0.38	15	2.09	0.053
	Treatment Group 4	1.37	0.43	15	3.1	0.0062**
	Date	-1.25	0.037	1363	-33.9	<0.001***
	Outside Temp	0.029	0.0029	1363	9.99	<0.001***
	Activity	0.038	0.026	1363	1.4	0.14
	Treatment Site	0.054	0.092	1363	0.59	0.55
	Control Site 2	1.03	0.12	1363	8.5	<0.001***
	Spray	1.28	0.091	1363	13.9	<0.001***
	Crop Death	-0.61	0.061	1363	-10.06	<0.001***
	Date^2	-0.96	0.023	1363	-41.4	<0.001***
	Date^3	-0.39	0.017	1363	-22.6	<0.001***

Table G.2: Comparisons of internal temperature of all treatment and control colonies prior to the spray event (or move event for the control colonies).

Model/response ~ Explanatory variables considered	Fixed effect	Estimate	SE	df	T-value	p-value
Hive Temp ~ Treatment + Date + Outside Temp + Activity + Date^2+ Date^3+ (1|colony)	Intercept	32.7	0.26	17	123.3	<0.001***
	Treatment Group 1	1.71	0.35	14	4.86	<0.001***
	Treatment Group 2	1.20	0.31	14	3.81	0.0018**
	Treatment Group 3	1.40	0.31	14	4.41	<0.001***
	Treatment Group 4	1.29	0.35	14	3.64	0.0026**
	Date	-0.15	0.02	1998	7.23	<0.001***
	Outside Temp	-0.009	0.004	2001	-1.98	0.046*
	Activity	0.22	0.045	2002	4.89	<0.001***

Table G.3: Comparisons of internal hive temperature between all treatment and control colonies, post-spray event on the treatment site but prior to complete crop death (25th February).

Model/response ~ Explanatory variables considered	Fixed effect	Estimate	SE	df	T-value	p-value
Hive Temp ~ Treatment + Date + Outside Temp + Activity + Date^2+ Date^3+ (1|colony)	Intercept	33.1	0.17	15	186.2	<0.001***
	Treatment Group 1	0.82	0.24	14	3.32	0.0049**
	Treatment Group 2	0.25	0.22	14	1.14	0.27
	Treatment Group 3	0.57	0.22	14	2.57	0.022*
	Treatment Group 4	0.40	0.24	14	1.62	0.12
	Date	0.095	0.0084	6462	11.2	<0.001***
	Outside Temp	0.01	0.0017	6464	5.9	<0.001***
	Activity	-0.028	0.013	6465	-2.04	0.040*

Table G.4: Post-crop death comparisons of interal hive temperature between all treatment and control colonies.

Model/response ~ Explanatory variables considered	Fixed effect	Estimate	SE	df	t-value	p-value
Hive Temp ~ Treatment + Date + Outside Temp + Activity + $Date^2$+ $Date^3$+ (1|colony)	Intercept	30.7	0.67	14	45.2	<0.001***
	Treatment Group 1	0.09	0.96	14	0.10	0.919
	Treatment Group 2	-0.71	0.85	14	-0.84	0.415
	Treatment Group 3	-1.1	0.85	14	-1.35	0.198
	Treatment Group 4	0.48	0.95	14	0.51	0.618
	Date	-1.6	0.03	4666	-48.4	<0.001***
	Outside Temp	0.058	0.0061	4657	9.40	<0.001***
	Activity	-0.22	0.057	4659	-3.94	<0.001***

Appendix H

Appendix 8

Table H.1: Two weeks immediately after crop death (25th February - 10th March), observing differences in internal hive temperature between the control and treatment colonies.

Model/response ~ Explanatory variables considered	Fixed effect	Estimate	SE	df	T-value	p-value
Hive Temp ~ Treatment + Date + Outside Temp + Activity + $Date^2$+ $Date^3$+ (1|colony)	Intercept	33.4	0.31	16	105.3	$<0.001^{***}$
	Treatment Group 1	0.038	0.43	14	0.089	0.93
	Treatment Group 2	-0.90	0.38	14	-2.3	0.033^{*}
	Treatment Group 3	-0.090	0.38	14	-0.23	0.81
	Treatment Group 4	-0.079	0.43	14	-0.18	0.85
	Date	-0.60	0.031	1857	-19.2	$<0.001^{***}$
	Outside Temp	-0.0061	0.0056	1851	-1.10	0.27
	Activity	0.21	0.054	1853	3.92	$<0.001^{***}$

Table H.2: Two to fours weeks after complete crop death on the treatment site (11th March - 24th March), showing differences in internal temperature between all control and treatment colonies.

Model/response ~ Explanatory variables considered	Fixed effect	Estimate	SE	df	T-value	p-value
Hive Temp ~ Treatment + Date + Outside Temp + Activity + $Date^2$+ $Date^3$+ (1|colony)	Intercept	30.7	0.87	16	35	<0.001***
	Treatment Group 1	0.94	1.25	14	0.75	0.46
	Treatment Group 2	0.14	1.11	14	0.12	0.90
	Treatment Group 3	-0.56	1.10	14	-0.51	0.618
	Treatment Group 4	1.80	1.37	14	1.30	0.21
	Date	-0.35	0.038	1513	-9.26	<0.001***
	Outside Temp	0.0074	0.0073	1513	0.89	0.37
	Activity	0.079	0.071	1513	1.10	0.27

Table H.3: Four to six weeks after complete crop death on the treatment site (25th March - 7th April), showing temperature differences within all treatment and control colonies.

Model/response ~ Explanatory variables considered	Fixed effect	Estimate	SE	df	T-value	p-value
Hive Temp ~ Treatment + Date + Outside Temp + Activity + $Date^2$+ $Date^3$+ (1|colony)	Intercept	28.6	1.24	11	23.0	<0.001***
	Treatment Group 1	-0.34	1.95	11	-0.17	0.86
	Treatment Group 2	-1.46	1.63	11	-0.89	0.38
	Treatment Group 3	-2.94	1.63	11	-1.89	0.085
	Treatment Group 4	1.02	1.94	11	0.52	0.60
	Date	-1.27	0.06	1412	-18.3	<0.001***
	Outside Temp	0.14	0.013	1412	10.8	<0.001***
	Activity	-0.50	0.10	1412	-4.92	<0.001***