

Understanding the Mechanisms of Honey Bee (*Apis mellifera* L.)
Grooming Behaviour in Relation to its Effectiveness as a Defence
Against *Varroa destructor*

By

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To Jenna,
without whose unwavering love and encouragement
this thesis would not have been possible.

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Table of Contents

Dedication	i
Acknowledgements	ii
List of Figures	v
General Abstract	1
General Introduction	2
Chapter 1. Literature Review	4
Introduction	4
The Western Honey Bee, <i>Apis mellifera</i> Linnaeus	4
Taxonomy and Economic Importance	4
Biology and Life Cycle	5
Communication Among Bees	6
Pheromones	6
Dance Language	7
Vibroacoustic Components	9
Trophallaxis	11
Honey Bee Pests, Pathogens, and Parasites	12
<i>Varroa destructor</i> Anderson and Trueman	12
Taxonomy and Host-shift	12
Biology and Life Cycle	13
Impact on <i>Apis mellifera</i>	14
Control of <i>Varroa destructor</i>	15
Social Immunity Behaviours of Honey Bees	16
Grooming Behaviour	17
The Grooming Invitation Dance	18
Grooming in <i>A. cerana</i> vs <i>A. mellifera</i>	18
Damaged Mites as an Indicator of Grooming Behaviour	19
Mite Mortality Rate to Measure Grooming	20
Individual Bee Assays to Assess Grooming Responses	21

Heritability	22
Other Factors Influencing Grooming Behaviour	22
Environmental Factors	23
Queen Pheromone	23
Age and Reproductive Status of Mites	23
Grooming Specialists	23
Impact of Other Pathogens.....	24
Potential Biological Cost Associated with Increased Levels of Grooming Behaviour	24
Study Objectives	24
Preface to Chapter Two	28
Chapter 2. Heightened Sensitivity in High-grooming Honey Bees (<i>Apis mellifera</i>).....	29
Abstract	29
Introduction.....	30
Materials and Methods.....	32
Results.....	39
Discussion	41
Preface to Chapter Three	49
Chapter 3. Examination of Communication Responses in High-grooming and Low-grooming Honey Bees (<i>Apis mellifera</i>).....	50
Abstract	50
Introduction.....	50
Materials and Methods.....	54
Results.....	59
Discussion	61
Chapter 4. General Discussion.....	74
Summary and Conclusions	81
References	84

List of Figures

Fig 1.1: Current model of the known and predicted mechanisms of grooming behaviour in <i>A. mellifera</i> against <i>V. destructor</i>	27
Fig 2.1: Mean daily mite mortality rates (DMMR) for (A) high- and low-grooming groups of colonies selected based upon DMMR, and (B) high- and low-grooming groups of colonies with the high-grooming colonies further divided into high- and moderate-damage subcategories.....	46
Fig 2.2: Mean proportion of damaged mites for (A) high- and low-grooming groups of colonies selected based on daily mite mortality rate; and (B) high- and low-grooming groups of colonies with the high-grooming colonies further divided into high- and moderate-damage subcategories	47
Fig 2.3: Proportion of time spent intense grooming by selection & treatment for high-DMMR/high-damage colonies, high-DMMR/moderate-damage colonies, and low-DMMR colonies	48
Fig 3.1: (A) Experimental setup showing the ring stands holding the iPhones used to record video, as well as the condenser microphones held in place by the mic stands—positioned to record audio from the ventilation holes in the top of the cages. (B) A zoomed in photo of an iPhone recording video of a cage of bees	67
Fig 3.2: Mean daily mite mortality rates for selected high- and low-DMMR groups of colonies	68
Fig 3.3: Mean proportion of damaged mites for (A) high- and low-DMMR groups of colonies	68
Fig 3.4: Mean auto-grooming scores for high-DMMR colonies and low-DMMR colonies by stimulus-level (with varroa and chalk dust stimuli pooled).....	69
Fig 3.5: Mean number of allo-grooming events by stimulus-level for high- and low-DMMR colonies (stimulus-type pooled).....	70
Fig 3.6: Mean dance score by stimulus-type and stimulus-level for high- and low-DMMR colonies	71
Fig 3.7: Mean trophallaxis score by stimulus-level for each group.....	72
Fig 3.8: Mean number of worker piping noises by stimulus period for high- and low-DMMR colonies.	73
Fig 4.1: An updated model of the known and predicted mechanisms of grooming behaviour in <i>A. mellifera</i> against <i>V. destructor</i>	83

General Abstract

The parasitic mite *Varroa destructor* is a major driver of global honey bee (*Apis mellifera*) colony losses. The grooming behaviour of bees is a social immunity trait that provides some resistance to varroa. However, the biological mechanisms of the behaviour remain poorly understood, and thus selective breeding for grooming currently relies on imperfect, often indirect measures, such as mite mortality rate or mite damage. This study aimed to elucidate the different mechanisms involved in grooming behaviour by bees, leading to improved breeding methods. I first compared the sensitivity of individual bees from high- and low-grooming colonies after a stimulus of varroa or an alternative stimulus of chalk dust applied to different body regions. I found that high-grooming bees, selected using both the mite mortality rate and rate of mite damage, had heightened responses to both varroa and to chalk dust applied to the head or thorax body regions, compared to unstimulated control bees, and that bees from the low-grooming colonies showed no difference among treatment groups. Further, when high-grooming colonies were selected only based on mite mortality, bees still showed heightened responses to chalk dust on the thorax, however, the responses of bees to mites were not different than control bees. Although chalk dust was a useful alternative irritant to use in place of mites in assays, the increased sensitivity to varroa in the high-grooming bees with high mite damage showed that the use of live varroa in assays may help select for colonies with more precise sensitivities to varroa. Second, I studied high- and low-grooming cohorts of caged bees, examining their responses to varroa and chalk dust. I quantified auto-grooming, allo-grooming, grooming invitation dances, trophallaxis, and acoustic responses before and after stimuli were applied. I found high-grooming bees exhibited more auto-grooming, increased allo-grooming at low levels of either stimulus, increased grooming invitation dancing behaviour at low levels of varroa stimulus, and produced more worker piping noises. The results of this study contribute valuable information to the wider body of knowledge on the biological mechanisms of honey bee grooming behaviour and offer new avenues for further research.

