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Parasites and pathogens at honey bee mating sites, and the implications for monitoring colony health.

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Abstract

Western honey bees (*Apis mellifera*) are an insect species of high environmental, economic, and cultural importance due to their provision of pollination services to cultivated and native plants and production of honey. Rapid, international dispersal of the parasitic mite, *Varroa destructor*, has played a central role on colony health. The host-parasite relationship between *V. destructor* and immature (brood) and adult worker honey bees has been researched extensively. However adult drone (male) honey bees and their relationship with *V. destructor* has been underexplored. I used a choice test to investigate *V. destructor* host preference between nurses, foragers, and drones. I investigated the prevalence and abundance of *V. destructor* and pathogens at drone congregation areas compared to apiaries to explore the role of drones and drone congregation areas in monitoring of pests and pathogens. *Varroa destructor* selected drones as hosts in equal proportion to foragers, suggesting that drones are important phoretic hosts for the dispersal of *V. destructor.* I found that there was a significant positive relationship between the *V. destructor* abundance at DCAs and the *V. destructor* abundance at the nearest apiary. There was also a significant positive relationship between the abundance of DWV in drones and the abundance of DWV in colonies the nearest apiary. The abundance of DWV was found to be correlated with the abundance of *V. destructor*, with more viral copies of DWV found in drones and colony bees that had higher infestations of *V. destructor*. These results support the potential of drones as agents of dispersal for *V. destructor* and highlights the opportunity that drone congregation areas present for population-scale monitoring of honey bee pests and pathogens.

To my Grandad, Brian. Thank you for sharing your passion with me.

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DCA	Drone Congregation Area
PCR	Polymerase Chain Reaction
RT-PCR	Real-Time Polymerase Chain Reaction
BQCV	black queen cell virus
CBPV	chronic bee paralysis virus
DWV	deformed wing virus
SBV	sacbrood viruses
APV	acute paralysis virus
KBV	Kashmir bee virus
IPV	Israeli paralysis virus
AKI complex	Collective grouping of acute paralysis virus (APV), Kashmir bee virus (KBV), and Israeli paralysis virus (IPV)

CHAPTER 1

Literature Review

Honey bees are one of the most culturally significant insects in the world today. Archaeological evidence indicates that honey bees have been kept in hives from as early as 5000 and 3000 BC in ancient Egypt (Crane, 1999), with beekeeping in the New World beginning in the 1600s (Crane, 1999).

There are nine known species in the honey bee (*Apis*) genus: of which two are giant bees (*Apis laboriosa* and *Apis dorsata*), two are dwarf bees (*Apis florea and Apis andreniformis)* and five are cavity-nesting (*Apis cerana*, *Apis koschevnikovi*, *Apis nuluensis*, *Apis nigrocincta*, and *Apis mellifera)* (Koeniger et al., 2010). Of these nine species, eight are native to Asia, and only one, the Western honey bee *Apis mellifera*, is native to Africa, Europe, and the Near East (Koeniger et al., 2010., Ruttner, 2013). *Apis mellifera* can be further divided into 33 distinct subspecies which are organised into four geographically defined lineages (African, Western Asian, Middle Eastern, and European) (Ilyasov et al., 2020). Of these lineages, several of the European subspecies of *Apis mellifera* such as *A. m. carnica* and *A. m. ligustica* are popular strains for beekeeping due to their high honey production and gentleness and have thus been spread by humans to every continent of the world except Antarctica (Leclercq et al., 2017). Admixture between the subspecies of *A. mellifera* is extremely common (Schiff & Sheppard, 1995., Petersen et al., 2021). For example, the introduction of the African honey bee, *A. m. scutellata* into the Americas has caused the development of a hybrid population of African and European honey bees known as ‘Africanized honey bees’ (Winston, 1992).

Varroa destructor: A Threat to European Honey Bees

Varroa destructor is a parasitic mite of *A. cerana* and *A. mellifera* honey bees. *Varroa destructor* causes damage to honey bees by feeding on the fat body tissue of both mature and developing honey bees (Ramsey et al., 2019) and vectoring several honey bee viruses through the feeding process. Fat bodies are multifunctional organs, unique to insects, that are critical to the storage and utilisation of energy and nutrients, as well as the production of hormones and immune responses (Arrese & Soulages, 2010). Damage to the fat bodies of honey bees by *V. destructor* can substantially weaken nutrient stores and immune function, and increase sensitivity to pesticides (Arrese & Soulages, 2010, Blanken et al., 2015). The immune suppression caused by *V. destructor* infestation is a significant stressor on honey bees, with Yang and Cox-Foster (2005) finding that *V. destructor* infested bees produce significantly fewer antimicrobial peptides in response to an *E. coli* infection compared to uninfested bees. The suppression of immune responses in the honey bee caused by *V. destructor* causes honey bees to be more susceptible to infection by viruses that would not normally be of concern, many of which are vectored by the mite itself.

Historically *Varroa* spp. parasitised *A. cerana,* exclusively. However, sometime during the 1950s and 1960s, *Varroa* spp. made a host shift and began parasitising *A. mellifera* colonies that had been introduced into the native range of *A. cerana* (Peck, 2021). In the late 1900s, *Varroa* sp. began to spread rapidly through *A. mellifera* colonies across the world. At the time it was widely accepted that a single species of *Varroa, V. jacobsoni*, was infesting *A. mellifera* colonies (Oldroyd, 1999., Delaplane, 2001). However, a second *Varroa* species, *V. destructor*, was formally described in 2000 and discovered to be the species that had experienced the global spread formerly

accredited to *V. jacobsoni* (Anderson and Trueman, 2000). In contrast, *V. jacobsoni*'s infestation of *A. mellifera* is still largely confined to Indonesia and causes substantially less damage to colony health that *V. destructor* does (Traynor et al., 2020). *Varroa destructor* has become a problematic parasite for *A. mellifera* colonies worldwide, with only a few isolated islands left uninvaded. Its dispersal across the world was facilitated by the global honey bee trade and took place in less than half a century (Traynor et al., 2020).

Despite being a significant threat to *A. mellifera* colonies, *V. destructor* populations remain relatively low in number in the colonies of its original host, *A. cerana*. This is due to the timing of brood emergence (Spivak, 1996), and the defensive adaptations of *A. cerana,* such as grooming and removal (hygienic) behaviours. *Varroa destructor* are only able to reproduce in the drone brood cells of *A. cerana* (Koeniger et al., 1983). This substantially limits the growth of *V. destructor* populations within *A. cerana* hives, as drone rearing ceases when drones are expelled from the hive by their sisters in Autumn (Free & Williams, 1975., Cicciarelli, 2013), drones only make up 5 – 10% of the colony during the mating season (Seeley & Morse, 1976). While *V. destructor* demonstrates a preference for reproducing in drone brood cells in *A. mellifera* colonies (Fuschs, 1990., Boot et al., 1995), *V. destructor* can reproduce in both drone and worker brood cells of *A. mellifera* colonies (Ritter & de Jong, 1984), allowing for more rapid population growth that can occur year-round. The loss of brood specificity for reproduction enables *V. destructor* populations in *A. mellifera* colonies to greatly exceed its potential in *A. cerana* colonies, thus risking death in highly infested *A. mellifera* colonies (Ritter, 1981., Martin, 1994).

Furthermore, the co-evolution of *Apis cerana* alongside *Varroa* spp. has resulted in defensive behaviors in *A. cerana* that *A. mellifera* does not exhibit to the same extent. *Apis cerana* have proven to be remarkably efficient at grooming themselves and their nestmates to remove mites, with one study finding that 88.6% of bees commenced auto-grooming behaviours within one minute of contact with a *V. destructor*, and 75% successfully removed the mite in the process (Buchler et al, 1992). *Apis cerana* is also well adapted to detecting and removing infested brood, with 90% of the infested brood being uncapped and removed within 96h (Rath & Drescher, 1990). These adaptations of *A. cerana* mean that *V. destructor* infestations do not significantly affect the health of the colony. Unlike *A. cerana*, *A. mellifera* has not evolved alongside *Varroa* spp. (Ritter, 1981), so the host switch of *V. destructor* from *A. cerana* to *A. mellifera* has had a significant impact on the health of *A. mellifera* population worldwide. *Apis mellifera* has not had the opportunity to evolve mite defense behaviours to the extent that *A. cerana* has. Compared to *A. cerana*, *A. mellifera* performs grooming behaviours at a significantly lower frequency and with significantly lower success at removing mites (Peng et al., 1987., Buchler et al., 1992). This underdeveloped parasitic relationship between *V. destructor* and *A. mellifera* renders *V. destructor* one of the most pressing threats to apiculture globally.

The rapid pace at which *V. destructor* colonised managed honey bee *A. mellifera* colonies has contributed to a widespread decline in honey bee populations (Le Conte et al., 2010., Abbo et al., 2017., Stahlmann-Brown et al., 2022), as well as a pressing need for effective miticide treatments. The ability of *V. destructor* to successfully disperse

globally can be attributed to the ‘phoretic’ stage of its life cycle; in which the mite survives for extended periods of time on adult honey bees, outside of the brood cells.

Phoresy and *Varroa destructor*

Dispersal to new hosts is risky for parasites as the individual must forfeit the resources provided by the current host and seek out a new host (Ward, 1987), thus leaving the dispersing individual vulnerable to decreased fitness and death if it fails to find a new host (Ward et al., 1998). One strategy to minimise dispersal risk is to exploit your host’s own movements. This is referred to as phoresy and is defined as when one organism attaches itself to another, more mobile organism for the purpose of dispersal (Bartlow & Agosta, 2021). Phoresy is observed in a wide range of arthropods (e.g., mites, ticks, and millipedes), tardigrades, and nematodes (White et al., 2017; Binns, 1982; Bartlow & Agosta, 2021). Phoresy is a common behaviour among parasites, especially parasitic species who have limited mobility, or parasitises of host species that are widely dispersed (Bartlow & Agosta, 2021).

Phoresy is most often defined as a form of commensalism where there is no harm to the host (Bartlow & Agosta, 2021). However, in practice the dynamics and consequences of phoretic relationships are not always readily apparent. Parasite dispersal via phoresy can have a negative effect on the host species, even if there is not direct damage to the host during the phoretic transport itself. Host dispersal patterns can change in response to parasitism in search of habitat with reduced parasitic pressure (Lion et al, 2006). This is exemplified by *Hemisarcoptes cooremani* (Houck & Cohen, 1995) and *Telenomus Euproctidis* (Arakaki et al, 1997): two parasites that hijack their host’s dispersal patterns to invade new habitats and supporting uninfested hosts

and resources. Moreover, Houck and Cohen (1995) hypothesise that parasitic relationships can evolve from a formerly phoretic relationships, thus blurring the lines between parasitism and phoresy. They found this to be the case in the relationship between a mite species, *Hemisarcoptes cooremani* and its host beetle *Chilocorus cacti* (Houck and Cohen 1995). Conversely, host-parasite interactions described as phoretic, have later been discovered to include parasitism of the host.

Varroa destructor and its host honey bees exemplify the complexity of phoretic interactions. During its dispersing life stage *V. destructor* feeds directly on the fat bodies of the adult honey bees it is using as a means of dispersal (Ramsey et al, 2019). This phase is distinct from the reproductive life phase of *V. destructor* that is recognised as the most damaging to honey bee health where *V. destructor* feeds on developing honey bee brood (larvae and pupae) under the wax cappings of brood combs in the hive. Because *V. destructor* has specifically adapted to parasitise adult honey bees during this life stage, it is unclear whether this can truly be regarded as phoresy (Ramsey, 2019). However, due to the distinction between the damage incurred and the biological function of these two life phases, ‘phoretic phase’ is still commonly used to differentiate between the dispersing (‘phoretic’) and reproductive life phases of *V. destructor* and there has not yet been consensus as to the appropriate descriptor for this life stage.

Dispersal of *Varroa destructor*

Varroa destructor can move freely but rely heavily on honey bees for dispersal both within and between colonies (Boot, 1993), and the behaviour of that host bee influences the opportunity for intra- or inter-colony dispersal (Peck 2021). To reproduce, a female mite will detach from the adult bee and enter the brood cell of a mature honey

bee larvae just before pupation. Within the capped brood cell, the foundress mite will lay 2-5 eggs, one of which is male. When hatched, the young mites mate within the brood cell and feed on the developing honey bee pupae (Nazzi & Le Conte, 2016). The male mite dies in the cell and the mature females emerge with developed honey bee. The reproductive stage is supported by intra-colonial dispersal of *V. destructor*, and it is widely accepted that *V. destructor* prefer hosts that remain in the brood area of the hive caring for the developing brood for this purpose (Kraus, 1993., Kraus, 1994., Xie, 2016). However, population success of *V. destructor* is reliant on dispersal to new colonies and the selection of honey bee hosts that exit the hive is hypothesised in to support extra-colonial dispersal.

During the dispersal phase of *V. destructor*'s life cycle, honey bee movements outside the hive facilitate the spread of *V. destructor* between hives. These dispersal methods are usually separated into two categories: vertical and horizontal transmission. Vertical transmission occurs during swarming, where mites from the hive travel with the swarming colony to infest the new hive (Peck, 2021). Swarming can also be a mechanism for accidental human-mediated *V. destructor* dispersal, with the accidental international transport of infested swarms on ships and trains attributed to *V. destructor*'s worldwide colonisation (Owen, 2017). However, in regions where *V. destructor* is already established and standard colony management includes swarm control, horizontal transmission represents the main form of dispersal (DeGrandi-Hoffman et al., 2017).

Horizontal transmission is characterised as *V destructor* moving to a new hive, via human transport (such as beekeepers moving frames of brood between colonies),

robbing, drift, or the mixing of bees at shared floral resources (Peck, 2021., Peck et al., 2016). Beekeeper movement of mites occurs occasionally but drifting and robbing are recognised as key modes of *V. destructor* dispersal (Degrandi-Hoffman et al, 2017). During robbing, a honey bee either transports *V. destructor* from its colony to the colony its robbing, or a honey bee returns to its hive with new *V. destructor* it acquired from the colony it was robbing. Colonies that are heavily infested *by V. destructor* that are nearing death often become targets of robbing as their weaken workforce can no longer adequately defend their food stores int eh hive from intruders. Thus, creating what is commonly referred to as “mite-bombs” that increase *in V. destructor* infestations in nearby colonies (Peck & Seeley, 2019).

In contrast to robbing, drift occurs when a bee simply returns from a flight to a hive that was not their own (Peck & Seeley, 2019). Foragers are often highlighted as the key drivers of *V. destructor* dispersal through drifting and robbing. Undoubtedly, foragers do play an important role in the dispersal of *V. destructor*, as evidenced by a study carried out by DeGrandi-Hoffman et al (2016). They found that the increase in the mite population of two colonies during autumn was much higher than what they predicted based on the number of mites already existing within the colony at the end of summer. This increase in mites was correlated with an increase in mite-carrying foragers entering the hive, a trend which they attributed to an influx of mites dispersing from other hives via drift. They hypothesise that a low-level entry of mites into the hive over the foraging season via drift is responsible for the unexpectedly large population growth of mites they observed in their study hives.

In addition to foraging worker bees, drone honey bees drift between colonies when returning from mating flights. *Varroa destructor* have been discovered on drone honey bees collected from mating sites (Mortensen et al, 2018., Galindo-Cardona et al., 2020). Peck and Seeley (2019) conducted a study on drift and robbing between highly infested colonies (mite donor colonies, MDC's) and colonies with low infestation rates (mite receiver colonies/MRC's). They found that the percentage of MDC foragers at the hive entrances of MRC colonies didn't exceed 7%, but the percentage of MDC drones at the hive entrances of MRC colonies was 21.5% ± 16.9%. Moreover, Free (1958) found that drones tended to drift 2 - 3 times as much as workers. The contribution of drones to *V. destructor* dispersal between colonies could be of equal, or greater importance to that of foragers.

Honey Bee-Associated Viruses

Viruses affect almost all living things including bacteria, plants, and animals. Insects are often regarded as viral vectors, facilitating transmission of viruses between plants and animals alike. For example, plant-feeding insects are often the main means of transmission for viruses that infect immobile plants (Roossinck, 2013), and mosquitoes are well known vectors of several viruses that infect humans (Vasilakis & Tesh, 2015). However, insects themselves are also vulnerable to viral infections.

The western honey bee is vulnerable to several viruses. Presently, 23 viruses have been identified that infect honey bees (Chen & Siede, 2007, McMenamin & Genersch, 2015, Gisder & Genersch, 2015). These honey bee-associated viruses represent two viral families, Iflaviridae (notably: deformed wing virus (DWV) and sacbrood viruses (SBV); Valles et al., 2017., Amiri et al., 2020), and Dicistroviridae

(notably: black queen cell virus (BQCV), Kashmir bee virus (KBV), acute paralysis virus (APV), and Israeli paralysis virus (IPV); Amiri et al., 2020), or are not a member of a named viral family (notably: chronic bee paralysis virus (CBPV) (Amiri et al., 2020).

Evidence for vertical transmission of honey bee-associated viruses (DWV, BQCV, CBPV, SBV, KBV and ABPV) was presented by Chen et al. (2006) by analysing the profiles of honey bee queens in comparison to the offspring of those queens. When viruses were present in the tissues (including ovaries and spermatheca) of queens, those viruses were also present in her offspring. Furthermore, honey bee-associated viruses are horizontally transferred between bees by sharing contaminated food (Chen & Siede, 2007), faecal contact/consumption (Chen & Siede, 2007), physical contact (Chen & Siede, 2007), and mating (Chen et al., 2005., Yue et al., 2006., Chen & Siede, 2007).