General Introduction

The varroa mite (*Varroa destructor* Anderson and Trueman) is a devastating ectoparasite of honey bees (*Apis mellifera* Linneaus) that harms colony productivity and survival. Varroa is commonly cited as the most damaging pest, pathogen, or parasite that honey bees face (FAO, 2018; Guzmán-Novoa et al., 2010; Rosenkranz et al., 2010). Varroa mite parasitism is associated with the underdevelopment of immature bees (Amdam et al., 2004), desiccation of adult bees (Bowen-Walker & Gunn, 2001), decreased metabolic function (Van Dooremalen et al., 2013), reduced lifespans (Boecking & Spivak, 1999), precocious foraging (Rosenkranz et al., 2010), reduced flight duration and homing ability (Kralj & Fuchs, 2006), as well as suppressed immune response functioning (Nazzi et al., 2012), pesticide detoxification capacity (Drescher & Schneider, 1988), and thermoregulation ability (Blanken et al., 2015). Varroa mites also vector several honey bee viruses and increase the bees' susceptibility to viruses and a range of other pathogens (Emsen et al., 2015). Varroa is a leading cause of increased winter mortality (Amdam et al., 2004; Guzmán-Novoa et al., 2010) and if not controlled, a honey bee colony infested with varroa will usually die within two to three years (De Jong, 1996; Korpela et al., 1992).

Controlling varroa mite infestations is commonly achieved with chemical acaricides, however, these have several disadvantages including cost of application, environmental pollution associated with their disposal, chemical residues remaining in bee products (e.g., wax and honey), and the rapid development of acaricide resistance in mites (Elzen et al., 1999; Milani, 1999; Rinderer et al., 2010; Wallner, 1999). Thus, finding alternative methods to control varroa infestations is increasingly important.

One alternative method of controlling varroa that would help decrease or eliminate reliance on chemical acaricides is the selective breeding of bees for varroa resistance behaviours (Currie & Tahmasbi, 2008; Guarna et al., 2017; Ibrahim et al., 2007; Rinderer et al., 2001). Grooming behaviour is an important behaviour in honey bees that has potential to be developed and used in selective breeding regimes (Guzman-Novoa et al., 2012) but has not been fully exploited by breeding programs.

The objective of my thesis is to better understand the overall mechanisms of grooming behaviour—specifically, how honey bees detect and respond to varroa at both the individual

(auto-grooming) and group (allo-grooming) level. In this thesis, groups of honey bee colonies were first separated into high- and low-grooming groups based on an initial analysis of 200 honey bee colonies involved in a separate experiment at the University of Manitoba. These colonies had their grooming behaviour analyzed at the colony level using a combination of screening methods including the mite mortality rate (when brood was absent) and the proportion of mites with visible physical damage collected on bottom boards. Chosen colonies were grouped into high- or low-grooming categories and worker bees from the selected colonies in each group were then tested to assess grooming responses to mites or standardized stimuli at the individual level, as well as at the group level using small cohorts of bees in cages.

This thesis is written in scientific paper style and consists of two different research studies. Chapter 1 is a literature review that provides relevant background information on the biology of the European honey bee, *Apis mellifera*, and the parasitic varroa mite *Varroa destructor*, as well as what is currently known about the overall mechanisms of grooming behaviour and the different methods of selection for grooming.

Chapter 2 is a study that tested the sensitivities of individual bees from selected high- and low-grooming colonies to a stimulus of either a live varroa mite, a standardized puff of chalk dust to the head, thorax, or abdomen, or no stimulus (control). Video of experimental trials was recorded, and the auto-grooming responses of individual bees were quantified using a grooming timer. This manuscript will be submitted for publication in the International Journal of Parasitology.

Chapter 3 is a study that examined the group grooming and communication responses of small cohorts of caged bees from selected high- and low-grooming colonies when a sequential stimulus of varroa or chalk dust was applied to individual bees through a small window. Video of experimental trials was recorded, and the visual responses measured included auto-grooming, allo-grooming, grooming invitation dances, and trophallaxis. Sound of each experimental cage was also recorded, and different sound responses were quantified, including worker piping responses. This manuscript will be submitted for publication in Behavioral Ecology and Sociobiology, or Animal Behaviour.

Chapter 1. Literature Review

Introduction

This study compares the grooming responses of honey bees (*Apis mellifera*) against parasitic mites (*Varroa destructor*) versus an alternative standardized irritant (chalk dust), both at the individual and group level. The following literature review provides a general overview of *Apis mellifera* and *Varroa destructor* biology, as well as what is currently known and unknown about honey bee grooming behaviour and its potential for controlling *Varroa destructor* infestations.

The European Honey Bee, *Apis mellifera* Linnaeus

Taxonomy and Economic Importance

Honey bees are insects classified under one genus, *Apis*, which includes up to 11 separate species (Crane, 2009; Engel, 1999). According to the United Nations Food and Agriculture Organization (FAO), six of these species are “managed” to some extent worldwide (FAO, 2018), however, only two honey bee species are known to be truly domesticated and intentionally kept in human-made structures for commercial honey and pollination services (Mason & Mason, 1984). These are the Eastern, or Asian honey bee, *Apis cerana* Fabricius, and the Western, or European honey bee, *Apis mellifera*. Of these two, *Apis mellifera* (mellifera = honey bearing) produces larger colonies and thus a larger crop of honey and is the most commonly managed species around the world (Gruszka & Currie, 1998; Winston, 1987).

Several subspecies, or races, of *A. mellifera* exist, which were a product of natural selection in their respective homelands, but have been distributed broadly by humans in different regions of the world (Graham, 1992). Each subspecies has distinctive population-related characteristics such as colony size and seasonal growth patterns that are adapted to their unique regions (Winston, 1987) as well as morphological differences in size, colour, hair coverage, wing venation, and tongue length (Graham, 1992).