Foodborne transmission is a common route of virus transmission between honey bees (Chen & Siede, 2007). Viruses are detected in the stored honey and pollen in the hive (Shen et al., 2005., Chen et al., 2006(b), Chen & Siede, 2007), and viral concentrations are highest in the gut of individual bees compared to other tissues (Chen et al., 2006; de Miranda et al., 2012). Moreover, food sharing, via trophallaxis, is fundamental to the coordination and regulation of colony behaviour and physiology, and if therefore continuously occurring within the hive (Crailsheim, 1998). This creates an environment where contaminated food is consumed and shared readily between bees with the additional exchange of any viral contamination that each bee brings to the interaction.

Under normal circumstances honey bees leave the hive to defecate. However, faeces can be found inside the hive when a colony outgrows the available space in its hive or the colony is symptomatic for non-viral pathogens, such as *Nosema apis*, that can cause dysentery (Bourgeois et al., 2010). Young, adult, worker bees carry out housekeeping tasks and brood care within the hive (Johnson, 2008., Mortensen et al., 2015) and are exposed to viruses when cleaning contaminated faeces within the hive (Chen & Siede, 2007). In addition to faecal contamination, crowded conditions increase physical contact between bees and creates more opportunity for viruses, such as KBV and CBPV, to be shared between bees through the cuticle or broken hairs (Chen & Siede, 2007).

Finally, viruses are sexually transmitted to queens during mating (Chen & Siede, 2007). Male (drone) honey bees carry viruses in their semen that are transferred to the queen during mating (Chen et al., 2005., Yue et al., 2006., Chen & Siede, 2007). Each drone only ever mates once and dies during copulation (Koeniger, 1986), whereas each queen mates with upwards of 50+ drones in the first weeks of her adult life and stores that sperm in her spermatheca (and potentially associated viruses) for the rest of her life (Withrow & Tarpy, 2018), thereby, creating the conditions for vertical transmission of those viruses present in the spermatheca to future offspring (Chen et al. 2006).

Honey bee-associated viruses are widespread and typically persist within honey bee colonies at low levels, in an inactive or inapparent state, where no overt symptoms are present (Anderson & Gibbs, 1988., Bailey et al., 1981., Chen et al., 2005, Tentcheva et al., 2004). Historically, when active infections did occur, only a small proportion of the colony would be affected, and the effects on colony health would

include symptoms like reduced honey production (Shimanuki, 1983). Very rarely, infections of CBPV, BQCV, or SBV can cause clinically significant infections that could lead to colony death (Shimanuki, 1983, McMenamin & Genersch, 2015). Based on this historical understanding of viral effects on colony health, it is generally accepted that viruses alone do not drive colony death. However, the interaction of viruses with other stressors, such as pesticides and parasite infestations, can lead to the death of a colony (de Miranda et al., 2012).

The introduction of *V. destructor* into *A. mellifera* colonies has profoundly changed the viral landscape and caused both the prevalence and virulence of key viral infections to increase in *A. mellifera* colonies worldwide (Martin et al., 2012., Mondet et al., 2014., Noël et al., 2020). Most honey bee viruses will not cause colony death on their own, their presence in the colony, in addition to other stressors such as parasitism and viral vectoring by *V. destructor* can contribute to colony loss (Roberts et al., 2017). For example, prior to the introduction of *V. destructor* into the honey bee-virus paradigm, DWV was not known as a pathological agent (de Miranda et al., 2012) as it was not effectively transmitted between bees, and only occurred in colonies at low levels without causing overt symptoms (de Miranda & Genersch, 2010., Highfield et al., 2009). Because *V. destructor* can harbour DWV at significantly higher titres than honey bees, bees parasitised by *V. destructor* are exposed to dangerously high viral loads (Bowen-Walker et al, 1999). Now visually apparent symptoms of DWV are commonly recognised as a sign of severe *V. destructor* infestation and a precursor of colony death (de Miranda & Genersch, 2010). Similarly, vectoring of ABPV, KBV, and IPV by *V.*

destructor has resulted in visual disease manifestation of these previously inconsequential viruses (de Miranda et al., 2010).

Vectoring of viruses by *V. destructor* has been explicitly confirmed for DWV and APV (Noël et al., 2020). The significant increase in DWV and KBV prevalence following the introduction of *V. destuctor* into Aotearoa-New Zealand has reflected this relationship, with DWV prevalence increasing from ~5% to almost 80%, and KBV prevalence increasing from 5 – 10% to almost 40%. Moreover, BQCV, SBV, KPV, and CBPV have been identified in both *A. mellifera* and *V. destructor* (Mondet et al., 2014) and indirect evidence of increasing prevalence of SBV following the introduction of *V. destructor* cumulatively suggest that *V. destructor* contributes to the dispersal and virulence of a diversity of honey bee-associated viruses (Levin et al., 2019, Herrero et al., 2019, Mondet et al., 2014). However, *V. destructor* is unlikely to vector all honey bee-associated viruses, as incidences of BQCV and CBPV appear to be independent of *V. destructor* infestations (Mondet et al., 2014., Tentcheva et al., 2004., de Miranda et al., 2012).

Viral infections further impact colony health by interacting with the effects of other stressors like pesticides, non-viral pathogens, nutritional availability, and climate extremes (van Engelsdop et al., 2009., Roberts et al., 2017). One example of this is the detection of DWV and *Nosema ceranae* in the remains of mass colony death events in the United States (Zheng et al., 2015). *Nosema ceranae*, is a microsporidian parasite of honey bees that also host-shifted from to *A. mellifera* from *A. ceranea* around 2006 (Higes et al., 2006). *Nosema ceranae* lives in the gut of honey bees and is usually transmitted between honey bees via spore contaminated food and faecal-oral

transmission while cleaning contaminated hive surfaces (Traver et al., 2011). *Nosema ceranae* does not present overt visual symptoms of infection (Fries, 2010). However, there is evidence of reduced honey production of infected colonies (Higes et al., 2006), and eventual colony death in extreme cases (Higes et al., 2008., Martín-Hernández et al., 2007).

Monitoring Honey Bee Health

The viral load of colonies is not often monitored by beekeepers. Monitoring viral infections of colonies can be expensive and time consuming, and viruses alone are rarely troublesome for the functioning of a colony (Roberts et al., 2017). Because viral infection is usually constrained by the presence of *V. destructor*, beekeepers' generally focus efforts on monitoring and controlling *V. destructor* populations within their colonies. However, monitoring of *V. destructor* is often a neglected practice due to the time and labour involved (Peck, 2021). In the absence of regular monitoring, chemical treatments are routinely applied to hives without knowledge of the severity of mite infestation, leading to overuse and mismanagement of miticide treatments and the development of miticide resistant in *V. destructor* populations (Jack & Ellis, 2021). Moreover, chemical miticide treatments are not without risk to the honey bees themselves. Amitraz and fluvalinate have been shown to negatively impact on the reproductivity and honey production of honey bees (Colin et al., 2020, Lim et al, 2021). Therefore, strategic, and effective use of miticides is both economical and beneficial to the health of the honey bee.

Monitoring is an important principle in integrated pest management (Bottrell, 1979, and one that is equally important in the management and control of *V. destructor*.

Monitoring *V. destructor* has applications such as managing productive honey bees, preventing colony death, identifying miticide resistant mites, identifying mite resistant or tolerant honey bees, and preventing waste and mismanagement of chemical treatments. Most importantly, regular monitoring allows the beekeeper to identify whether their hives have a *V. destructor* infestation, thus preventing the unnecessary use of miticides (Jack & Ellis, 2021). In this way, monitoring can be a means of identifying colonies that have mite-resistant adaptations. In the absence of monitoring these mite-resistant bees, which are valuable breeding stock, would go unnoticed (Peck, 2021). Monitoring also allows a beekeeper to time mite treatments before mite populations have exceeded the treatment threshold to best prevent colony death (Imdorf & Charrière, 2003). Finally, monitoring a hive before and after treatment is useful for recognising whether the treatment is working effectively (Peck, 2021). Rapid reinfestation or little change in mite infestation allows the beekeeper to implement a secondary treatment, thus preventing colony death.

While there are several methods used to monitor *V. destructor* infestation of colonies, bee sampling is widely regarded as the most useful and accurate mite monitoring method (Peck, 2021). The bee sampling method involves taking a sample of approximately 300 adult worker bees (~½ a cup) from the brood frames of a colony, dislodging the mites from the bees, and counting the dislodged mites. *Varroa destructor* can be removed from the bees using the ‘sugar shake’ method, or an ethanol wash. The ‘sugar shake’ method involves putting the 300 bees in a jar with a 2 mm grating at the top, pouring powdered sugar on top of the sampled bees, shaking the jar to coat the bees in sugar, and waiting for two minutes for the bees to groom themselves and

dislodge any attached mites (Dietemann, 2013). The mites are then shaken out of the grate at the top of the jar and counted, and the bees are released back into their colony. An alcohol wash works similarly, where bees are collected into a jar with ethanol, shaken for 20 seconds to dislodge the mites, and then thoroughly sieved with warm water or more ethanol to separate the bees from the mites (Dietemann, 2013). These methods provide the beekeeper with a ratio of mites to bees that can be used to estimate the condition of the entire colony.

As with any method of monitoring, the bee sampling method comes with limitations. The most obvious limitation is the time and labour involved with sampling individual colonies, especially in large commercial apiaries (Peck, 2021). In some cases, it is simply not feasible to monitor each colony in an apiary. Hence, often only a sample of colonies in an apiary may be taken, or *V. destructor* is not monitored at all. In this thesis, I propose an alternative approach to direct monitoring of *V. destructor* on an individual colony basis that utilises honey bee mating sites, known as drone congregation areas (DCAs), as a means of monitoring *V. destructor* at the population level.

Drone Congregation Areas

Drones first leave the hive at 6-9 days old to perform short orientation flights, and then perform longer mating flights to DCAs from 21 days old onward (Reyes et al., 2019). Drone congregation areas are 7- 30m above ground (Zmarlicki & Morse, 1963, Koeniger, 1986) where drone honey bees from multiple colonies/apiaries gather in large numbers for the purpose of mating with a queen honey bee (Ruttner & Ruttner, 1966). Tens of thousands of drones, from upwards of 200 different colonies, may gather at a

given DCA during the flight time (Reyes et al., 2019). DCAs occur at fixed locations, with some recorded to persist for over 15 years (Koeniger, 1986). Why DCAs persist in the locations that they do is not well understood, but Ruttner (1966) found that they tend to be found in clear, open areas towards depressions in the horizon. Drones tend to fly to the nearest DCA possible (Koeniger et al., 2005b), and the maximum drone flight range is estimated to be about 3.75 km (Utaipanon, 2019) with most drones flying 0.5 km or less to a DCA (Rowell et al., 1992).

Drones perform regular mating flights throughout their lifetime, which only lasts through one mating season (spring and summer). Drones are either excluded from the colony at the end of the mating season or die immediately after mating with a queen (Page Jr & Peng, 2001).

The presence of *V. destructor* at DCAs could have practical implications for the monitoring of *V. destructor* in apiaries. *Varroa destructor* monitoring is an important beekeeping practice, but one that is often neglected due to limitations of the current methods. The standard method for monitoring *V. destructor* in managed colonies is to take a sample of ~300 adult honey bees from individual colonies and count the number of *V. destructor* found in each sample (Peck, 2021). While this is feasible on a small scale, monitoring becomes a time consuming and tedious task when applied to commercial beekeeping operations with large quantities of colonies. Many commercial apiaries lack the resources to carry out mite monitoring on a large scale, and, therefore, the practice is often neglected. Because a DCAs attracts drones from multiple colonies, and drones tend to fly to the DCA nearest to their colony, DCAs present an opportunity to monitor *V. destructor* at the population level. DCAs have already been applied to

monitor honey bee health and genetics (Jaffe et al., 2009., Bertrand et al., 2015., Baudry et al., 1998). For example, DCAs were used to monitor and influence the level of Africanisation of Western honey bee populations by African honey bees (*Apis mellifera scutellata*) in the USA (Mortensen and Ellis, 2016., Loper & Fierro, 1991). Similarly, Galinda-Cardona et al (2020) used DCAs to monitor population level differences in *V. destructor* infestation across different eco-climatic regions of Argentina. The applicability of DCAs for monitoring *V. destructor* density for informing practical decision-making regarding management of nearby honey bee colonies is yet to be investigated.

Thesis Aims

In this thesis I aim to investigate the role that drone honey bees play in the dispersal of the parasite *V. destructor* and honey bee-associated viruses between colonies during mating flights to and from drone congregation areas. To do this, I investigated the relationship between the prevalence of *V. destructor* and associated viruses at DCAs versus in colonies in the nearest apiary. To make this comparison, I also investigated *V. destructor* preferences for different honey bee host types and the regional timing of drone flights in Aotearoa-New Zealand My findings expand our knowledge of the contribution of drones to colony health and fitness and explore the practical application of DCA monitoring as an opportunity to monitor regional *V. destructor* infestation risk.

In **chapter two** I investigate the host-parasite relationship between honey bee drones and *V. destructor.* I perform a choice test to determine *V. destructor* preference for drone honey bees over nurse and forager bees. Determining whether *V. destructor* parasitises adult drones during the dispersal phase in relation to foragers and s is an

important step for interpreting the relationship between *V. destructor* infestation rates at DCAs compared to honey bee colonies managed nearby.

In **chapter three** I note my observations on drone flight time in Waikato, Aotearoa-New Zealand. I frame this knowledge in the context of what is currently known about drone flight time across the world and discuss the significance of understanding drone flight time across different geographical areas.

In **chapter four** I compare *V. destructor* infestation rates and pathogen loads of drones captured at DCAs to *V. destructor* infestation rates and pathogen loads of colonies at nearby apiaries. Investigating these relationships help us to understand the applicability of DCAs for monitoring *V. destructor* infestation and pathogen burden at the population level, gives insight into the dispersal strategy of *V. destructor*, and highlights the previously unconsidered contributions of drone mating behaviour to the ecology of honey bee pests and diseases.

In **chapter five** I discuss my findings and how *V. destructor* host preferences, the presence of *V. destructor* at DCAs, and the prevalence of viruses at DCAs compared to apiaries contribute to our understanding of how honey bee parasites and pathogens spread, and the application of DCAs in honey bee research and monitoring going forward.

CHAPTER 2

Varroa destructor Host-Choice

Abstract

Varroa destructor has been known to select honey bee hosts based on differences in age and function within the colony (Kuenen & Calderone). While *V. destructor* is known to prefer drone brood over worker brood during the reproductive phase of its life cycle (Fuchs, 1990., Boot et al., 1995., Calderone, 2005), *V. destructor* preference for adult drones has not been investigated in detail. Because drone honey bees drift 2-3 times as much as workers (Free, 1958), I hypothesise that *V. destructor* will select drones as phoretic hosts. I found that *V. destructor* chose drone and forager hosts in equal proportion. Additionally, I found that *V. destructor* chose drones as hosts significantly more frequently when the drones available were mature drones, and not randomly selected drones from within the colony. This infers that drones play a role that is equally important to that of foragers in the spread of *V. destructor,* and that mature drones are preferentially chosen as hosts for the purpose of dispersal.

Introduction

Varroa destructor is reliant on honey bees, *Apis meliffera*, for dispersal both within and between colonies, but for both dispersal needs to be met, *V. destructor* depend on the different behaviours of their immediate honey bee host.

There are several castes of bees within a colony, each of which is associated with a distinct behavioural repertoire inside and/or outside the hive. Firstly, there are the castes underpinning the reproductive division of labour in the colony: queens

(reproductive females), drones (reproductive males), and workers (non-reproductive females, Seeley, 1985). Workers are further distinguished into 'temporal castes' (Wilson, 1968) that each worker progress through a stereotyped pattern with age. This progression through temporal castes is referred to as age (or temporal) polytheism; and describes the tendency of eusocial organisms to change their role within the social structure of their colony as they age (Seeley 1982). For honey bees there is typically a progression from tasks within the hive to higher risk tasks at the hive entrance and then finally tasks outside the colony (Hölldobler & Wilson, 1990). Four temporal castes are defined in honey bee workers; cell cleaners (1-3 days old), nurses (4-12 days old), house bees (13-20 days old), and foragers (>21 days old) (Seeley, 1982., Johnson, 2008).

Because of the different behaviours and tasks carried out by each reproductive caste and temporal caste of honey bee, it follows that certain castes would be more favourable hosts for *V. destructor* over others (Del Piccolo et al., 2010., Pernal et al., 2005). It is widely accepted that the preferred host type of *V. destructor* is the nurse honey bee (Kraus, 1993., Kraus, 1994., Xie, 2016) because nurses spend most of their time tending brood cells where *V. destructor* reproduces, and do not leave the safety of the hive. However, the success of *V. destructor* is also reliant on the ability of the parasite to disperse to new colonies, therefore forager hosts are hypothesised to be a preferred host for *V. destructor* during their dispersal phase (Kuenen & Calderone, 1997).

Olfaction and taste are imperative to the host finding behaviour of *V. destructor* (Pernal et al., 2005). Functional pit organs on the front legs of *V. destructor* are used to

detect critical sensory cues that shape their behaviour responses to their environment (Nganso et al., 2020, Dillier et al., 2006). Receptor cells in the pit organ showed an increase activity in the presence of honey bee volatiles (Dillier et al., 2002, as cited in Dillier et al., 2006). Moreover, Nganso et al., (2020) found that when the front legs of *V. destructor* were covered with nail varnish, significantly less mites were able to locate a honey bee host compared to mites without nail varnish, or mites with a varnished idiosoma. It follows that different castes of honey bee must have differing chemical profiles that allow them to be distinguished by *V. destructor* (Plettner et al., 1997., Iovinella et al., 2018).