Honey bees are kept around the world to produce honey and other hive products (e.g., pollen, wax, and propolis) but are also used for pollination services. In Canada, Agriculture and Agri-food Canada (AAFC, 2020) state that roughly 10,000 beekeepers and 775,000 honey bee colonies produce about 80–90 million pounds of honey annually with an estimated economic

value of about CAD \$173 million. However, the additional harvest yields of orchard fruits, berries, vegetables, forage, and hybrid canola seed that is attributed to honey bee pollination services is valued at roughly 30 times that at CAD \$5.5 billion (AAFC, 2020). Worldwide, or at least in developed countries, honey bees are estimated to contribute to the production of about one-third of the food humans consume through their pollination of important agricultural crops (Delaplane et al., 2000; McGregor, 1976). Thus, the maintenance of strong, healthy, productive honey bee colonies is of great importance for the global economy as well as the world's food supply.

Biology and Life Cycle

A honey bee colony is made up of three castes: a single fertile female queen, up to 60,000 sterile female workers, and a varying number of males called drones. These castes differ in their size, shape, sex, and specific functions within the colony (Gruszka & Currie, 1998).

The queen plays a major role in the colony and is typically the only fertilized female in a colony. A honey bee queen mates with an average of 7–17 drones during flight and then spends the rest of her life in the hive, laying eggs in wax cells constructed by worker bees (Winston, 1987). Honey bees reproduce sexually but under a sex-determination system called haplodiploidy, whereby the queen, through controlling the release of sperm prior to egg laying, can choose whether to lay unfertilized (haploid) or fertilized (diploid) eggs. Normally, haploid eggs (which have 16 single chromosomes) develop into drones and diploid eggs (with 32 chromosomes) develop into workers or queens (Guzman-Novoa, 2007; Winston, 1987).

All eggs hatch after three days and develop into larvae. Fertilized eggs that hatch and are destined to become workers have a larval period that lasts 5–6 days, during which the larva moults four times, moving from 1st to 5th instar. Adult workers in the colony then seal the 5th instar in the cell with a wax capping. The sealed brood stage lasts 11–12 days, during which the larva spins a cocoon, moults a fifth time turning into a pupa, then undergoes a final moult before finally chewing through the wax capping and emerging as an adult. Similar developmental processes occur with unfertilized eggs that are destined to become males. The entire development process generally takes 21 days for a worker and 24 days for a drone, with some variation in development times attributed to temperature, nutrition, and genetics (Winston,

1987). Immature workers and drones are the major sources of breeding sites for varroa mites in the colony (Rosenkranz et al., 2010).

In contrast to the queen, diploid but unfertilized adult workers carry out most of the tasks within the hive. Some major tasks include nursing and feeding brood, tending the queen, cleaning cells, building comb, carrying out hygienic tasks (e.g., removing dead brood), defending the colony, and foraging for nectar and pollen. Worker bees follow an age-based division of labour (known as temporal polyethism), moving from specialized tasks within the hive to tasks outside the hive as they age (Winston, 1987).

Communication Among Bees

Honey bees communicate with nestmates through a combination of pheromones, dances, shaking/vibrational signals, sounds, and trophallaxis (food sharing) (Kirchner, 1993a; Winston, 1987). Together, these processes convey tremendous amounts of information to other colony members and serve to control and direct bee activity, behaviour, and physiological processes as described further below.

Pheromones

A large array of pheromones emitted by queens, workers, and developing brood control many behavioural and physiological processes within the hive. For example, the queen's retinue pheromone (QRP) has multiple functions including both releaser and primer effects such as attracting workers to the queen, functioning as a long-distance sex pheromone to attract drones during mating flights, and inhibiting the ovary development of worker bees (Trhlin & Rajchard, 2011; Wanner et al., 2007). Workers also produce and use several releaser pheromones. Their alarm pheromones associated with sting glands modulate defensive behaviour (Pankiw, 2004). The worker Nasanov gland also produces a pheromone that acts as an attractant, which is used for aggregation, especially during swarming and to attract bees to odourless forage sources (Trhlin & Rajchard, 2011; Wells et al., 1993). Research by Thom et al. (2007) also suggests that pheromones are given off by waggle dancing bees, serving as attractants that recruit more foragers to the dance floor, increase the number of foragers leaving the hive, and likely pass on more informative cues about the forage source. Pheromones given off by developing brood commonly have primer-like functions, stimulating nurse bees' hypopharyngeal glands,

regulating the behavioural maturation of workers, further inhibiting worker ovary development, and triggering the capping of brood cells (Pankiw, 2004; Trhlin & Rajchard, 2011). Some primer-like pheromones also may be produced by older worker bees with evidence of compounds that delay the onset age of foraging in younger workers (Leoncini et al., 2004).

Dance Language

The honey bee dance language serves many functions: to recruit other worker bees to resources—nectar- or pollen-producing flowers—or new nesting sites, stimulate foraging or swarming, provide cross-inhibition signals (i.e. stop signals) to discourage further recruitment to lower quality food sources or nesting sites, or solicit social grooming from nestmates (Land & Seeley, 2004; Seeley et al., 2012; Von Frisch, 1967; Von Frisch & Ilse, 1955; Winston, 1987). The “round dance”, for example, essentially informs other bees in the colony about resources that are in close proximity to the colony—usually within 15 m (Von Frisch, 1967; Winston, 1987). The waggle dance informs other bees of resources further away from the colony, however, much more information about the distance, direction, and quality of the resources are also conveyed (Von Frisch, 1967). Waggle dances on average last about 10 minutes (Seeley, 1995). Other known dances fall under the categories of shaking signals (Allen, 1956), tremble dances (Seeley, 1992), and grooming invitation dances (Haydak, 1945), which may be shorter in duration.