The reproductive and temporal castes of honey bees each have specific olfactory profiles that are available to *V. destructor* to inform host-choice behaviour. Cervo et al (2014) demonstrated differential host preferences of *V. destructor* under low and high infestation scenarios. At low infestation rates the olfactory profiles of nurses and foragers were distinguishable and *V. destructor* preferred nurses as hosts over foragers from colonies. Additionally, Del Piccolo et al (2010) found that foragers have higher concentrations of a compound (Z)-8-heptadecene in their cuticular hydrocarbons which appears to repel *V. destructor*. In contrast, at high infestation rates the distinction in the olfactory profiles of nurses and workers was lost and *V. destructor* no longer selected nurse hosts over foragers (Cervo et al., 2014). This inability to distinguish between nurses and foragers at high mite infestation rates may underpin the dispersal of *V. destructor* outside the hive on foragers. These studies emphasise the reliance of *V. destructor* on subtle chemical differences to distinguish between types of honey bee, and the potential importance of *V. destructor* choice in dispersal.

To date, choice tests of adult bee host preferences in *V. destructor* have only compared the temporal castes of workers, overlooking to potential for adult drones to act as viable hosts. It is known that drone brood is the preferred host for reproductive *V. destructor* (Fuchs, 1990., Boot et al., 1995., Calderone, 2005). Boot et al (1995) found that *V. destructor* invaded drone brood 11.6 times more frequently than they invaded worker brood, and Calderone (2005) found that removing frames of drone brood from a colony significantly reduced the mite-bee ratio of the colony over the course of the mating season. Despite the research conducted on *V. destructor* preference for drone brood, *V. destructor* preference for mature drone honey bees in relation to nurses and foragers during the dispersal phase is unknown.

As preference for nurse bees is advantageous for reproductive mites to remain close to the developing brood (Xie et al., 2016), a preference for drones over foragers may be advantageous for dispersal to new colonies due to increased drifting behaviour in drones compared to foragers (Free, 1958). Drones tend to drift 2 – 3 times as much as foragers (Free, 1958). *Varroa destructor* has been found on drones at honey bee mating sites, drone congregation areas (DCAs), confirming that adult drones are a viable host for *V. destructor* (Mortensen et al., 2018). The presence of *V. destructor* on drones at DCAs challenges the current thinking around *V. destructor* dispersal and highlights the question of whether *V. destructor* displays preferences for drones over other host types. Understanding *V. destructor* preference for different honey bee host types, first steps to better understanding the role of drone honey bees in *V. destructor* dispersal, and for exploring the potential DCAs hold for monitoring *V. destructor*. In this

chapter, I aim to explore *V. destructor* preference for drone honey bees compared to other castes by carrying out a choice test between nurses, drones, and foragers.

Methods

Choice tests were carried out over two weeks in December 2021 and early January 2022 in Kirikiriroa-Hamilton, Waikato, Aotearoa-New Zealand and all *V. destructor* and honey bees were sourced from colonies in the research apiary on site. Preliminary sampling was carried out to identify *V. destructor* infestation rates of the source colonies.

Varroa destructor collection

Varroa destructor were collected from a single colony with a high infestation rate (>20 mites per 300 bees) via the 'sugar shake' method (Nganso et al, 2020). A tablespoon of icing sugar was added to a jar of 300 bees with a course screen lid. After several minutes, the jar was shaken upside down and the loose *V. destructor* were collected into a tray. Sugar was cleaned from the mites with a damp paper towel. *Varroa destructor* were stored in a glass petri dish at room temperature for no more than three hours prior to the start of the tests (Singh et al, 2020). Unused *V. destructor* were euthanised via freezing (-20°C) and disposed of. New *V. destructor* were collected for each day of testing.

Honey bee collection

All honey bees used in the choice tests were collected from colonies with low mite density (<5 mites per 300 bees). Mature drones were collected from hive entrance

whilst leaving or returning from mating flights during drone flight time (2 pm - 6 pm), and frozen overnight (-20°C) for use the next day. Drone honey bees of mixed ages were taken from frames inside a hive several hours before use in choice tests. Workers observed poking their heads into brood cells were collected as nurse bees (Singh et al, 2015), and forager bees were collected from the hive entrance (Singh et al, 2015). All bees were freeze killed at-20°C for 2 hours, thawed for 30 minutes, and then used for the bioassay (Nganso, et al 2020). Mixed age drones, nurses, and foragers were collected fresh each day of testing.

Choice test bioassay

Bioassays were conducted in 9 cm diameter, 2 cm height glass petri dishes (Singh et al, 2015). A damp piece of filter paper was placed at the bottom of each petri dish to maintain humidity (Nganso et al, 2020). The petri dishes were kept in darkness and incubated at ~35 degrees Celsius throughout the experiment to best replicate the conditions inside a beehive (Le Doux et al, 2000).

A freeze-killed nurse, drone, and forager were evenly spaced apart around the edge of the filter paper in each petri dish (Figure 2-1). A single *V. destructor* mite was taken from the petri dish of collected mites and placed in the centre of the filter paper using a paintbrush. The petri dishes were put in the incubator and a *V. destructor*'s position was checked every hour for three hours. If a mite was on or underneath a bee, a choice was recorded. If the mite crawled off or under the filter paper, or was lying on its back, it was relocated in the centre of the filter paper. If the mite did not decide in the allocated three hours, it was recorded as "no choice". A total of 149 *V. destructor* were individually tests in the bioassay.

Figure 2-1. Picture of the experimental set up of choice test bioassays. A nurse (top left), a drone (top right), and a forager (bottom) are evenly spaced apart in a petri dish, with a *V. destructor* placed in the centre and able to move freely.

Statistical analysis

All data analysis was conducted in R version 4.2.2 and R Studio (R Core Team, 2022). All *V. destructor* that did not make a choice were omitted from analysis (57 no-choice mites omitted from the 149 total tested). To determine whether *V. destructor* preferentially chose drones over nurses and foragers, the choice test data was analysed using an analysis of variance (ANOVA). The frequency of choices for each honey bee host type was compared with the choices made at each time interval (1, 2, or 3 hours), and with the choices made in tests using mature drones compared to mixed drones.

Results

A total of 149 *V. destructor* were used in the choice tests. Of these, 92 made a choice during the 3 hr period, 57 made no choice, and 13 died. An ANOVA showed that *V destructor* did not show a significant preference for one type of honey bee over another (F = 2.913, DF = 2, p = 0.09303, Figure 2-2).

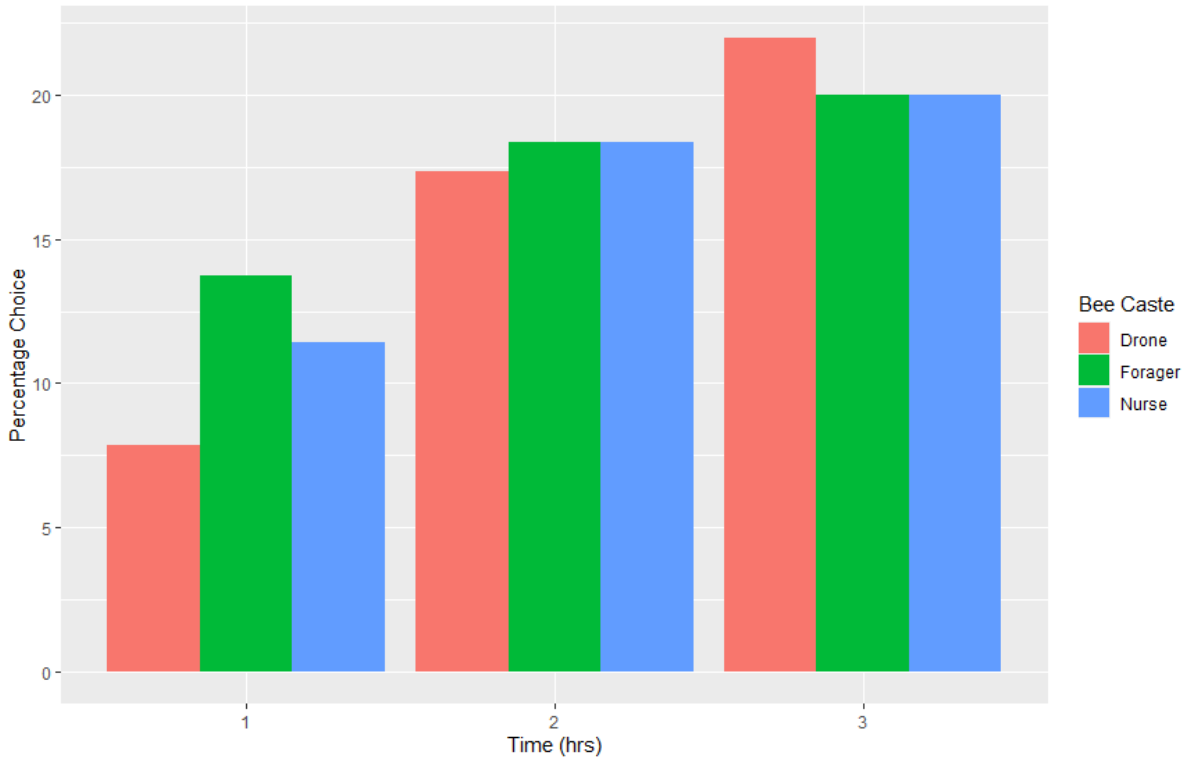

Figure 2-2. Host choice selection of *Varroa destructor*. Data are the percent of *Varroa destructor* that selected a host that chose drone, nurse, or forager honey bees at 1, 2 and 3 hr observations (*Varroa destructor* that made a choice: n = 92, no choice: n = 57).

Though there was not a significant difference in *V. destructor*'s choice of different honey bee host types, mature drones were chosen as hosts by *V. destructor* significantly more than mixed drones (DF = 1, F = 9.895, p = 0.008; Figure 2-3).

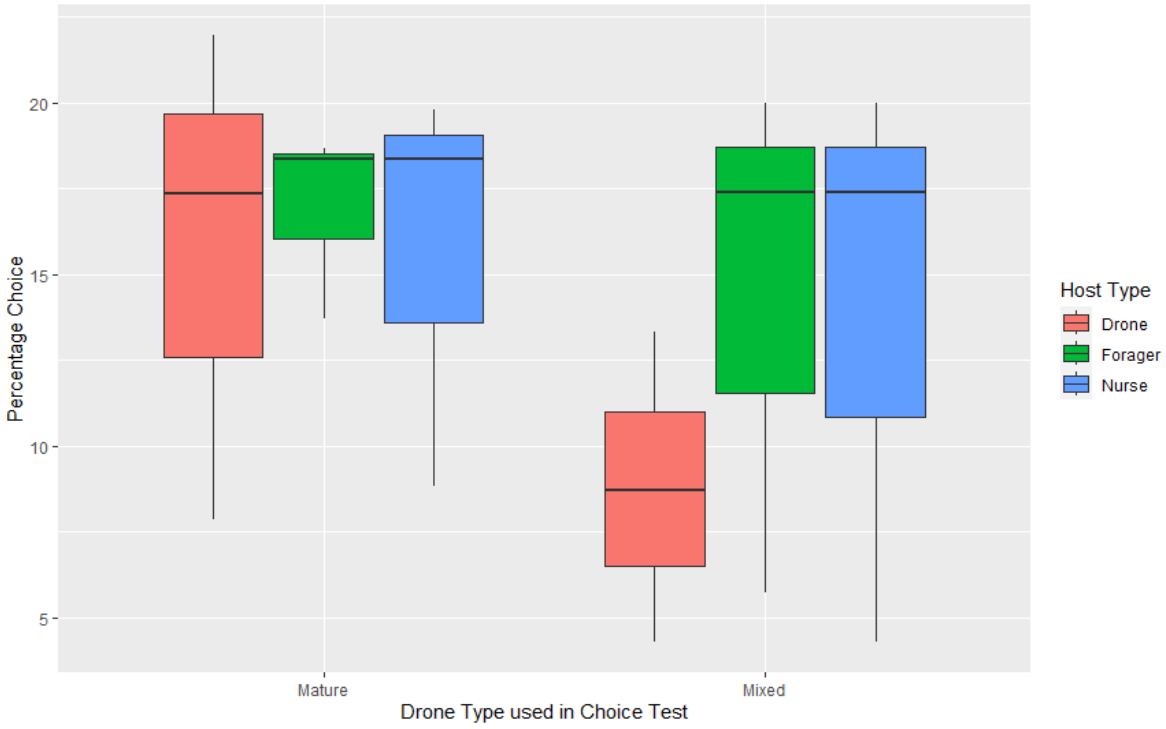

Figure 2-3. Host-choice decisions made by *Varroa destructor* with mature (n = 66) or mixed age (n = 26) drones. Data are the percent of *V. destructor* that selected a host that chose drone (mature or mixed age), nurse, or forager honey bees at each time interval. The box represents the first quartile, median, and third quartile, and the whiskers represent the minimum and maximum.

Discussion

In this study, I found evidence that drones likely play an important role in *V. destructor* dispersal between different colonies via mating flights to and from DCAs. This has implications for the transfer of *V. destructor* between colonies within an apiary, and the monitoring of *V. destructor* within those apiaries. The importance of drones in *V. destructor* dispersal was first evidenced by the choice tests. *Varroa destructor* taken from highly infested colonies did not exhibit a preference for workers over drones, nor between nursing-age or foraging-age workers. The lack of preference for drones over foragers suggests that drones play a role in *V. destructor* dispersal that is at least equally important to that of foragers. The percentage of times a drone was chosen by *V.*

destructor was significantly higher when the drone present was sexually mature (collected from the hive entrance during mating flights) rather than of mixed age (collected from within the hive). This supports my hypothesis that drones are selected by *V. destructor* as a means of transport outside the hive for *V. destructor.*

Drones, foragers, and nurses each play an important role during the dispersal life phases of *V. destructor*, but the role played by each host type differs slightly. It is widely known that *V. destructor* tend to choose nurse honey bees as their preferred host (Kraus, 1993., Del Piccolo et al., 2010., Xianbing, 2016), and this is thought to be because *V. destructor* reproduce in brood cells constantly tended by nurse bees. Therefore, parasitising nurse bees provides increased reproductive opportunities, safety within the hive, and increased overall fitness and fecundity to *V destructor* (Xianbing, 2016). However, the role of foragers and drones as hosts is equally important. Foragers and drones transport *V. destructor* to new, un-infested hives; a role that cannot be carried out by the nurse host type. While nurse honey bees are often regarded as the preferred host of *V. destructor*, it is important to note that the success of *V. destructor* relies heavily on their ability to select foragers or drones as hosts as a means of dispersal, especially when their colony is highly infested and nearing collapse.

There was no difference in *V. destructor* preference between drone honey bees and forager honey bees. The selection of drone honey bees in equal proportion to foragers and nurses provides evidence that drone honey bees play a role in honey bee dispersal, if not a more important one than foragers. The increased drifting of drones compared to foragers likely increases the success of dispersal for *V. destructor* that choose to parasitise them. Knowing that drones do play a role in *V. destructor* dispersal,

we can infer that DCAs could be a useful means of exploring the infestation and spread of *V. destructor*.

While my results show that *V. destructor* selected drones as often as foragers and nurses, the mechanisms influencing choice are complex, and there are additional variables at play. Though *V. destructor* is reliant on olfaction to differentiate between types of honey bee, the Cervo et al (2014) has shown that presence of *V. destructor* in a hive can itself have an influence on the chemical profiles of honey bees, with foragers and nurses becoming indistinguishable at high mite densities. In my choice tests, the honey bees collected were from colonies with relatively low mite densities. Repeating the same experiment using honey bees from a colony with high mite density could help determine if *V. destructor* are choosing differently based on the infestation rate of their hive, or whether the main mechanism for choice is the indistinguishable chemical profiles lack of different host types at high mite densities as suggested by Cervo et al (2014).

Olfaction is likely not the only stimulus that *V. destructor* uses to distinguish between honey bee host types. Other stimuli such as temperature and vibration have been shown to influence *V. destructor* behaviour (Le conte & Arnold, 2021). Differences in the vibrations made by workers and drones could also be a means of differentiating castes used by *V. destructor*. By using dead, previously frozen bees, my choice test was limited to olfactory and tactile cues but using live bees in a choice test could be a useful means of determining whether *V. destructor* utilise other stimuli to differentiate between host types.

Age differences in both worker honey bees, and in *V. destructor* have been shown to influence *V. destructor* preferences for adult honey bee hosts, and our results suggest that the age of drone honey bees may also affect *V. destructor* preference. *Varroa destructor* selected drones significantly more when presented with only mature drones in the choice test, rather than a randomly selected drone from a group of drones of mixed ages. I hypothesise that this is because mature drones leave the hive to go on mating flights regularly, presenting the opportunity for *V. destructor* to disperse. Drones remain within the hive until they are 6 - 9 days old, at which point they leave the hive to go on brief orientation flights (Reyes et al., 2019). Drones begin to perform 30-minute mating flights at 21 days old in spring and 13 days old in summer (Reyes et al., 2019). The mixed drones used in our experiment were collected from within the hive, meaning that they could have been either mature or newly emerged. Xianbing et al (2016) found that *V. destructor* significantly preferred to parasitise nurse or forager workers compared to newly emerged workers, and that mites that parasitised newly emerged workers during their dispersal phase had reduced fecundity compared to *V. destructor* that parasitised older bees. Though drones were not included in their dataset, my results suggest that a similar trend exists with drone honeybees. From this we draw that the age of drones is an important variable in *V. destructor* host choice.

Additionally, *V. destructor* of different ages may exhibit different preferences for honey bees. Older *V. destructor* mites with a lower reproductive value are more likely to partake in risk taking behaviours such as parasitising drones and foragers, which will likely remove them from the safety of being within the colony (Nolan & Delaplane, 2017). The preferences of *V. destructor* mites of different ages for different honey bee

hosts have been relatively understudied. *Varroa destructor* of mixed ages were used in my choice test but comparing the preferences of *V. destructor* of different ages for drones would also contribute to our knowledge of *V. destructor* dispersal via drones.

The preference *V. destructor* displayed for mature drones over mixed drones suggests that *V. destructor* choice for drones was not purely random, but that drones do play an important role in the dispersal of *V. destructor* between colonies. Given this information, further research on the relationship between drone honey bees, mating flights, and *V. destructor* dispersal could be useful for monitoring the transmission of *V. destructor* and associated pathogens between colonies.

CHAPTER 3

Identifying the Honey Bee Mating Flight Time in the Waikato Region of Aotearoa

Abstract

During the mating season, drones honey bees (*Apis mellifera*) make daily flights to drone congregation areas (DCAs) for the purpose of mating with a virgin queen honey bee. While drone flight time has been known to vary temporally between countries and regions, the spatial scale over which this variation occurs and the mechanisms contributing to this variation are poorly understood. The drone flight time in Waikato, Aotearoa-New Zealand was determined by observing the number of drones leaving the hive entrance per minute at 15 different colonies in an apiary in Kirikiriroa-Hamilton, Waikato. Drone flight time was found to be between 14:00 and 17:00, with peak flight time occurring around 16:00. This research contributes to our knowledge of drone flight time in Aotearoa-New Zealand and contributes to our larger scale understanding of how drone flight time varies in different parts of the world.

Introduction

The timing of mating is an essential component to the success of reproduction in many species. Favourable environmental conditions, receptivity and abundance of mates, and the presence of pollinators/hosts are all variables that contribute to the importance of timing in reproduction (Crews & Moore, 1986., Hubbel & Johnson, 1987., Frankel & Galen, 2012). All three of these variables relate to the reproduction of the European honey bee (*Apis mellifera*) for which timing is a crucial element to reproductive success.

Honey bees mate mid-flight at drone congregation areas (DCAs). Drone congregation areas (DCAs) are areas 15-30m in the air where drones from multiple colonies gather in large numbers for the purpose of mating with a queen (Zmarlicki & Morse, 1963., Ruttner, 1966). Though drones (male honey bees) go on many mating flights throughout their life, 60% of honey bee queens will make two or less mating flights, with 30% only making one mating flight (Woyke, 1964). Each mating flight poses a risk of mortality to the queen, thus jeopardising her whole colony (Koeniger & Koeniger, 2007). Up to 20% of queens do not return to their colony from mating flights, leaving their colony queenless with no means of raising a replacement queen (Ruttner, 1980., as cited by Koeniger & Koeniger, 2007). Therefore, coordination of the timing of mating flights is essential to ensure optimal mating success, thereby reducing the number of flights a queen must undertake.

Honey bee mating flights are seasonal, occurring in the spring, summer, and early autumn (depending on the local climate) when colonies are producing drones (Koeniger et al, 2014). Mating flights take place during the day when the weather is mild and clear. Generally, drones carry out mating flights for a longer time than queens, beginning mating flights earlier and continuing to fly later than queens each day (Koeniger & Koeniger, 2004). During the drone flight time, drones fly to and from nearby drone congregation areas and their hive to eat and rest. As such, notable drone activity can be observed at hive entrances during the flight time.

Many aspects of the honey bee mating system, including DCA formation, DCA identification by drones and queens, and regional mating flight times, are not yet well understood. Drone flight time is thought to be determined by environmental conditions

such as temperature, humidity, and light intensity (Rowell et al, 1986., Howell & Ursinger 1933., Bertholf 1931., Luckiesh, 1922). When the temperature is higher, drone flight time tends to occur later in the day (Rowell et al, 1986). Howell and Ursinger (1933) found that peak drone flight time occurred when relative humidity was at its lowest during the day. They also hypothesised that ultraviolet light may affect the flight time of drones. The stimulative efficiency of ultraviolet light on honeybees increases rapidly from 297 μμ to 365 μμ (Bertholf 1931). Luckiesh (1922) found that ultraviolet wavelength increased throughout the day, from 295.5 μμ at 10:30 to 307 μμ at 16:38, potentially providing a cue for drones to fly.

Specific drone flight time has been found to vary between regions, presumably due to geographical variation in weather and climate. Ruttner (1966) originally cited drone flight time as occurring from 12:15 until 17:00 from DCA research conducted in Germany, but since then, other publications have reported a variety of different drone flight times. For example, the drone flight time in France was reported as 14:00 until 18:00 (Reyes et al, 2019), in Japan it was reported as 11:30 until 15:00 (Yoshida et al, 1993), in Kansas, USA it has been reported as 15:00 and 17:30 (Rowell et al, 1986), and in Malawi it has been reported as 11:20 until 16:00 (Lahner, 1998., as cited in Koeniger & Koeniger, 2000).

Determining the drone flight time is essential to carrying out research involving DCAs. DCAs are applicable for the study of drones and honeybee mating, as well for studying genetics (Jaffe et al, 2009), relatedness of local colonies (Baudry et al, 1988), and population density of honeybees (Beaurepaire et al, 2014., Utaipanon et al, 2021). Drone flight times in Aotearoa-New Zealand are yet to be documented. The aim of this

study was to determine the drone flight time in the Waikato region of Aotearoa-New Zealand.

Methods

Drone flight behaviour was monitored in December 2020 (late spring/early summer in the southern hemisphere) at a single apiary in Kirikiriroa-Hamilton, Waikato, Aotearoa-New Zealand (-37.774898 S, 175.313943 E). All honey bee colonies were of comparable health and strength status: European-derived (*Apis mellifera* spp.), queen-right, free of visible signs of disease, and had completed a standard Bayvarol® (flumethrin) miticide treatment in November 2020.

Hive entrances of 15 colonies were observed for one minute every hour for four consecutive days. During the first two days, hives were observed from 10:00 until 16:00. Because the drone counts made on these days were still high at 16:00, observations were made from 11:00 until 18:00 for the next two days. Observations were made on fine days when weather conditions were suitable for mating flights (low wind, moderate to little cloud cover). During each one-minute observation, drone honey bees leaving the hive were counted (drone activity).

Drone activity in relation to time was averaged across hives and days and plotted to identify the regional drone flight time.

Results

A total of 708 drones were observed leaving their colonies over the four observation days (max 222 per day, min 109 per day). An ANOVA showed that there was not a significant difference in drone count between the four days (DF = 1, F =

4.273, p = 0.0393). Drone activity occurred between 14:00 and 17:00, with peak flight time occurring at 16:00. These flight times were used for drone collection around the Waikato region through spring and summer of 2020, 2021, and 2022 and remained accurate through all three seasons.

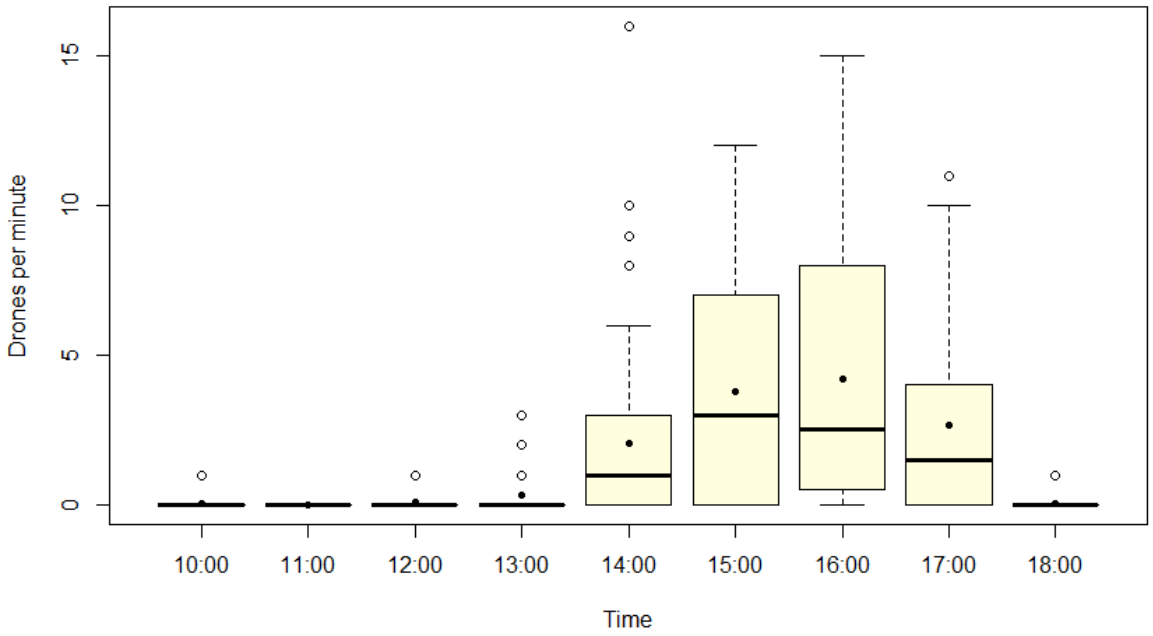

Figure 3-1. Drone honey bee activity by time of day. Data are the average count of drones exiting the hive in minute. Boxes indicate the median (interior line) and upper and lower quartiles, black dots represent the mean, bars represent the minimum and maximum (excluding outliers), and open circles represent outliers (included in analyses).

Discussion

Drone flight time in the Waikato region of Aotearoa-New Zealand was found to be from approximately 14:00 until 17:00. Not only does this result aid us in completing further research on DCAs in the Waikato region, but it also contributes to our

understanding of the global pattern of drone flight time, and how it can vary between countries and regions.

The characteristics of DCAs and mating flights remain remarkably similar across time, with some DCAs recorded to occur in the same place for over 50 years (Jean-Prost, 1957., Tribe, 1982., as cited by Koeniger et al., 2005b). However, mating flights have been shown to vary across space. The extent to which drone flight time is variable across different regions and climates is not yet well understood.

As with many fields, the literature on drone congregation areas has been heavily dominated with texts from Europe. The overrepresentation of the Northern hemisphere in scientific literature has led to generalisations being extended to the Southern hemisphere, where they do not necessarily apply. For example, Catchpole and Slater (1995) published a book stating that birdsong was performed largely by male birds, which was true of the temperate North climate, but incorrect when applied to the tropics (Terborgh, 1996., as cited by Slater & Mann, 2004). Similarly, most of the pioneering research on drone congregation areas was carried out in Germany and Austria by Zmarlicki and Morse (1963), Koeniger and Koeniger (2004), and Ruttner (1966). In the last decade, publications involving DCA publications from Southern hemisphere countries like South Africa (Jaffe et al, 2019), Argentina (Ayup et al., 2021), and Australia (Ataipanon et al., 2019) have made a significant contribution to the literature, providing insight from the Southern hemisphere into the global pattern of variation in drone flight time. Our study of drone flight time from Waikato, Aotearoa-New Zealand, will contribute to this pool of knowledge, allowing for more thorough research on the patterns of drone flight time across both hemispheres.

In addition to environmental factors such as weather and climate, biotic factors such as the presence of other honey bee species (*Apis* sp.) may have an impact on the timing of mating flights. Koeniger and Wijayagunasekera (1976) found that the drones and queens of three species of honey bee found in Sri Lanka (*Apis dorsata, Apis cerana,* and *Apis florea*) all have distinct mating flight times that do not overlap. This is hypothesized to be because the sex pheromone, 9-oxodec-trans-2-enoic acid, is common among all three of these honey bee species, and *Apis mellifera* as well (Shearer et al., 1970). The separation of mating flights ensures mating success for each species, with minimal reproductive effort wasted on pursuing a queen of the wrong species. Species of honeybee that are not normally sympatric may not have evolved this separation of flight time. For example, when Ruttner et al. (1972) took *Apis cerana* from Pakistan and tried to naturally mate them in Germany, they found that the *A. cerana* drones visited the same DCAs and *A. mellifera drones* (Ruttner 1972., as cited by Koeniger and Wijayagunasekera, 1976), and were unable to naturally mate unless their colonies were isolated from *A. mellifera colonies*. It is likely that populations of *A. mellifera* living sympatrically with other species of honey bee have more specific or alternative flight times than *A. mellifera* populations in places where other honey bee species are not present, such as Aotearoa-New Zealand (Howlett & Donovan, 2010), providing another plausible hypothesis for why variability exists between mating flights.

Our current understanding of how environmental factors, location, and interspecific competition affect the time of drone mating flights is limited. As more geographically diverse work is done on DCAs, the variability and factors involved in determining drone flight time will become clearer. Though drone mating flights are often

reported to occur during mid-afternoon from approximately 14:00 until 17:00 (Drescher, 1969., as cited by Koeniger et al, 2005a., Witherell, 1971., Reyes et al., 2019., Galinda-Cardona et al., 2012), the findings of others such as Yoshida (1993) and Rowell et al (1996) show that spatial variation in drone flight time does occur, and that the drone flight time of a location cannot be assumed. Therefore, determining the drone flight time in an area where DCAs have not previously been studied, such as my study area in Waikato, Aotearoa-New Zealand, is an essential preliminary step for carrying out research on drone congregation areas within this region. Determining the local flight time also remains broadly relevant for contributing to our understanding of how drone flight time varies across regions and ecosystems.

CHAPTER 4

Honey Bee Mating Sites Facilitate Admixture of More Than Genetics

Abstract

In this chapter, I aim to explore the potential of DCAs for monitoring honey bee health at the population level and expand on our current knowledge of the role drone honey bees play in the dispersal of honey bee parasites and pathogens. I sampled colonies from 16 apiaries around the Waikato region and drones from the nearest respective DCA. I counted the number of *V. destructor* found in each sample, and I also found the number of viral copies for the microsporidian parasite *Nosema ceranae*, and four common honey bee viruses: BQCV, CBPV, DWV, and the AKI complex. I found that there was a significant positive relationship between the *V. destructor* count on drones from DCAs and the *V. destructor* count on colony bees from the nearest apiary. There was no significant relationship between the number of viral copies of *N. ceranae*, AKI, BQCV, and CBPV in drones versus in colony bees, however, there was a significant positive relationship between the number of viral copies of DWV found in drones versus colony bees. The *V. destructor* count could also be used to predict the degree of DWV infection in both drones and colony bees. My results suggest that population level monitoring of *V. destructor* infestation using DCAs is feasible and could be developed to be used as an alternative monitoring strategy for commercial apiaries.

Introduction

Varroa destructor is an economically damaging parasite of European honey bees (*Apis mellifera* sspp.) that has become prevalent worldwide, with only a few isolated

islands left uncolonized (Boncristiani, 2020., Peck, 2021). *Varroa destructor* causes direct bodily damage to honey bees, reducing the body mass of emerging workers and drones that were parasitised during development by 7% and 11-19%, respectively (Rosenkranz et al., 2010). Moreover, parasitised foragers demonstrate decreased learning and navigational abilities (Rosenkranz et al., 2010). In addition to the direct feeding damage, *V. destructor* vectors several honey bee-associated viruses and dramatically changes the abundance and virulence of viruses in honey bees.

Varroa destructor is recognised as a leading cause of death for honey bee colonies globally (Peck, 2021), and host-colony death would be equally fatal to *V. destructor* without effective means of dispersal. As such, *V. destructor* has developed robust inter-colonial dispersal strategies that are demonstrated by its rapid international spread (Boncristiani et al., 2020., Peck, 2021., Traynor et al., 2020). Likely mechanisms by which *V. destructor* disperse to infest new colonies are colony swarming, foraging honey bees from multiple colonies visiting the same floral resources, human transport due to beekeeping practices such as moving frames of brood (combs of honey bee eggs, larvae, and pupae) between hives, foragers robbing resources of other hives, and adult bees drifting between hives.

Exploitation of honey bee robbing and drifting behaviours are currently considered key mechanisms for inter-colonial dispersal of *V. destructor* (Degrandi-Hoffman et al, 2017). Robbing is the process by which foraging honey bees enter a foreign hive to steal resources (typically nectar and/or honey) to take back to their own hive (Peck & Seeley, 2019). Drift occurs when an adult honey bee returns to a hive that is not its own (Peck & Seeley, 2019). *Varroa destructor* attached to adult honey bees

leaving the hive have the potential to be introduced to a new colony if their host bee engages in robbing or drifting. Both behaviours occur commonly and increase in frequency in managed apiaries where hives are kept near one another (Jay, 1966., Seeley & Smith, 2015).

Both worker and drones (males) have the propensity to drift when they return to the colony. Workers typically drift during orientation flights or foraging, and drones typically drift during orientation flights (6-9 days post emergence) or mating flights (21 days post emergence onward, (Reyes et al., 2019). While workers represent higher proportion of the total individuals in a colony (Seeley & Morse, 1976), drones express a higher frequency of drift than workers (Free 1958).

Adult drones routinely fly to drone congregation areas (DCAs) for the purpose of mating with a queen (Zmarlicki & Morse, 1963, Reyes et al., 2019). Drones tend to frequent the nearest DCA possible (Koeniger et al., 2005b), and make repeated trips between the hive and the DCA each day with each trip lasting approximately 30 minutes (Reyes et al., 2019). During mating flight times, tens of thousands of drones, representing all nearby managed and feral/wild colonies, are present at any given DCA (Reyes et al., 2019). *Varroa destructor* have been discovered on drones at DCAs (Mortensen et al, 2018), and Peck and Seeley (2019) noted that drifting drones represented an average of 21% of the drones entering monitored hives.

The presence of *V. destructor* at DCAs could have offer practical opportunities for monitoring regional *V. destructor* population levels. Sampling drones at DCAs has been utilised by research scientists to explore honey bee population genetics (Jaffe et al., 2009., Bertrand et al., 2014., Baudry et al., 1998; Mortensen and Ellis, 2016., Loper

& Fierro, 1991), and more recently, Galindo-Cardona et al (2020) compared *V. destructor* abundances at DCAs in different eco-climatic regions of Argentina. However, profiling the relationship between the health status drones at DCAs with that of nearby colonies has not yet been attempted.

Monitoring is foundational to managing colony health but is often neglected due to constraints on time, accessibility, and cost (Peck, 2021). The standard methods for monitoring *V. destructor* in honey bee colonies require someone to quantify the mites present in a sample of about 300 adult workers from each colony (Peck, 2021, Dietemann et al., 2013). While this is feasible on a small scale, monitoring becomes a time consuming and tedious task when applied to commercial beekeeping operations with large numbers of colonies across numerous apiaries.