A variety of different vibrational signals are described in the literature. Shaking signals have been previously referred to as dorsoventral abdominal vibrations (Milum, 1955), jerking dances (Von Frisch, 1967), shaking dances (Gahl, 1975), or vibration dances (Schneider, 1986). However, the term dorsoventral abdominal vibration is not appropriate since it was learned that in performing the signal a bee shakes its entire body (Gahl, 1975). Similarly, Seeley et al. (1998) argued that calling these signals dances is unfitting due to their brief nature compared to the much longer round and waggle dances. Allen (1956) coined the term ‘shaking’ or ‘shaking signal’ for these responses, which may be most appropriate (Seeley et al., 1998). Regardless of their name, the “shaking signal” is when a worker bee grasps a nestmate and “vibrates her own body dorsoventrally for 1-2 seconds at about 16 Hz” (Seeley et al., 1998). The bee will usually then continue to shake other bees or may also shake the comb, and it is thought that these signals are used to stimulate other bees to prepare for periods of higher activity, for example during

times of abundant foraging or prior to swarming. Seeley et al. (1998), for instance, found that when they removed and then reintroduced a food source to a colony, the first bees to find the food and return to the colony produced shaking signals, and it was only later that the bees began to produce waggle dances. They concluded that shaking signals and waggle dances function together to stimulate a colony's foraging activity. Shaking dances may also serve to stimulate the activity of queens before swarming or leaving the colony on a mating flight, again supporting the theory that the message of the signal is to prepare for increased activity (Allen, 1956; Schneider, 1991).

A different form of vibrational signalling are tremble dances that are described as when “a forager shakes her body back and forth, at the same time rotating her body axis by about 50° every second or so, all the while walking slowly across the comb” (Seeley, 1992). Vibrational movements in the tremble dance have about the same frequency as waggle dances—10 to 15 Hz—and the dances may last for many minutes; Seeley (1992) recorded average tremble dance durations of 27 minutes. These dances occur in great frequency when the time it takes foragers to find nectar receiving bees to offload their nectar is prolonged, suggesting an important role in regulating foraging (Kirchner & Lindauer, 1994; Seeley, 1992). However, it is thought that tremble dances may have multiple meanings, serving to both increase the number of nectar receiving bees as well as inhibit further recruitment to food sources by waggle dancers, thus matching the nectar processing capacity of the colony with its nectar intake during times of increased nectar influx (Kirchner, 1993b; Seeley, 1992; Seeley et al., 1996).

Another known signal is the grooming invitation dance, which is performed by worker bees to solicit social allo-grooming responses from nestmates (Bozic & Valentincic, 1995; Haydak, 1945; Land & Seeley, 2004; Milum, 1955; Pettis & Pankiw, 1998). This dance resembles a tremble dance; however, it has subtle differences and conveys much different information. Land and Seeley (2004) describe the dance as when a worker bee “stands stationary and vibrates her whole body from side-to-side at a frequency of 4.2 ± 0.2 Hz.” Thus, the dancing frequency is much lower and the dances are also relatively short, often lasting only a few seconds. Land and Seeley (2004) noted an average duration of 9.3 ± 1.0 seconds in a study of grooming behaviour. More information on grooming invitation dances in response to varroa is covered below (page 18).

Vibroacoustic Components

The cavity nesting honey bee species, *Apis mellifera* and *Apis cerana*, perform most colony behaviours in the dark where intra-colony communication is partly achieved through a variety of vibrational and acoustic signals (Kirchner et al., 1991; Towne & Kirchner, 1989), collectively known as vibroacoustics (Hunt & Richard, 2013). Sound waves have both pressure and particle movement components (McNeil, 2015) and bees can hear by means of detecting the air-particle movements of airborne sounds (Towne & Kirchner, 1989) with the Johnston's organ—a chordotonal organ found in the pedicel of the antennae (Dreller & Kirchner, 1993; Snodgrass, 1956; Tsujiuchi et al., 2007) which is sensitive to vibrations of the antennal flagellum (Kirchner, 1993a). It is known that the acoustical sense of bees is sensitive enough to detect air particle movements within several millimeters of other sound-emitting bees (Towne & Kirchner, 1989). However, according to Tsujiuchi et al. (2007), the Johnston's organ is not fully formed at early adult stages (0-11 day old workers) and honey bee hearing matures with age, becoming more sensitive in 21-day-old foragers. Thus, Tsujiuchi et al. (2007) claim that only older foragers can effectively decode dance language. However, bees can also detect substrate vibrations with the highly sensitive subgenual organs located within the tibia of their legs and will regularly stop and freeze in response to artificially-generated comb vibrations, which may occur across a broader range of ages. (Autrum & Schneider, 1948; Frings & Little, 1957; Michelsen et al., 1986b). Bees as young as 3 days old, for example, engage in vibration (aka “shaking”) signals (Painter-Kurt & Schneider, 1998), indicating younger bees also participate in vibro-acoustical communication signals to some extent.

To quantify sound in biological systems like bee hives, sound frequency, amplitude, and velocity are used. Frequency is measured in Hertz (Hz) and different frequencies of sound waves are known as ‘pitch’, with higher frequencies producing higher pitch. Amplitude is a measure of the displacement of a medium from its equilibrium and is often measured in millimeters (mm) or micrometres (μm) from peak to peak. Velocity is a measure of the rate of displacement per unit time, often measured in mm/s. Bees generate vibrations producing sound in a wide range of frequencies—from less than 10 Hz to more than 1000 Hz—using their wings as well as their thoracic muscles (Kirchner, 1993a; McNeil, 2015). To a human ear, the sounds they generate range from lower-pitched buzzes to higher-pitched squeaks, beeps, and pipes

(Michelsen et al., 1986b; Schlegel et al., 2012; Thom et al., 2003). It has been shown that bees can detect sound frequencies in the range of about 10 Hz up to about 500 Hz (Kirchner et al., 1991) and the hearing threshold is roughly constant across all detectable frequencies, at velocities of about 100-300 mm/s (Kirchner et al., 1991).

Of the different sounds made by honey bees, some of the most well-known are the piping signals of the queens (Huber, 1792; Michelsen et al., 1986a). Often referred to generally as queen piping, these sounds produced by honey bee queens can be broken up into toots, which are produced by recently emerged virgin queens, or quacks, which are produced by queens still in their queen cells in response to toots (Kirchner, 1993a; Michelsen et al., 1986a). These low frequency (~400 Hz) sounds are produced by queens rapidly contracting their thoracic muscles against the honeycomb (Kirchner, 1993a) and are generally higher in amplitude than other dance sounds as they aim to be directed at a “broader audience” (Michelsen et al., 1986a; Nieh & Tautz, 2000). The piping signals allow newly emerged queens to locate non-emerged queens and they play a role in regulating swarming behaviour (Kirchner, 1993a; Michelsen et al., 1986a).

Sound and vibrational signals also play an important role in the honey bee dance language (Esch, 1961; Kirchner, 1993b; Michelsen et al., 1987; Thom et al., 2003; Wenner, 1962). It is known that bees use a combination of these cues (Nieh & Tautz, 2000), as well as other cues such as tactile contact (Rohrseitz & Tautz, 1999), odour (Von Frisch, 1967), and temperature cues (Stabentheiner & Hagmüller, 1991) to find and follow waggle dancers. Esch (1961) and Wenner (1962) were the first to show that bees performing waggle dances produce sound, and Kirchner et al. (1988) also found that sounds are produced during round dances. Dancing bees vibrate their wings and waggle their abdomens, producing substrate vibrations, near field sounds, and air jets (Dreller & Kirchner, 1993; Michelsen, 2003; Michelsen et al., 1986b; Nieh & Tautz, 2000). A dancer waggles her abdomen in 15 Hz wagging motions and produces near field dance sounds with her wings in the range of 200-300 Hz (Michelsen et al., 1986b; Spangler, 1991). These sound vibrations occur at much lower amplitudes than the tooting and quacking of queens due to being directed at a smaller, local audience of workers in close proximity (Michelsen et al., 1986a; Nieh & Tautz, 2000). Dance sounds have also been noted in the context of tremble dancing (Kirchner, 1993b; Nieh, 1993, 2010; Schlegel et al., 2012; Seeley et al., 2012; Thom et al., 2003). Originally referred to generally as worker piping, these high-

pitched noises serve as “stop signals” that inhibit further waggle dancing and thus further recruitment to lower-quality food sources (Thom et al., 2003) or nesting sites (Seeley et al., 2012). While it is still currently held that these stop signals exist, Schlegel et al. (2012) uncovered another distinct sound hidden within the stop signal—having a significantly different duration, fundamental frequency, and frequency modulation. They re-classified the lower frequency (407 Hz) stop signals as “beeping” and retained the term “piping” to describe the higher pitched (451 Hz) signals. The authors claim this newly discovered signal is used in similar fashion to the shaking signal, with a bee grasping onto a nearby resting bee and pressing her body against the other bee while vibrating her wing muscles. According to Schlegel et al. (2012), these piping signals cause excitation of quietly resting bees and, in the context of swarming, causes them to warm up their flight muscles in preparation for the swarm’s departure.

In *A. cerana*, other studies have found worker bees in colonies under attack by hornets produce alarm signals in the form of audible pipes (Ohtani & Kamada, 1980), group hissing (Kawakita et al., 2018), or specific anti-predator pipes that correlate with the arrival of hornets, as well as increased predator-specific activity at the nest entrance (Mattila et al., 2021).

Trophallaxis

Honey bee trophallaxis refers to the process of food transmission between workers, or between workers and queens or drones. Older nectar foraging bees that return to the hive with their honey stomachs full of nectar pass their nectar loads off to (multiple) younger nectar receiving bees who antennate each other and protrude their tongues (proboscises) to sip the regurgitated nectar (Winston, 1987). Many more trophallaxis interactions then occur between workers within the colony. Nixon and Ribbands (1952) showed that over half of a colony’s worker population contained radioactive nectar in their honey stomachs just 27 hours after only 6 foragers had brought it into the colony. Much information about the availability and quality of nectar can be passed on through trophallactic interactions (Graham, 1992). However, Korst and Velthuis (1982) demonstrated that many trophallaxis interactions result in very little or no actual food transfer and that most trophallactic interactions have a communicative function. Most interactions are brief, lasting only one to five seconds, and fewer last longer than 10 seconds (Istomina-Tsvetkova, 1953). Trophallaxis interactions involve substantial antennal contact and act as an important medium to pass pheromones (e.g. queen pheromone) throughout the colony

(Graham, 1992). However, while trophallaxis is obviously an important colony function, Geffre et al. (2020) demonstrated that honey bee colonies may also have adaptive responses to suppress transmission of viral infections by decreasing the number of trophallaxis interactions in the colony, thus limiting the spread of pathogens.

Honey Bee Pests, Pathogens, and Parasites

Though incredibly productive when healthy, a honey bee colony is also vulnerable to a variety of pests, pathogens, and parasites, including bacteria, fungi, viruses, mites, other insects, and animal predators that may act in combination with other stressors (e.g. nutritional stress, poor weather, and pesticides) resulting in colony losses and significant economic damage if not avoided or controlled (Currie et al., 2010; Pernal & Clay, 2013). It is widely agreed that the most damaging of these is the invasive ectoparasitic mite, *Varroa destructor* (FAO, 2018; Guzmán-Novoa et al., 2010; Rosenkranz et al., 2010).

***Varroa destructor* Anderson and Trueman**

Taxonomy and Host-shift

Until the year 2000, the genus *Varroa* was thought to be comprised of just three distinct species of mites: *Varroa jacobsoni*, *Varroa underwoodi*, and *Varroa rindereri* (Anderson & Trueman, 2000). However, researchers had previously noted that varroa mites were having different impacts on their bee hosts in different regions of the world (Delfinado & Baker, 1974). DNA sequencing by Anderson and Trueman (2000) confirmed that *Varroa jacobsoni* (originally described by Oudemans in 1904) was a species complex, consisting of individuals belonging to two separate species. Thus, the name *Varroa destructor* Anderson and Trueman was given to the larger less spherical, and more damaging species of mite. A much more virulent mite, *V. destructor* is the species found on *A. mellifera* as well as on its original host, *A. cerana*, while the less virulent *V. jacobsoni* remains restricted to *A. cerana*, and completely lacks the ability to reproduce in colonies of *A. mellifera* (Anderson, 1994; Anderson & Sukarsih, 1996).