I aim to expand our understanding of drones in the context of colony health and pest and disease dispersal and determine if DCA sampling presents a practical opportunity for predicting regional honey bee health parameters. Our understanding of drone biology and behaviour is only recently expanding beyond reproduction (Rangel & Fisher, 2019). In addition to *V. destructor*, drones are likely to transport pathogens, infecting the drone and/or being vectored by *V. destructor*, to DCAs and on to another colony if they drift. Drones have not yet been considered as modes of pathogen transmission, however if drones support inter-colonial dispersal of *V. destructor*, and DCA sampling offers a viable monitoring strategy, then the same may be true for inter-colonial dispersal and monitoring of pathogens. Test these assumptions and hypotheses I compared the *V. destructor* infestation rates and pathogen profiles (black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus

(DWV), the AKI complex (acute paralysis virus (APV), Kashmir bee virus (KBV), and Israeli paralysis virus (IPV)), and *Nosema ceranae*) of drones collected at DCAs and workers collected from colonies at nearby apiaries.

Methods

From January to March of 2021, and February and March of 2022, I sampled bees from a total of sixteen DCA – apiary pairs in the Waikato Region of Aotearoa-New Zealand (Figure 4-1). Each DCA was located within 1 km of the apiary pair (Figure 4-2). DCAs were sampled from 2 pm until 5 pm, as per the local drone flight time (see Chapter 3). Sites B, M, and N were sampled twice, once in 2021 and again in 2022.

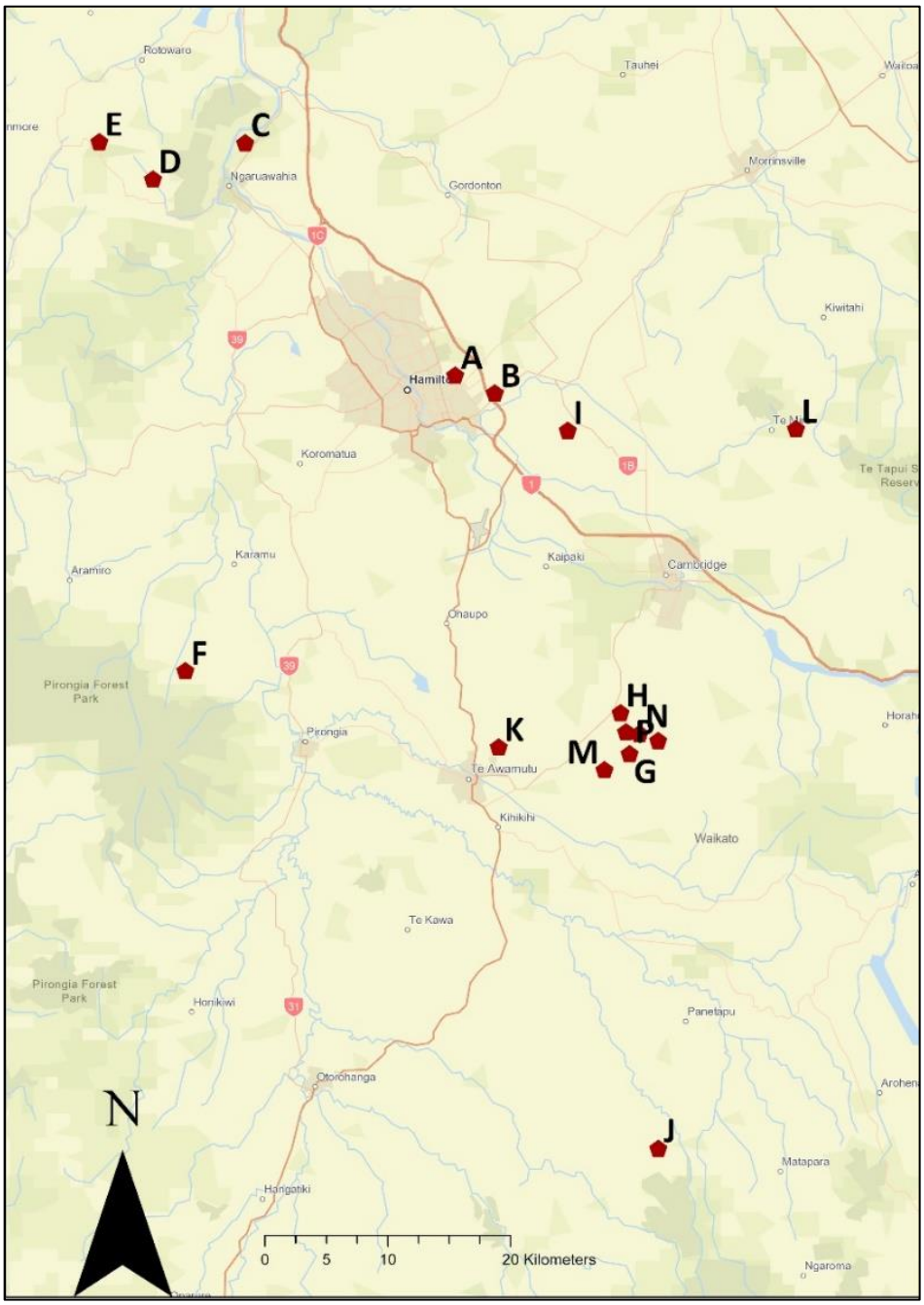

Figure 4-1. Map of the distribution of the paired study sites in the Waikato Region of Aotearoa-New Zealand. Each site consists of a drone congregation area (DCA) and an apiary of at least 20 honey bee colonies.

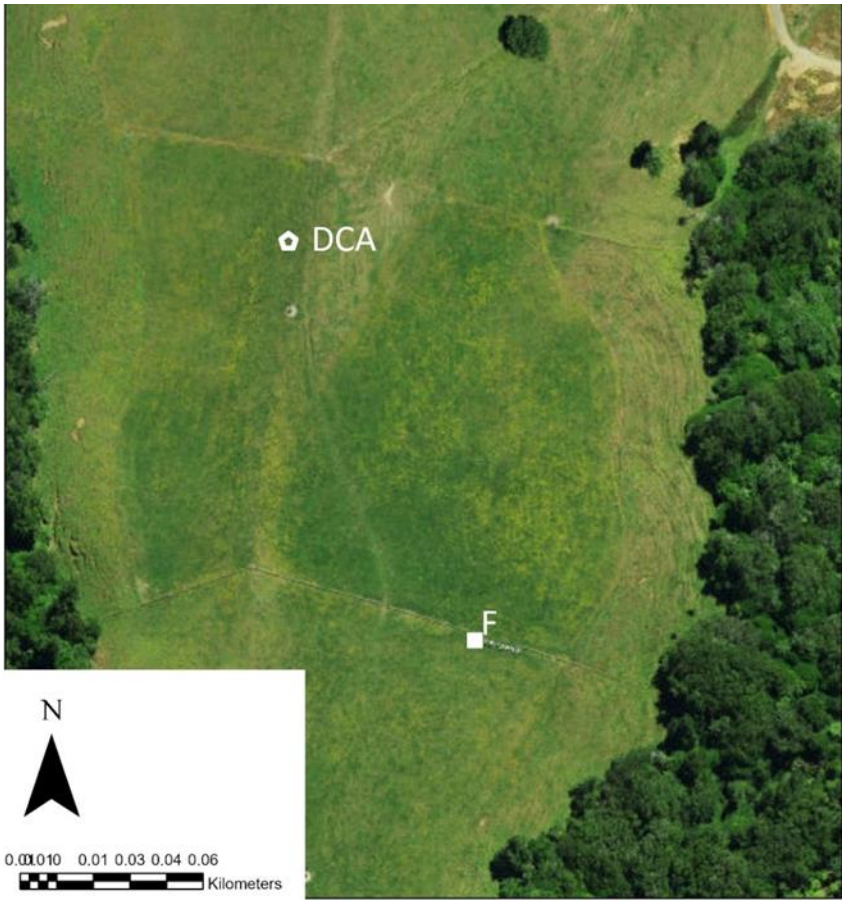

Figure 4-2. Map of paired site ‘F’ as an example of the proximity of the apiary collection site and a drone congregation area (DCA) collection site.

Drone congregation area sampling

A Williams (1987) trap was tied five metres beneath a white 1.2 m chloroprene balloon (Mortensen & Ellis, 2016) to trap drones from the DCAs (Figure 4-3). A live queen honey bee was suspended from the bottom of the trap as pheromone lures. The traps were suspended 15 m in the air above the ground. The drones initially were drawn to the trap by the pheromones of a caged virgin queen suspended below the trap opening and then were then lured up into the trap by visual ‘queen dummies’ made of cigarette filters dyed black.

Samples of drones for calculating *V. destructor* infestation rates were collected into plastic 50 ml conical centrifuge tubes filled with ethanol (95%) (approximately 60 drones per tube, Mortensen et al. 2018). Sampling continued at each DCA until eight 50 tubes for filled with drones, or the drone flight time ended. Samples were transported to the laboratory and stored at room temperature for 1-5 days until analysis. Any DCAs that yielded less than 100 drones were excluded from analysis and colony samples were not collected at the paired apiary (excluded sites: I, J, K, L, and O).

Samples of 20 drones for pathogen analysis was collected from each DCA into a sterile 15 ml conical centrifuge tube and stored in liquid nitrogen or dry ice (depending on availability) for transport to the laboratory where they were stored in a -80°C freezer until transport to the contracted diagnostic facility (no longer than 4 months).

Figure 4-3. A Williams drone trap in the air at a drone congregation area (DCA). The trap is elevated so that the base of the trap is 10-15 m above the ground. The caged queen is visible just below the base of the trap and approximately 6 visual queen dummies are mounted within the trap. Numerous drones are visible approaching the trap from below and clustered inside, at the top of the trap. Photograph by Erin J Steed.

Apiary sampling

Samples of workers for calculating *V. destructor* infestation rates were collected from each of the eight colonies. Workers were shaken from 2 - 3 brood combs into the lid of their hive. A ½ cup (approximately 300 workers) was collected from the lid transferred to a labelled glass jar containing ethanol (95%). Colony samples were

transported to the laboratory where they were and stored at room temperature for 1-5 days until analysis.

Samples of 20 workers for pathogen analysis were collected from each of the eight colonies into sterile 15 ml conical centrifuge tubes and stored in liquid nitrogen or dry ice (depending on availability) for transport to the laboratory where they were stored in a -80°C freezer until transport to the contracted diagnostic facility (no longer than 4 months).

Varroa destructor infestation rates

Sample containers (multiple tubes per DCA or single jar per colony) were manually shaken for one minute then poured into a double mesh sieve as washed as described by Dietemann (2013). Total number of bees and *V. destructor* were counted for each site and *V. destructor* per 100 bees calculated for by dividing the total count of *V. destructor* by the total count of bees and multiplying by 100.

Repetitive sampling

In September of 2022 repetitive sampling of a single DCA was carried out at 1 DCA. Sampling methods were as described above with the exception that 26 tubes (totaling 1378) drones were collected during a single drone flight period. The 26 tubes were divided into 13 pairs, then shaken and washed for quantification of bees and *V. destructor* as described above. *Varroa destructor* per 100 bees was calculated for each pair.

Pathogen analysis

Pathogen analysis was contracted to a diagnostic facility, dnature diagnostics & research ltd. Composite samples of 10 workers per colony for each of the 8 colonies in each apiary and individual samples of 10 drones from each DCA were screened for *N. ceranae* via dnature's 'Nosema Dou' test and DWV, CBPV, BQCV, and the AKI complex (APV, KBV, and IAPV) via dnature's 'ApiVirus Panel.'

Drone and colony samples couriered overnight to dnature's laboratory in Gisborne, New Zealand in a Styrofoam chilly bin of dry ice. Receipt of the samples and integrity of the chilly bin was confirmed the next day by dnature. Summary of the methods followed by dnature is as follows.

Extraction

A modified CTAB extraction method was used. For each sample the reference bee(s), two 6 mm stainless steel ball bearings, CTAB buffer (3% hexadecyltrimethylammonium bromide, 100 mM Tris, pH 8, 10 mM EDTA, pH 8, 8.3% NaCl, 2% PVP-40), and 30 µl sodium metabisulfite were placed into a centrifuge tube. For composite colony samples specific volumes were as follows: reference bees (10 workers), CTAB buffer (3 ml), and tube size (7 ml (labcon)). For individual drone samples specific volumes were as follows: reference bee (1 drone), CTAB buffer (1 ml), and tube size (2 ml (Sarstedt)). Tissues were homogenized for 1 min with a bead beating instrument (BioSpec, MiniBeadbeater-16) then samples were then incubated at 65°C for 15 min with occasional mixing.

For composite bee samples, 1 ml of the homogenised liquid was transferred to a 2 ml centrifuge tube. Individual drone samples remained in the 2 ml centrifuge tube from homogenisation. The 2 ml tubes of homogenate were then centrifuged for 5 min at

15,000 x g in a microcentrifuge (Eppendorf centrifuge 5425). An aliquot of 700 µl of supernatant was transferred to a new 2 ml centrifuge tube, and 700 µl of chloroform/isoamyl alcohol (24:1 v/v) was added to each. Sample were vortexed briefly and centrifuged for 5 min at 15,000 x g in a microcentrifuge. An aliquot of 500 µl of supernatant was transferred to a new 1.5-ml microcentrifuge tube and 350 µl isopropanol was added to each. Tubes were inverted several times to precipitate the nucleic acid and centrifuged at 15,000 x g for 10 min in a microcentrifuge. The supernatant was discarded from each sample and replaced by 300 µl 70% ethanol. Tubes were vortexed briefly and then centrifuged for 5 min at 15,000 x g in a microcentrifuge. The supernatant was discarded, and the pellet air-dried for approximately 15 min. Samples were resuspended in 100 µl elution buffer (10 mM Tris, pH 8).

Real-time PCR

For each sample, individual reactions were run for each target (DWV, CBPV, BQCV, the AKI complex, and *N. ceranae*). Details of the reaction mixes and cycling parameters for viral and *N. ceranae* reactions are presented in Table 4-1 and Table 4-2, respectively.

Table 4-1. The components and their respective volumes for viral and *Nosema ceranea* reaction mixes. Individual viral targets were: DWV, CBPV, BQCV, the AKI complex.

Volume (µl)	Viral Reaction Mix	*Nosema ceranea* Reaction Mix
0.3	forward primer, 10 µM	forward primer, 10 µM
0.3	reverse primer, 10 µM	reverse primer, 10 µM
0.15	probe, 10 µM	probe, 10 µM
5.0	Virus ToughMix (QuantaBio)	ToughMix (QuantaBio)
2.25	PCR-grade water	PCR-grade water
2	genetic template	genetic template

Note: all molecular analysis was contracted to dnature diagnostics & research ltd and conducted as per their standard ‘ApiVirus Panel’ and ‘Nosema Dou’ tests.

Table 4-2. Cycling parameters of viral and *Nosema ceranea*. real-time PCRs. Reactions were run on a Mic PCR instrument (Bio Molecular Systems) and both viral and *Nosema ceranea*. RT-PCRs included 43 cycles. Individual viral targets were: DWV, CBPV, BQCV, the AKI complex.

	Viral		*Nosema ceranea*	
Stage	**Temp (°C)**	**Duration**	**Temp (°C)**	**Duration**
Hold	50	10 min	-	-
Hold	95	30 sec	95	2 min
Cycle (phase 1)	95	5 sec	95	5 sec
Cycle (phase 2)	60	20 sec	60	20 sec

Note: all molecular analysis was contracted to dnature diagnostics & research ltd and conducted as per their standard 'ApiVirus Panel' and 'Nosema Dou' tests.

Statistical analysis

All data analysis was conducted in R version 4.2.2 and R Studio (R Core Team, 2022).

Repetitive sampling

To determine the number of tubes of drones that must be sampled from a DCA to accurately represent the *V. destructor* load of the DCA, the repetitive sampling data was analysed using a polynomial curve model. The asymptote (where the slope dy/dx = 0) and 95% confidence intervals were calculated using this model. The asymptote is assumed to indicate the point where the sample size is large enough to accurately predict the actual *V. destructor* load of the DCA.

Relationship between Varroa destructor at DCAs and apiaries Varroa

To determine the correlation between *V. destructor* per 100 drones at a DCA and *V. destructor* per 100 bees from a colony, the DCA data was analysed using a Poisson

generalised linear model. The relationship between *V. destructor* per 100 drones over *V. destructor* per 100 colony bees was investigated.

Pathogens

Raw data was received from dnature as a spreadsheet with estimated copy numbers for the four viruses and *N. ceranae*. To determine whether there was a significant relationship between the number of viral copies in drones captured at DCAs and the number of viral copies in colony bees from apiaries, the data for each virus was fitted to a quasipoisson model.

Relationship between Varroa destructor and pathogens at DCAs and Apiaries

Additionally, a quasipoisson model was run to test the relationship between *V. destructor* count on viral copy number for both colony bees and drones. Models were fitted for both colony bee and drone groups individually, and a third model was run testing the relationship between copy number, type of sample (colony bees or drone), and *V. destructor* count.

Results

Repetitive sampling

The asymptote of the growth model representing the relationship between *V. destructor* frequency and number of sampling tubes was at 13.36, indicating that 13 tubes is the minimum number of tubes that need to be collected to accurately represent the ratio of *V. destructor* to drones at a DCA. However, 6 tubes (approximately 300

drones) are enough to predict the ratio of *V. destructor* to drones at 95% confidence (Figure 4-4).