Though the details are unclear, it is believed that *V. destructor* underwent a host shift from *A. cerana* to *A. mellifera* during the mid-twentieth century when colonies of *A. mellifera* were introduced into Asia (Anderson & Trueman, 2000; Dietemann et al., 2012). Through global trade and natural dispersal, the mite then spread quickly around the world and can now be found

in almost all corners of the globe where honey bees are present, except for (at the current time) Australia and the province of New Brunswick in Canada, both of which are under strict quarantine (DPIRD, 2015; NLBKA, 2020). While *A. cerana* has established a stable host-parasite relationship with *Varroa* mites over a long history of co-evolution (Rath, 1999), *V. destructor* found a much more susceptible host in *A. mellifera*, and is now considered the number one problem for honey bee colonies and the beekeeping industry worldwide (Invernizzi et al., 2016; Le Conte et al., 2010).

Biology and Life Cycle

Varroa destructor (hereafter referred to simply as varroa) is an obligate ectoparasitic mite of honey bees (Rosenkranz et al., 2010). There are two distinct life-phases in the life cycle of a female varroa mite: a phase on adult bees commonly referred to in the honey bee literature as the “phoretic” phase and a reproduction phase. During the phoretic phase, female varroa mites feed on adult bees and are usually found on the abdomen (metasoma), wedged underneath the overlapping terga or sterna (abdominal plates) (Fernández et al., 1993)—particularly on the left ventral side of the bee, underneath the sternite of the third metasomal segment (Ramsey et al., 2019). Varroa mites may also be found on the mesosoma, however, Ramsey et al. (2019) found that all mites collected from the mesosoma showed no evidence of feeding in this area. On adult bees, varroa mites feed primarily but not exclusively on fat bodies with a preference for selecting younger nurse bees, likely because of an increased fitness benefit from their larger fat bodies (Ramsey et al., 2019; Xie et al., 2016). During periods of brood production, varroa mites remain attached to adult bees between 1 and 13 days, with an average of about 7 days (Beetsma et al., 1999; Martin, 1998; Ramsey et al., 2019). Because of the inherent risks that exist with a prolonged phoretic stage (e.g. grooming), it is believed that this stage must confer some physiological benefit (Xie et al., 2016). Häußermann et al. (2016) suggested that the phoretic stage is required for spermatozoa capacitation (maturation) which generally takes 5 days. The phoretic phase can also last much longer, however, as varroa mites overwinter readily on adult bees for months in temperate climates (Boecking & Genersch, 2008).

Varroa mites reproduce in the brood of honey bee colonies, either in worker or drone brood, but show a stronger preference for drone brood (Boot et al., 1995; Calderone & Kuenen, 2001; Fuchs, 1990). During honey bee development, a female “foundress” mite enters the cell of

a 5th instar larva, just before the cell is capped over by worker bees (Rosenkranz et al., 2010). The mite slips down between the cell wall and the developing larva to the bottom of the cell and becomes stuck within the brood food. About 5h after the cell is capped and the larva has finished consuming the remainder of its food, the mite moves and begins feeding on the developing larva, starting to lay eggs approximately 60-70h after the cell is capped (Ifantidis, 1983; Steiner et al., 1994). The first egg is normally unfertilized and will develop into a male mite, whereas the remainder are fertilized female eggs, laid in 30h intervals (Ifantidis, 1983; Rehm & Ritter, 1989). Normally, up to 5 eggs are laid in worker brood and up to 6 in drone brood (Garrido & Rosenkranz, 2003; Martin, 1995a; Martin, 1994).

The mother mite creates a single feeding hole in the pupa for the nymphs to feed, generally on the 5th segment of the bee pupa, near to the “fecal accumulation site” at the base of the cell (Donzé & Guerin, 1994). Male mites are smaller than females (Rosenkranz et al., 2010). The male mite reaches sexual maturity about 20 hours before the first female and begins to mate with his sisters as they each become sexually mature (Donzé et al., 1996). Male mites do not emerge from the cells and die soon after mating. The average number of mites produced under natural conditions, calculated by Martin (1994) and Martin (1995b) are about 1.3-1.45 in worker brood and about 2.2-2.6 in drone brood, due to the longer developmental time during capping for drones. Varroa mother mites also carry out multiple reproductive cycles throughout their lifetime (usually two or three) (Martin & Kemp, 1997), thus each mite would produce approximately 3 to 4 offspring in worker brood and 4 to 8 offspring in drone brood.

Impact on *Apis mellifera* L.

Varroa has many negative effects on immature bees and adults that individually and cumulatively impact honey bee colonies. For example, immature bees infested with multiple mites during their development show degenerated fat bodies and underdeveloped hypopharyngeal glands (Boecking & Spivak, 1999). Parasitized bees exhibit significant weight loss at emergence (De Jong et al., 1982; Duay et al., 2003), deformed wings, and decreased flight performance (Duay et al., 2002). Bees infested as immatures also begin foraging earlier (Janmaat & Winston, 2000), and have significantly reduced lifespans (Amdam et al., 2004; De Jong et al., 1982; Schneider & Drescher, 1987). Mites feeding on adult bees result in a reduction in fat body and suppress a honey bee’s natural immune response (Annoscia et al., 2019; Yang & Cox-Foster,

2007; Yang & Cox-Foster, 2005), pesticide detoxification ability (Blanken et al., 2015; Drescher & Schneider, 1988), and thermoregulation capacity (Amdam et al., 2004), thus making them more vulnerable to secondary pathogens (e.g. viruses) and chemical pesticides. This may decrease a colony's overwintering success if infestations are high enough (Ramsey et al., 2019). Varroa mites are known to vector several honey bee viruses (Emsen et al., 2015) and mites can also affect the flight duration and homing ability of honey bees by impairing their orientation ability (Kralj & Fuchs, 2006).

The varroa mite originally parasitized and evolved on *A. cerana* where a balanced host-parasite relationship developed (Rath, 1999). *Apis cerana* has evolved multiple resistance mechanisms to varroa mites including heightened grooming behaviour (Peng et al., 1987) and suppressed mite reproduction in worker brood (Spivak, 1996). In contrast, varroa found a much less resistant host in *A. mellifera*, and if varroa mites are not regulated by the beekeeper on this species, an infested colony will usually die within one to three years (Fries et al., 1996). Varroa has had a profound effect on the economic costs associated with producing honey and offering pollination services, through diminished honey yields (Currie & Gath, 2006), increased costs associated with controlling mite populations (Rinderer et al., 2010), and the replacement of colonies lost as a result of varroa infestation. Sanford (2001) states that the varroa mite has killed millions of colonies worldwide and has caused the loss of billions of dollars in agricultural crops.

Control of *Varroa destructor*

Controlling varroa infestations is most commonly achieved with chemical acaricides. However, these treatments not only increase cost of beekeeping, but chemical residues can also remain in wax and honey (Mullin et al., 2010; Wallner, 1999), potentially resulting in sublethal impacts on bees (Lodesani & Costa, 2005; Pettis et al., 2004) or contamination of food products (Bogdanov et al., 1997; Wallner, 1999). Moreover, the repeated use of these chemicals can quickly lead to the development of acaricide-resistant mites (Elzen et al., 1999; Lodesani & Costa, 2005; Milani, 1999) resulting in a repeated need for the development of new treatments.

Alternative, non-synthetic treatments have been developed that rely on organic acids (e.g., formic acid and oxalic acid) or essential oils (e.g., thymol). These treatments provide good, albeit often less reliable control of varroa mites sometimes targeting mites in different ways (Rosenkranz et al., 2010). Formic acid, for example, is the only known acaricide that can

effectively kill varroa mites within capped brood cells (Fries, 1991; Roth et al., 2020). However, these “soft chemicals” are still costly and not without side effects. For example, organic acid and essential oil treatments can be hazardous to the beekeeper during application. Formic acid and thymol can have detrimental impacts on developing brood or cause queen supersedure (replacement) when applied at high doses or high temperatures (Elzen et al., 2004; Fries, 1989; Underwood & Currie, 2004; Vandervalk et al., 2014), and thymol and oxalic acid may impact honey taste if applied during the honey flow (Bogdanov et al., 1999). Oxalic acid may also be toxic to bees or brood at high doses (Higes et al., 1999; Toomemaa et al., 2010). Thus, researching and developing non-chemical methods of control is extremely important to decrease reliance on chemical acaricides.

Some of the better known non-chemical control methods include: mite-trapping through the removal of capped drone brood containing mites, using screened bottom boards, interruption of brood rearing, and the development of varroa-tolerant or resistant strains of honey bees through selective breeding (Roth et al., 2020). The latter involves capitalizing on natural social immunity behaviours of honey bees and is most ideal as it has the greatest potential to reduce or eliminate the need for additional varroa control measures and would have low costs associated with it once resistance is established (Rinderer et al., 2010).

Social Immunity Behaviours of Honey Bees

A honey bee colony has at its disposal a variety of mechanisms and behaviours that help it fend off unwanted pests, pathogens, and parasites—ranging from underlying “constitutive” mechanisms to “inducible” behavioural traits (Simone-Finstrom, 2017). On the “constitutive” end of the spectrum, polyandry (multiple mating), where a queen mates with multiple drones, contributes to an increase in genetic diversity within the hive, which is known to improve the overall health of gut microbial communities, as well as resistance to pathogens and parasites (Bailey, 1999; Desai & Currie, 2015; Tarpy, 2003; Tarpy & Seeley, 2006). Task allocation also relates to a form of social immunity within the hive. The division of labour structured around the age of worker bees (also known as temporal polyethism), whereby younger workers perform “inside” tasks and older workers outside tasks, helps shield younger bees, as well as the queen and brood being tended to by young nurse bees, from diseases, parasites, and other dangers in outer regions of the colony beyond the brood nest or from those outside the colony (Simone-

Finstrom, 2017). The collection of resin (i.e. propolis) with antimicrobial properties that bees use to line their nests also contributes to lower overall microbial load in the colony (Simone et al., 2009) and can have direct effects against brood pathogens like American Foulbrood bacterium and chalkbrood fungus (Borba & Spivak, 2017; Simone-Finstrom & Spivak, 2012).

On the “inducible” end of the spectrum, social fever is a behavioural mechanism where bees increase the temperature of the brood nest to inhibit the development of pathogens such as chalkbrood fungal spores in infected larvae (Starks et al., 2000). Two other known inducible behavioural defenses that can defend against varroa mites more directly are hygienic brood removal behaviour and adult grooming behaviour (Boecking & Spivak, 1999).

Boecking and Spivak (1999) show that hygienic brood removal behaviour is the primary mechanism by which *A. mellifera* colonies resist brood diseases such as American Foulbrood and chalkbrood. Hygienic workers detect diseased brood, uncap the wax covering the brood cells, and remove the infected larvae or pupae from the colony (Boecking & Spivak, 1999). Varroa Sensitive Hygiene is a form of hygienic behaviour that targets brood infected with varroa more directly (Danka et al., 2011). This behaviour involves workers selectively uncapping cells infested with varroa mites and removing the infested larvae or pupae from the colony (Rinderer et al., 2010). During this process, it is possible for mites to be expelled, although it is more likely that adult female mites will transfer onto the bees removing the brood or become free on the combs, making them vulnerable to attack by bees or exposed to grooming (Boecking & Spivak, 1999). Whether expelled or not, however, removing mites from brood cells also leads to an interruption in the reproductive cycle of the mites (Rosenkranz et al., 2010), which can suppress the rate at which mite populations increase within a honey bee colony.

Grooming Behaviour

Grooming behaviour involves biting and licking with the mouthparts and movement of the pro and/or mesothoracic legs, whereby bees can remove dust and pollen, as well as ectoparasites from their bodies or those of their nestmates (Boecking & Spivak, 1999). Worker bees often clean themselves using a process referred to as auto-grooming; for example, using the antenna cleaner (strigilis) on their prothoracic legs to clean their antennae (Winston, 1987). Auto-grooming activities can often be witnessed at flowers, in flight, and in the hive (Boecking & Spivak, 1999). Bees may also clean each other (referred to as allo-grooming), and bees in need

of grooming can recruit one or more nestmates by means of a specific grooming invitation dance (Haydak, 1945; Land & Seeley, 2004).