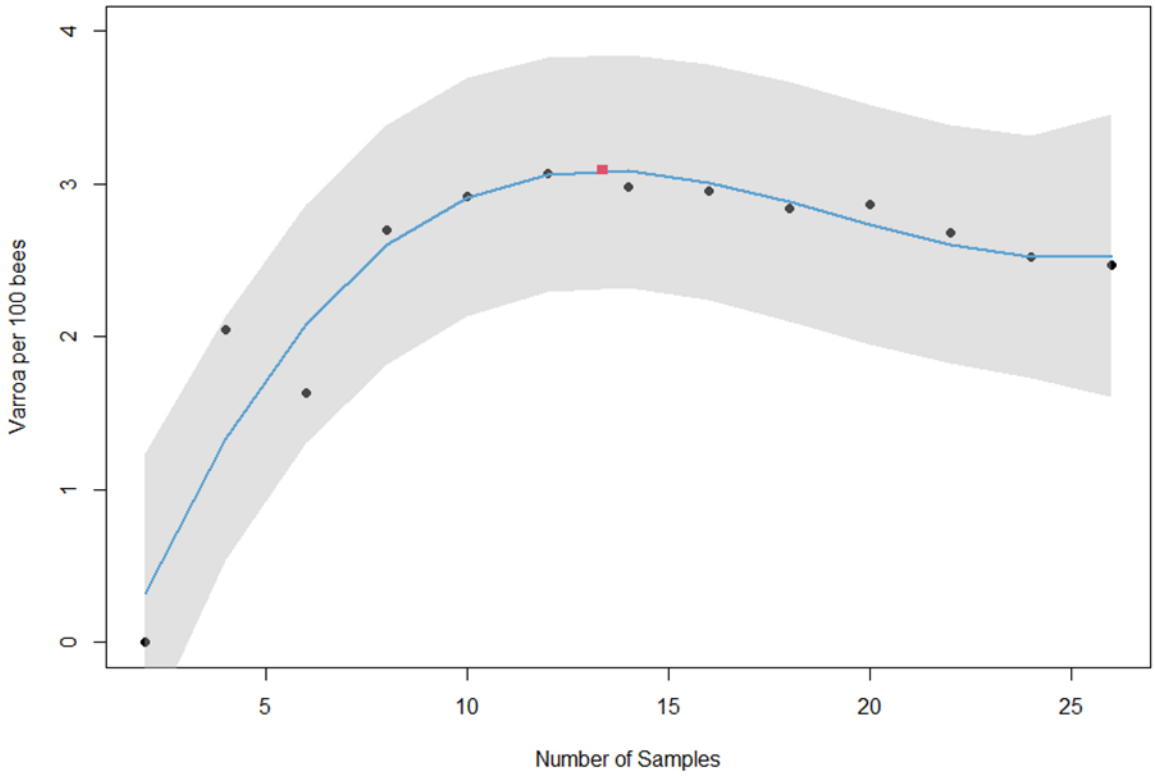

Figure 4-4. Ratio of *Varroa destructor* to drones sampled from a DCA. Drone samples are a 50 ml tube containing approximately 50 drones. The asymptote is marked in red, with error margin showing 95% confidence. Adjusted R squared = 0.8624.

Comparison of *Varroa destructor* abundance in apiaries and DCAs

The relationship between apiary *V. destructor* and DCA *V. destructor* infestation rates appear to be quite variable between sites. Most sites where there were high *V. destructor* infestation rates at the DCA also had relatively high *V. destructor* infestation rates within the colonies at the paired apiary. However, DCAs where little to no *V. destructor* were found varied drastically in the *V. destructor* infestation rates of their paired apiaries (Figure 4-5).

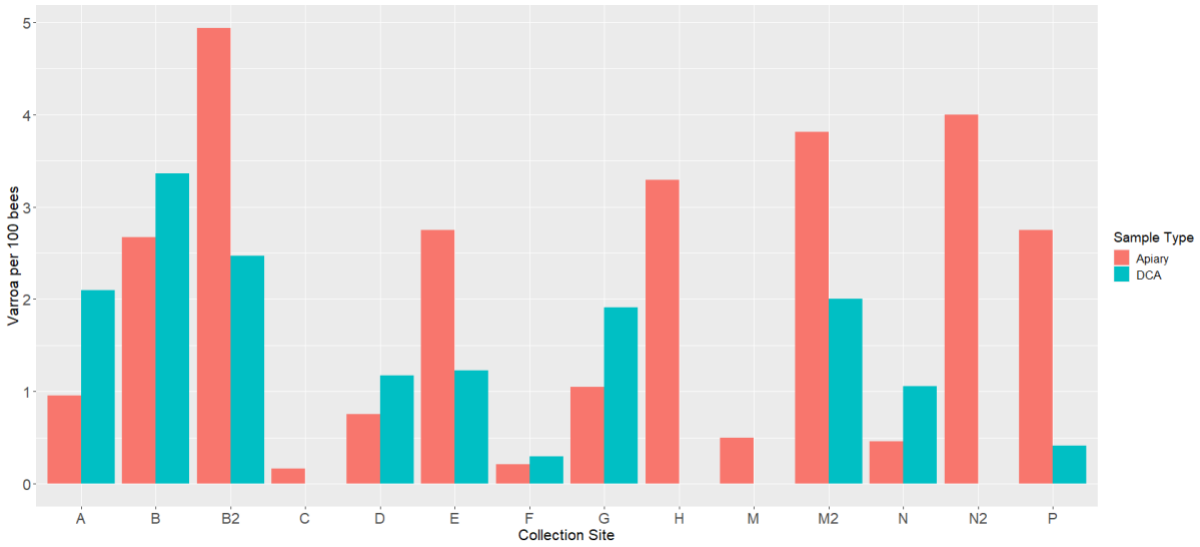

Figure 4-5. *Varroa destructor* count per 100 bees from DCAs (blue) and their nearest apiary (red). Sites B, M, and N were sampled in both 2021 and 2022, with B2, M2, and N2 representing data from the sampling of the same site during a second year.

There was a greater variation in *V. destructor* per 100 bees between apiary samples than DCA samples (Figure 4-6). The range of DCA *V. destructor* was 3.358 (min = 0, max = 3.358), and the range of the apiary *V. destructor* was 4.773 (min = 0.167, max = 4.939). The apiary samples had a median of 1.170 and the DCA samples had a median of 1.8572. The apiary samples had a higher *V. destructor* count on average (2.021 ± 1.615) compared to the DCA samples, which had an average *V. destructor* count of 1.230 ±1.088 per 100 bees.

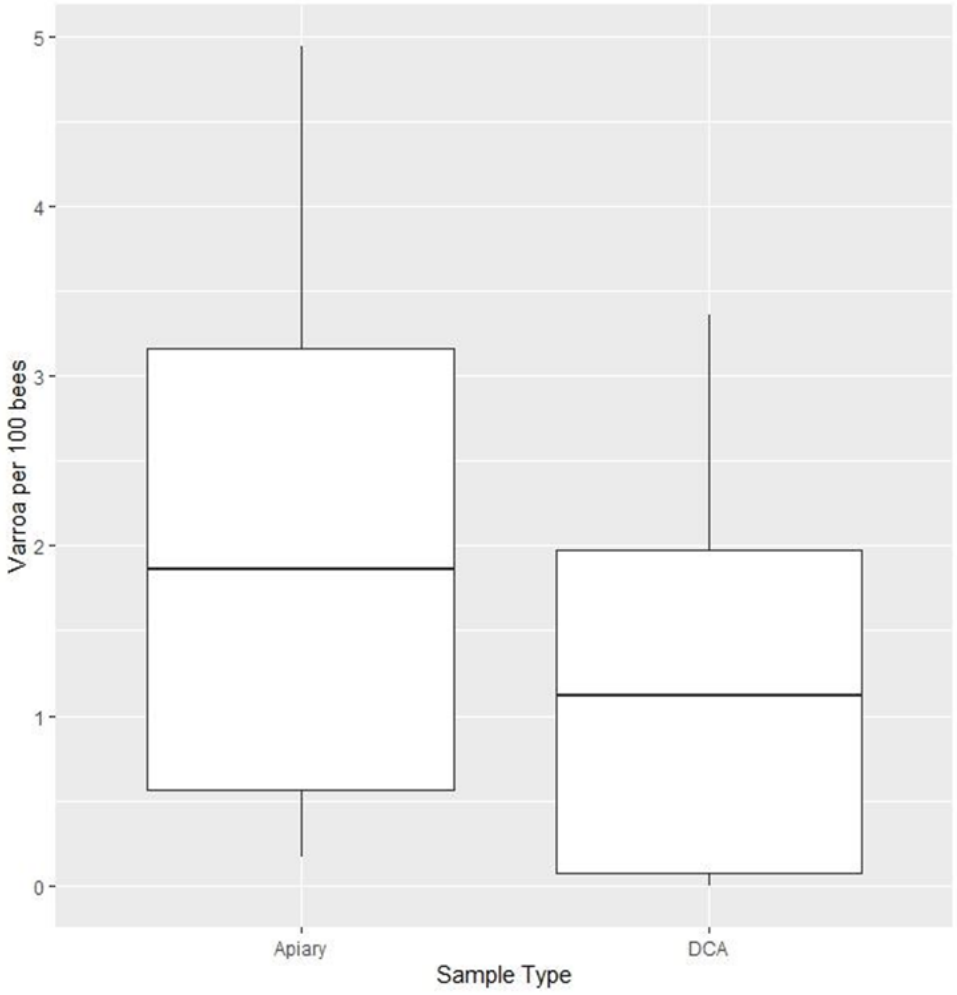

Figure 4-6. Comparison of *Varroa destructor* infestation rates between apiaries and DCAs. The centre line of the box represents the median, and the upper and lower ends of the box represent the upper and lower quartiles.

There was a significant positive relationship between the number of *V. destructor* per 100 drones found at a DCA and the number of *V. destructor* per 100 bees found in colonies in an apiary (B = 0.296, SE = 0.0101, t = 2.93, *p* = 0.00981; Figure 4-7).

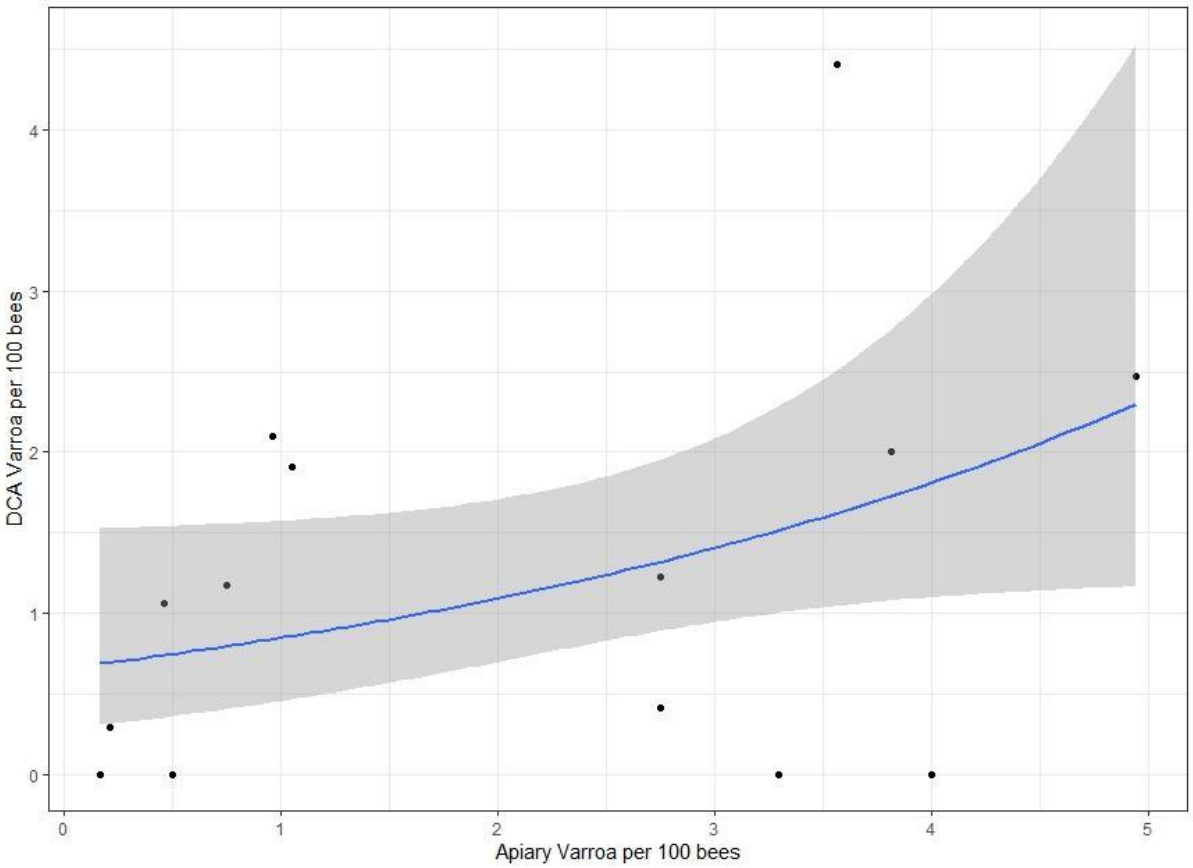

Figure 4-7. Poisson generalised linear model of the *Varroa destructor* infestation rates of paired DCAs and nearby apiaries. Black dots represent the individual DCA-Apiary pair data and the 95% confidence interval is indicated by the grey polygon.

Pathogens

DWV was the most prevalent virus, being present in both colony bees and in drones at every site (Figure 4-8). While DWV was present in drones at every DCA, most other viruses were absent or only present in drones in very low numbers. Colony bees tended to have lower copy numbers of DWV than drones. *Nosema ceranae* was found predominantly in colony bees, with its presence detected in 8 out of 10 apiaries, whereas *N. ceranae* was only present at 6 of the 10 in very low numbers. BQCV was

detected in low levels at all the sites, whereas AKI and CBPV were not detected in most samples (Figure 4-9, Table 4-3).

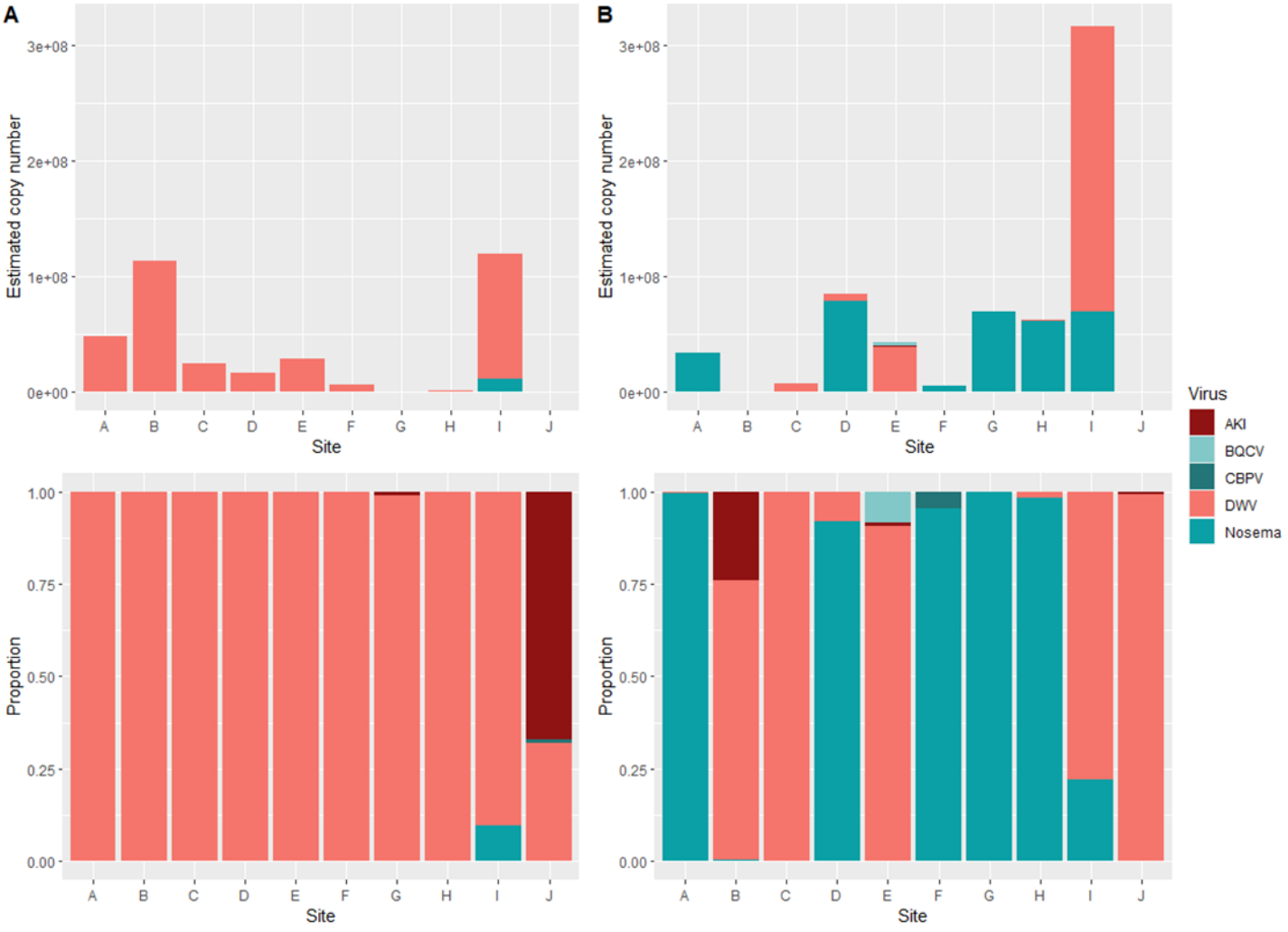

Figure 4-8. Estimated copy number and relative ratios of DWV, CBPV, BQCV, AKL complex, and *N. ceranae* in drones at DCAs (A) and colonies at nearby apiaries (B) from 10 paired sites in the Waikato Region.

A quasipoisson model found that there was a significant difference between BQCV infection in drones from DCAs and colony bees (se = 0.698, t = -2.695, p = 0.008). The estimated copy number of BQCV in colony bees was significantly higher

than that of drones (Figure 4-10, A). There was no significant difference between DWV infection in drones from DCAs and colony bees (se = 0.472, t = -0.172, *p* = 0.864) (Figure 4-10, C). Colony bees had a significantly higher estimated copy number of *Nosema ceranae* compared to drones collected at DCAs (se = -3.534, t = -3.254, *p* = 0.001) (Figure 4-10, E). *Nosema ceranae* was only present at 6 out of the 10 sampled DCAs, whereas it was present in all 10 of the apiaries where colonies were sampled. AKI and CBPV were absent or present in very low numbers at most of the sites, and therefore were difficult to compare.

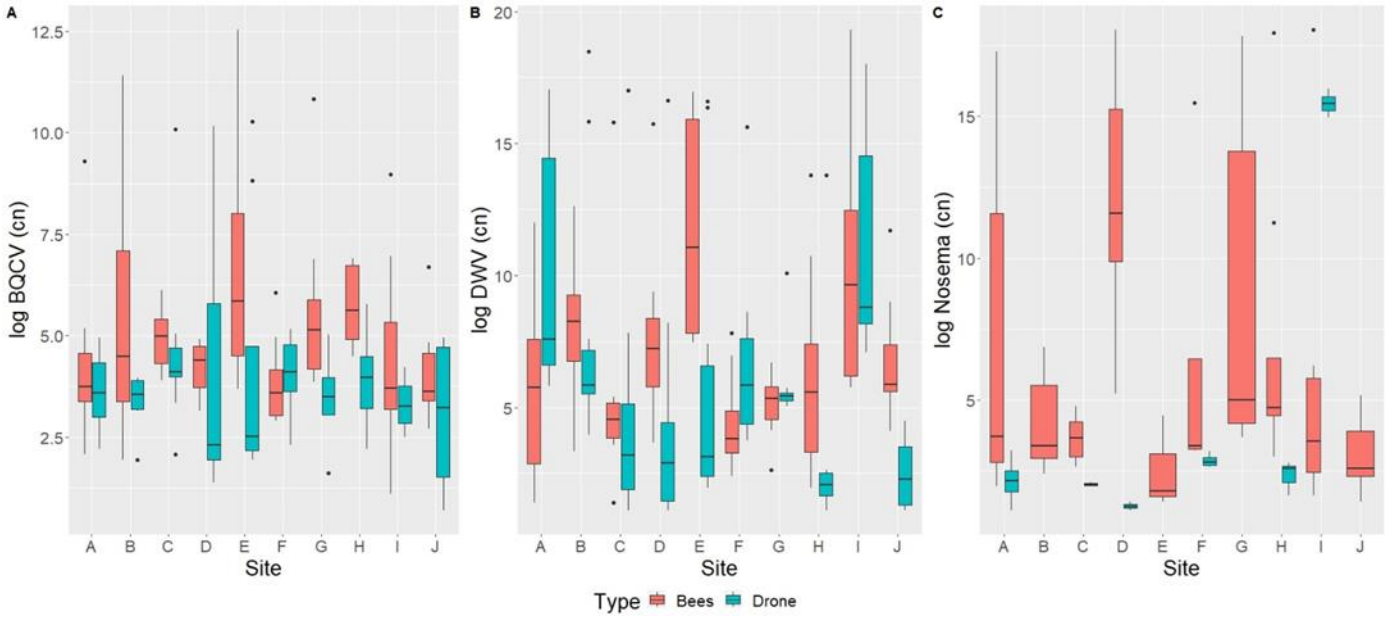

Figure 4-9. Abundance of BQCV (A), DWV (B), and *N. ceranae* (C) at each DCA (blue) and apiary (red). Data are the log transformed copy number. The middle of the box represents the median, the top and bottom of the box represent the upper and lower quartiles, and the top and bottom lines represent the maximum and minimum excluding outliers. Outliers are represented as dots.

The generalised linear model (GLM) showed that there was a significant positive relationship between DWV in drones at DCAs versus colony bees from apiaries (se = 1.162, t = 2.619, p = 0.031). GLMs of all other viruses showed that there was no

significant relationship between the copy number of the virus in drones at DCAs and the copy number of viruses for colony bees from apiaries.

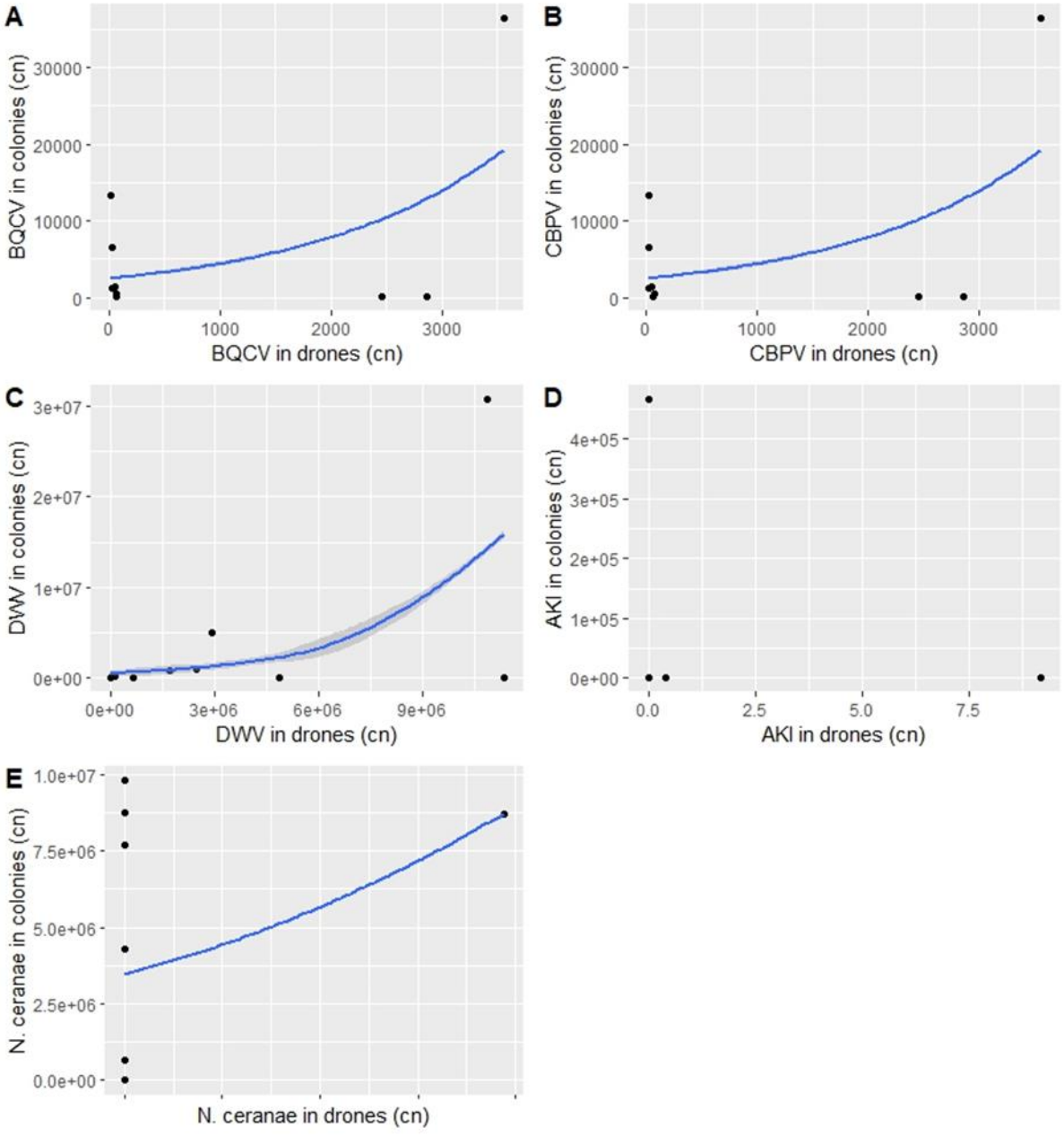

Figure 4-10. Quasipoisson GLM regressions for each pathogen of pathogen copy number at DCAs compared to the nearest apiary. Plots represent different viruses; BQCV (A), CBPV (B), DWV (C), AKI (D), and *N. ceranae* (E).

Table 4-3. Mean estimated copy number +/- standard deviation by pathogen for colonies and drones at each site. Colony data represent a composite sample of 10 workers from each of eight colonies per site. Drone data represent individual analysis of 10 drones. Non-detection of a pathogen is denoted as 'n/d'. Pathogens are, black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), the AKI complex (acute paralysis virus (APV), Kashmir bee virus (KBV), and Israeli paralysis virus (IPV)), and *Nosema ceranae*.

		BQCV	**CBPV**	**DWV**	**AKI**	***N. ceranae***
Site	**Type**	**Mean +/- StDev**	**Mean +/- StDev**	**Mean +/- StDev**	**Mean +/- StDev**	**Mean +/- StDev**
A	Colony	1,430 +/- 132	0 +/- 9.7	21,200 +/- 2,550,000	0 +/- 0.7	4,290,000 +/- 41.9
	Drones	51.3 +/- 7570	2.6 +/- 19.7	4,850,000 +/- 7,720,000	9.2 +/- 1.3	8.1 +/- 1.3
B	Colony	13,400 +/- 17900	0.4 +/- 1,560	42,400 +/- 269	4.9 +/- 0	188 +/- 19,600,000
	Drones	20.5 +/- 47.6	0.2 +/- 7.1	11,300,000 +/- 7,470	n/d	n/d
C	Colony	174 +/- 389	4.6 +/- 439	902,000 +/- 343,000	0.3 +/- 8.5	38.2 +/- 21,700,000
	Drones	2,460 +/- 97.7	8.3 +/- 40	2,440,000 +/- 276,000	0.4 +/- 0	1.5 +/- 0
D	Colony	79.4 +/- 44.6	2 +/- 5.5	850,000 +/- 2,400,000	n/d	9,800,000 +/- 24,300,000
	Drones	2,860 +/- 8140	n/d	1,680,000 +/- 5,330,000	n/d	0.7 +/- 0
E	Colony	36,500 +/- 98500	6.2 +/- 15.6	4,940,000 +/- 8,130,000	468,000 +/- 1,320,000	11.8 +/- 29.3
	Drones	3,560 +/- 9110	n/d	2,910,000 +/- 6,170,000	n/d	n/d
F	Colony	93.5 +/- 270	31,600 +/- 3.3	470 +/- 42,300	10.6 +/- 1.9	665,000 +/- 60.3
	Drones	63.7 +/- 69.3	0 +/- 1.9	619,000 +/- 40.2	0 +/- 0	7.1 +/- 0
G	Colony	6,590 +/- 2740	558 +/- 2.7	278 +/- 86,700,000	0 +/- 1.8	8,770,000 +/- 24,700,000
	Drones	26.3 +/- 21.5	3.3 +/- 10.4	2,580 +/- 22,800,000	0 +/- 1.3	0 +/- 1.3
H	Colony	457 +/- 31300	230 +/- 1.1	128,000 +/- 104,000	3 +/- 13.8	7,700,000 +/- 348
	Drones	69.2 +/- 22.1	24.5 +/- 0.7	97,000 +/- 33,200,000	n/d	3.4 +/- 0
I	Colony	1,150 +/- 140	1.8 +/- 89,300	30,800,000 +/- 890	0.6 +/- 6.8	8,740,000 +/- 1,880,000
	Drones	29.2 +/- 61.1	7.7 +/- 0	10,900,000 +/- 1,950,000	0.4 +/- 0	1,170,000 +/- 0
J	Colony	146 +/- 3880	1.2 +/- 0	16,400 +/- 56,300	1.3 +/- 0	35.5 +/- 11,200,000
	Drones	62.7 +/- 41.4	1 +/- 4.3	29.6 +/- 9,440,000	0 +/- 20.6	0 +/- 20.6

Note: The method of pooling for genetic extraction of colony samples generated template material that was ~3.3 times more concentrated than that of individual drone samples.

Relationship between *Varroa destructor* at DCAs and apiaries Varroa

While there was no significant relationship between *V. destructor* count and copy number for BQCV, CBPV, AKI, or *N. ceranae*, there was a significant relationship between the *V. destructor* count at DCAs and the number of viral copies of DWV present in drones (se = 0.290, t = 2.677, p = 0.009). The same was true of the *V. destructor* count of colonies and the number of viral copies present in colony bees (se = 0.227, t = 9.469, p = 1.33 × 10-14). The higher t statistic and p value suggests that the relationship between *V. destructor* prevalence and DWV copy number is a lot stronger in the colony bees than in the drones. Taken together, *V. destructor* count has a significant positive relationship with the copy number of DWV in both colony bees and drones, but the degree to which DWV copy number is affected differs significantly between colony bees and drones (se = 0.260, t = -2.119, p = 0.0356; Figure 4-11).

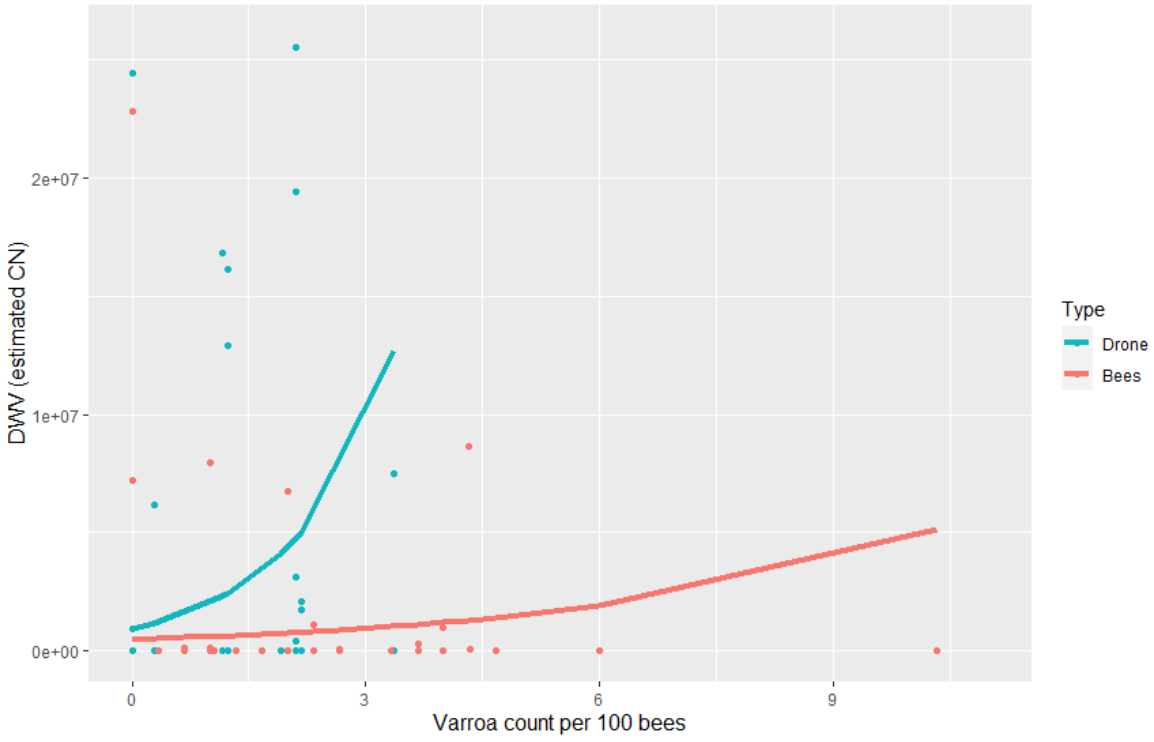

Figure 4-11. Quasipoisson model of DWV copy number over the *Varroa destructor* infestation rates of colonies (bees, red) and DCAs (drones, blue).

Discussion

In this study, I found evidence that drones do play an important role in *V. destructor* dispersal between different colonies via their mating flights to and from DCAs. This has implications for the transfer of *V. destructor* between colonies within an apiary, and the monitoring of *V. destructor* within those apiaries. This data was further supported by our findings from the DCA sampling. There was a positive correlation between the *V. destructor* infestation rate at DCAs and the *V. destructor* infestation rate of colonies at a nearby apiary. This demonstrates the potential for the use of DCAs in population level *V. destructor* monitoring and reinforces the important role drone honey bees play in *V. destructor* dispersal.

While the application of DCAs for *V. destructor* monitoring was reinforced by my findings, the use of DCAs for monitoring viral infection and spread was appeared less viable. No statistically significant relationships were present between AKI, BQCV, CBPV and *N. ceranae* copy numbers at DCAs versus apiaries. Additionally, colony bees had more viral copies of BQCV, CBPV, and *N. ceranae* across majority of my sites. However, there was a significant, positive relationship between DWV viral copies in drones compared to colony bees, which I hypothesize to be a product of the vector relationship between *V. destructor* and DWV. I also hypothesize that drones with severe viral infections die before reaching maturity, or present with symptoms that prevent them from carrying out mating flights, thus causing viruses to be underrepresented at DCAs compared to host colonies.

Effects of variable DCA sample sizes

I compared the *V. destructor* load of DCAs and apiaries at 16 sites in the Waikato region to investigate the correlation between *V. destructor* at DCAs and *V. destructor* in apiaries. When directly comparing *V. destructor* counts at individual DCAs to their respective apiaries, I found substantial variation between the *V. destructor* a count observed at a DCA and the average *V. destructor* count observed in the apiary (Figure 4-5). The DCA sites where a high abundance of *V. destructor* was observed were usually found near an apiary with a high *V. destructor* abundance, but the DCA sites where very few or no *V. destructor* were counted could have either a low or high *V. destructor* abundance at the corresponding apiary. While we were able to collect a standard 300 bees from each of 8 colonies within the apiaries, the number of drones sampled from the DCAs was quite variable, from 107 drones up to 450, suggesting sample size could be problematic. This begs the question - how many drones need to be sampled from a DCA to accurately represent the *V. destructor* load at the DCA? To answer this question, we carried out repetitive sampling at one DCA.

I conducted repetitive sampling of one DCA to determine the number of drones that must be collected to accurately estimate the *V. destructor* load of a DCA. Twenty-six 50 mL tubes, each of which holds about 60 drones, were collected from one DCA. We found that 13.36 tubes (approximately 800 drones) were the estimated minimum number of tubes required to give an accurate representation of the *V. destructor* count at a given DCA. However, the 95% confidence intervals were quite widely spread, indicating that as few as 6 tubes could be sufficient to give an accurate representation of the number of *V. destructor* per 100 bees at a DCA. While we aimed to collect 6-8 tubes (360 - 480 drones) from each DCA we sampled, any DCA where over 100 drones

were collected was included in the analysis. However, these results suggest that more than 100 drones are necessary to give an accurate representation of the *V. destructor* load of a DCA. It is also worth noting that repetitive sampling was only carried out at one site, so the scope of this data is very narrow. While it gives a useful indicator of the required sample size from a DCA, repetitive sampling from other sites should be carried out to validate the pattern we observed from a single site.

While gathering 6 - 8 tubes from each DCA is ideal for gaining an accurate understanding of the prevalence of *V. destructor* at these sites, the practicality of this must be considered. Four of our 19 sites were excluded from our analysis because less than 100 drones could be captured from the DCA, and less than 200 drones were collected from a further four sites that were included. There were several reasons for this, such as the weather impacting how many drones were flying, wind interfering with the trap, and difficulty locating the DCA. It is also worth considering that while some DCAs have up to 16,000 drones congregating, others may only have several hundred (Koeniger et al., 2011), and sampling many drones could have an impact on the stability of the DCA. Therefore, for this study where there is already a limited sample size, including sites with a count over 100 drones seems acceptable.

Differences in *Varroa destructor* infestation rates at DCAs and apiaries

In support of my suggestion to collect fewer tubes of drones to balance the practicalities of sampling at DCAs, I also found that even with a substantial sample size from the DCA, there was a higher *V. destructor* count in the apiary than at the DCA. This trend seemed to persist across most of our study sites (Figure 4-6). Given the results of my choice test, where there was no significant difference between *V.*

destructor preference for drones over the other castes of honey bee, it makes sense that a larger proportion of *V. destructor* would be found on nurse and forager bees within the hive when I did my sampling. Drone honey bees usually only make up 5 - 10% of a honey bee colony (Seeley & Morse, 1976., Page & Metcalf, 1984), though this is influenced by environmental factors, as well as decisions made by the queen and workers. Assuming that the proportion of drones in a colony is substantially lower than that of workers such as nurses and foragers, it is logical that colony *V. destructor* counts were consistently higher than DCA *V. destructor* counts.

Application of DCAs for monitoring *V. destructor* infestation in apiaries

Sampling of drones and honey bees from DCAs and their nearest apiaries demonstrated potential for DCAs to be used as a predictor *V. destructor* prevalence in nearby apiaries. There was a significant positive relationship between *V. destructor* per 100 drones found at a DCA and *V. destructor* per 100 bees found in colonies in an apiary. This has implications for *V. destructor* monitoring, particularly by providing the opportunity for population level *V. destructor* monitoring. While monitoring practices currently only provide information on individual colonies, utilising DCAs for the purpose of *V. destructor* monitoring allows the beekeeper to get a snapshot of what is going on in their apiary.

Using DCAs for monitoring could be a useful tool in biosecurity, allowing us to identify regions where *V. destructor* to try and contain it and prevent further spread. For example, could be used to monitor and track the spread of *V. destructor* across Australia following the recent introduction of *V. destructor* into the country (New South Wales Government, 2022). Given the application of DCAs for *V. destructor* monitoring,

there is the potential for monitoring the spread of other honey bee parasites or pathogens, as they threaten to spread to new regions across the world.

As well as the potential for monitoring the spread of pathogens and parasites, DCAs can also provide information on potential sources of *V. destructor* and associated pathogens, such as feral colonies. Because unmanaged feral colonies do not receive the regular miticide treatments as managed colonies do, they are especially vulnerable to *V. destructor* infestation and can consequently become a significant source of *V. destructor* to other colonies. When colonies die because of mite infestation, there is often a spike in *V. destructor* infestations of neighbouring hives (Peck & Seeley, 2019). This is thought to be because colonies that are failing because of high *V. destructor* infestation are more susceptible to robbing (Peck & Seeley, 2019), thus offloading mites to the robbing colony. Therefore, feral colonies are an important factor to consider when assessing regional *V. destructor* risk. Though traditional methods can only give insight into the *V. destructor* infestation of individual colonies, monitoring a DCA can give insight into these other *V. destructor* sources in the neighbourhood. Williamson et al (2022) found that genotyping drones captured at a DCA is a viable method of estimating honey bee density in an area, and that their sampled DCAs included drones from two thirds of the known feral population. This validates the use of DCAs for sampling parasites and pathogens at the population level, including both managed and feral colonies.

Monitoring pathogens at DCAs

Though there is a clear application for DCAs in the monitoring of *V. destructor*, the same function may not be as applicable to the monitoring of pathogens. Though

there was a significant relationship between the copy number of DWV in drones and the copy number of DWV in colony bees, the copy numbers of BQCV, CBPV, AKI, and the fungus *N. ceranae* in drones at DCAs did not have a statistically significant relationship with the copy number of these viruses in colony bees from apiaries (Figure 4-9). Colony bees also tended to have more copies of *N. ceranae* and the viruses BQCV and CBPV than drones did.

A potential reason for the increased viral infection observed in colony bees is a change in flight behaviour that can sometimes be observed in honey bees infected with parasites or pathogens. *Nosema ceranae* and DWV are both known to significantly reduce the duration and distance of flights and reduce the homing abilities of honey bees (Wells et al., 2016., Iqbal & Mueller, 2007., Kralj & Fuchs, 2009). It is possible that the decreased fitness and flight ability of drones infected with DWV or *N. ceranae* prevents the more heavily infected individuals from performing mating flights to DCAs, resulting in the underrepresentation of infected drones at DCAs. However, DWV copy numbers were not significantly different between drones and colony bees, and BQCV is not known to affect adult workers (Chen & Siede, 2007), suggesting that something else is at play.

I hypothesize that the role of *V. destructor* as a viral vector is the reason for relatively similar DWV infection in drones and colony bees in contrast to the lower viral copies of BQCV and *N. ceranae* found in drones compared to colony bees. *Varroa destructor* has been shown to be a vector of DWV (Noël et al., 2020), and following the arrival of *V. destructor* into New Zealand, DWV has become the most prevalent and abundant virus affecting honey bees (Mondet et al., 2014., Lester et al., 2022). In

contrast, BQCV and *N. ceranae* infections are independent of *V. destructor* infestations (Mondet et al., 2014., Tentcheva et al., 2004., de Miranda et al., 2012) and rely on other means of transmission between bees such as foodborne transmission or fecal-oral transmission (Chen & Reinhold, 2007). Because drones do not participate in colony activities such as food collection, brood care, or hygiene, they are usually only exposed to horizontal viral transmission through trophallaxis from other bees (Goins & Schneider, 2013). Workers participate in foraging, nectar and pollen exchange, food production such as making honey and royal jelly, brood care where nurses feed and rear brood, and cell cleaning where dead or diseased brood is removed or cleared out of cells (Seeley, 1982., Johnson, 2008., Chen & Siede, 2007). In this way, workers are exposed to more sources of viral transmission than drones, which could be a reason why *N. ceranae* and BQCV were more prevalent in colony bees. Unlike *N. ceranae* and BQCV, DWV does not spread efficiently on its own, but the presence of *V. destructor* as a vector allows DWV to be transmitted between bees with the movement of the parasite. Moreover, *V. destructor* has shown a preference for drone brood over worker brood, facilitating the exposure of drone honey bees to DWV (Fuchs, 1990., Boot et al., 1995., Calderone, 2005). This could be an explanation for why DWV was present in relatively similar copy numbers in drones compared to apiary bees.

I found that there was a significant positive relationship between DWV copy number and *V. destructor* abundance in both drones and colony bees. Drones collected from DCAs where there was a high *V. destructor* count tended to have more copies of the DWV virus than those collected from a DCA with a lower *V. destructor* count. Therefore, drones at DCAs where there is a higher prevalence of *V. destructor* could

also be more exposed to DWV, thus implicating them in the spread of DWV between colonies. The relationship between DWV and *V. destructor* was significantly stronger within the colony bees, where colony bees from apiaries that had higher *V. destructor* infestations also tended to have more viral copies of DWV. Once again, it is likely that the most severely DWV infected drones were unable to fly to a DCA due to overt symptoms such as deformed wings, which could be why the relationship was much stronger within the colony group. Additionally, the mixing of drones from different colonies and apiaries at DCA's could 'dilute' the DWV infection of the DCA.

Given this information, DCAs are a site of potential virus transmission, but the use of DCAs for monitoring viral communities within apiaries does not appear to be viable for most viruses. However, the close link between *V. destructor* and DWV could allow us to predict the presence of DWV within a DCA and perhaps even the neighbouring apiaries. The results encompassing the relationship between *V. destructor* in apiaries and the presence of *V. destructor* at DCAs demonstrates the potential that DCAs hold for *V. destructor* monitoring in the future. The preference *V. destructor* displayed for mature drones over mixed drones suggests that *V. destructor* choice for drones was not purely random, but that drones at DCA's do play an important role in the dispersal of *V. destructor* between colonies. Given the relationship between *V. destructor* at DCAs and *V. destructor* in colony bees, using DCAs as a means of monitoring *V. destructor* infestation is a feasible proposition. Further exploring this relationship between *V. destructor* at DCAs and in apiaries and its applications to beekeeping is an exciting area for future research and could have implications beyond *V. destructor* monitoring.

CHAPTER 5

Conclusion

In this thesis I aimed to investigate the role of drone honey bees in the spread of parasites and pathogens via drone congregation areas (DCAs) and explore the potential that DCAs hold for monitoring apiary health. To achieve this, I investigated *V. destructor* preference for drone honey bees as hosts over foragers and nurses, I determined the local drone flight time in Waikato, New Zealand, and I examined the prevalence and abundance of *V. destructor*, *N. ceranae*, and four common viruses at DCAs compared to nearby apiaries. My findings suggest that drones are chosen as phoretic hosts by *V. destructor* for dispersal, and that there is a significant relationship between the *V. destructor* abundance observed at DCAs and the *V. destructor* abundance observed in apiaries. Here, I discuss the significance of these results for population level monitoring of parasite and pathogens infections in honey bees and summarise my findings on drone mediated dispersal.

Drones Facilitate *Varroa destructor* and Pathogen Dispersal During Mating Flights

The ectoparasite *V. destructor*, microsporidian parasite *N. ceranae*, and honey bee viruses BQCV, CBPV, DWV, and AKI complex were all detected in drones collected from DCAs we sampled. This means that drones can transport both parasites and pathogens to DCAs, and that drone drift between colonies during mating flights can act as a flux for these parasites and pathogens in and out of colonies. The tendency of drone honey bees to drift more than workers (Free, 1958., Currie & Jay, 2015) means that drones play an important role in the spread of both parasites and pathogens. The

role of drones in the transmission of viruses via venereal transmission is known to be an important source of viral transmission between colonies for viruses such as BQCV, DWV, ABPV, and SBV (Chen et al., 2006., Yañez et al., 2012., Prodělalová et al., 2019., Amiri et al., 2020). However, the role of drones in supporting horizontal transmission of pathogens via drift and in the dispersal of *V. destructor* have been vastly understudied (Peck et al., 2016., DeGrandi-Hoffman et al., 2016., Cervo et al., 2014). My research reinforces our knowledge around the role of drones in the spread of viruses, but it also highlights the importance of drones for the dispersal of *V. destructor*.

Despite drone brood being widely accepted as the preferred host of *V. destructor* during the reproductive phase, the role of adult drones as hosts for *V. destructor* during the dispersal phase has been somewhat neglected. In contrast, the role of adult workers such as foragers and nurses as dispersal hosts has been studied extensively. My results showed that drone honey bees are selected as dispersal hosts by *V. destructor* collected from highly infested colonies in equal proportion to both nurses and foragers. This supports the hypothesis that drones support dispersal of *V. destructor* and could be equally important to that of foragers. As an important piece of the puzzle for understanding intercolonial spread of *V. destructor*, I propose that drone-mediated dispersal deserves more investigation and representation in the literature. Understanding the preferences *V. destructor* display for drones of different ages, the preferences of *V. destructor* of different ages, collecting more data on the relationship between drones at DCAs and colony bees in apiaries, and further researching drone drift are all useful fields for better understanding the role of drones in *V. destructor* dispersal.

DCAs Provide Alternate Means of Monitoring *Varroa destructor*

My data supports the application of DCAs for population level monitoring of *V. destructor* in apiaries. I found that there was a statistically significant positive relationship between the *V. destructor* infestation of DCAs and the *V. destructor* infestation of colonies within the nearest apiary. The use of population level *V. destructor* monitoring at DCAs has advantages for monitoring in commercial beekeeping. Monitoring in commercial beekeeping is often neglected due to constraints on time and labour available to sample individual hives. The use of DCAs for monitoring allows for a glimpse into the overall *V. destructor* infestation and health of the apiary. Of course, information in the *V. destructor* infestation of individual colonies cannot be gleaned from the sampling of a DCA, and the mixing of bees from other apiaries or feral colonies in the area must be considered. However, highly infested colonies share *V. destructor* very effectively via drifting and robbing, especially in densely populated apiaries (Peck & Seeley, 2019., Seeley & Smith, 2015), suggesting that a population level assessment may be offer more valuable insights for informing treatment choices for colonies managed nearby.

In addition to monitoring *V. destructor* for routine management, my research opens possibilities for monitoring in other context, such as monitoring *V. destructor* spread to new regions, the spread of the *Tropilaelaps spp* around the world, and for monitoring feral colonies. Up until recently, Australia was the only major continent without *V. destructor*. However, *V. destructor* was discovered parasitising bees in commercial apiaries in New South Wales in June of 2022 (The Senate, 2022). In this context, DCAs can be used as a biosecurity tool to monitor the spread of *V. destructor*

across New South Wales and the rest of Australia, allowing for better preparation, management, and control. In a similar vein, DCAs could likely have similar applications for monitoring the spread of *Tropilaelaps mercedesae*, another damaging parasite of *A. mellifera* that is still largely confined to Asia (Chantawannakul et al., 2018). *Tropilaelaps mercedesae* has recently expanded its range to South Korea and China, and similarly to *V. destructor*, it has both a reproductive phase and a 'phoretic' phase during which it is thought to disperse (Chantawannakul et al., 2018). The successful use of DCAs as a tool in monitoring the spread and infestation of *V. destructor* could potentially be applied to monitoring emerging threats like *T. mercedesae*. Finally, DCAs sampling offers the unique benefit of monitoring all colonies in the area, including feral colonies, which can be difficult to locate and monitor compared to managed colonies (Kohl et al., 2022). Feral colonies have been shown to decline rapidly following the introduction of *V. destructor* but monitoring feral populations can lead to the discovery of mite-resistant feral colonies and provide insight into the genetic diversity of local honeybee populations (Lopez-Uribe et al., 2017., Seeley, 2007). Overall, the use of DCAs in monitoring *V. destructor* populations shows great potential for biosecurity and management, providing avenues for future research to consolidate my preliminary results.

Expanding our Understanding Pathogen - *Varroa destructor* Interactions

I found that the viral loads of drones at DCAs did not necessarily reflect the viral loads in nearby colonies. There was no apparent relationship between BQCV, CBPV, and AKI, or *N. ceranae* in drones and nearby colonies, suggesting that DCAs are less appropriate for direct inference of nearby colony pathogens, unlike the predictive

relationship we saw with *V. destructor*. However, there was a detectable relationship between DCA and Apiary DWV abundance. Moreover, there was a significant positive relationship between *V. destructor* infestation rate and DWV load in both DCAs and apiary samples. This is consistent with the close vector-virus relationship that has been observed between DWV and *V. destructor* (De Miranda et al., 2010., Bowen-Walker et al., 1999). My results suggest that *V. destructor* infestation rate could be used to predict the degree of DWV infection, or vice versa.

I hypothesise that we observed lower viral copy numbers in drones collected from DCAs compared to the workers collected from nearby colonies because drones may experience limited exposure to viruses due to their relatively inactive role within the hive, and/or the inability of drones with severe viral infections to fly to DCAs. Another potential factor could be sex-related differences in susceptibility to viral infections. It has been shown that drone honey bees infected with *N. ceranae* had a higher mortality rate and lower body mass compared to infected workers, even though drones had lower levels of *N. ceranae* spores (Retschnig et al., 2014). Drones have also been found to be more sensitive to abiotic stressors such as temperature and pesticides than workers (McAfee et al., 2022). Limited research has been done to determine whether viruses affect drones differently to workers, but if drones are more sensitive to viruses as they appear to be with other stressors, it is plausible that drones cannot tolerate as well as workers and therefore die or fail to carry out mating flights and may limit the contribution of drones to intercolonial dispersal of pathogens.

Final Remarks

In conclusion, I found evidence that drone honey bees may play an important role in the dispersal of *V. destructor* between colonies, but that more information is needed to determine just how influential drones are in the spread of pathogens. *Varroa destructor* preference for drone honey bees in equal proportion to foragers highlights this result. This knowledge presents potential for the use of DCAs in monitoring *V. destructor* in regional honey bee populations and presents an exciting challenge going forward to develop a practical method of DCA monitoring that is accessible and efficient for practical application in managing honey bee health.

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