The Grooming Invitation Dance

The grooming invitation dance, first described by Haydak (1945), and further characterized by Milum (1955) and Land and Seeley (2004), is described as a worker bee “standing with her legs spread and tightly gripping the comb while rocking her whole body side-to-side in a plane parallel to the comb” (Land & Seeley, 2004). Pettis and Pankiw (1998) demonstrated that grooming behaviour is an effective mechanism against honey bee tracheal mites (*Acarapis woodi* Rennie) in *A. mellifera*, and that young worker bees exhibiting higher levels of grooming dances acquire the lowest number of mites. Land and Seeley (2004) showed that *A. mellifera* bees stimulated with a puff of chalk dust groomed themselves in most trials and performed grooming invitation dances in about half, proving that small particles can be used to stimulate grooming behaviour. Such behaviours that have evolved as “generalized responses” to a variety of potential irritants may also play a role in defence against the “evolutionarily recent” exposure of *A. mellifera* to the parasite, *V. destructor*.

Grooming Behaviour Against Varroa in *A. cerana* vs *A. mellifera*

Grooming dances are also found in *V. destructor*'s original host *A. cerana* where they facilitate recruitment of bees for defence against varroa mites (Peng et al., 1987). Peng et al. (1987) were the first to show that grooming behaviour is one of the main mechanisms of resistance in *A. cerana* against varroa mites. They found that grooming results in the removal and death of varroa mites, and described grooming as a series of actions, including auto-grooming, dancing, allo-grooming, and group cleaning. In an experiment using glass-walled observation hives and artificial inoculation with varroa mites, specific instances of allo-grooming were witnessed, as well as the physical removal and subsequent damage of mites from nestmate bees' bodies using their mandibles. Additionally, they found 99.6% of mites were removed within 2 hours with 73.8% of them having visible physical injuries. They suggest that a combination of this strong grooming behaviour and varroa sensitive hygiene limits infestations in *A. cerana* and ensures the survival of the colony. In a study of 64 infested colonies in southern Thailand, Rath and Drescher (1990) found an average of just 70 mites per colony, with a

maximum number of 798 mites, which is well below damaging levels in strong colonies of *A. mellifera* (Currie & Gatién, 2006).

In *A. mellifera*, Peng et al. (1987) found that grooming behaviour is present but minimal, and that *A. mellifera* usually fail to remove mites. In the same experiment mentioned above, they found that only 16.6% of workers engage in auto- or allo-grooming actions, and of these only 0.3% of mites are successfully removed. Further studies, however, have found higher grooming success in *A. mellifera* colonies, though still not reaching levels found in *A. cerana* (Boecking et al., 1993; Buchler et al., 1992; Fries et al., 1996; Guzman-Novoa et al., 2012; Hamiduzzaman et al., 2017). For example, Buchler et al. (1992) found successful removal of 75% of mites in *A. cerana* versus 48% for *A. mellifera* in an experiment that artificially infested individual workers through small windows in the glass walls of observation hives. Other recent studies have also demonstrated a range of grooming effectiveness and intensity across different *A. mellifera* subspecies. Africanized subspecies (descendants of the subspecies *A. mellifera scutellata*), for example, have been shown to exhibit more efficient grooming than European subspecies (Aumeier, 2001; Guzman-Novoa et al., 2012). Similarly, Rinderer et al. (2001) showed that bees originating from the Primorsky region of Russia engage in more grooming activity than bees from Louisiana, USA; and Ruttner and Hänel (1992) and Thakur et al. (1997) showed evidence of enhanced grooming behaviour in *Apis mellifera carnica*, a subspecies originating from Slovenia. These studies show that there is potentially a range of genetic diversity through which selection could be used to improve this trait in bee stock.

Damaged Mites as an Indicator of Grooming Behaviour

As in Peng's study with *A. cerana*, damaged mites on bottom boards have also been found in varroa-infested colonies of *A. mellifera*, and it is commonly assumed that damage to mites occurs as a result of grooming (Arechavaleta-Velasco & Guzman-Novoa, 2001; Boecking & Ritter, 1993; Fries et al., 1996; Hunt et al., 2016; Moosbeckhofer, 1992; Ruttner & Hänel, 1992; Wallner, 1989). In a study using *A. mellifera carnica* bees, for example, Ruttner and Hänel (1992) found that in 12 colonies selected by a beekeeper for low varroa mite population growth, 30 to 50% of mites on bottom boards had damage to their legs or idiosoma (main body), and claimed that the damage was caused by the mandibles of workers. Thakur et al. (1997) also recorded video evidence of aggressive behaviour of *A. mellifera carnica* worker bees against

varroa mites causing damage. Additionally, Moosbeckhofer (1992) found a significant negative correlation between the percentage of damaged mites on bottom boards and the overall colony infestation, indicating that mite damage may be an effective tool to select for grooming behaviour. More recently, Guzman-Novoa et al. (2012) also found a link between the percentage of injured mites found on sticky boards and reductions in overall mite infestation at the colony level, as well as direct observations of heightened grooming intensity and successful mite removal by individual bees from the same colonies in laboratory assays using petri dishes. Since grooming behaviour is often difficult and time-consuming to measure through direct observation, damaged mites have often been used as an indirect measurement of grooming.

However, the use of mite damage as an indicator of grooming behaviour, has also been heavily criticized and debated. Some argue, for example, that damage to mites may be due to other factors, such as other predators in the hive (ants or wax moth larvae) (Bienefeld et al., 1999; Szabo et al., 1996), or the cleaning of cells with infested brood (hygienic behaviour) rather than through grooming (Boecking & Spivak, 1999; Rosenkranz et al., 1997). It is also possible that mites could be damaged by bees after they have died through mechanisms unrelated to grooming or that mites are damaged when detected on comb or on the bottom board after natural “mite fall” unrelated to grooming. Corrêa-Marques et al. (2000) found no correlation between the percentage of damaged mites found in the hive debris on bottom boards and the overall colony mite infestation—however, this study included dimples on the dorsal shields of mites as a classification of mite damage, which has since been discredited (Davis, 2009). Harbo and Harris (1999) also doubted whether grooming is effective at all after finding low heritability values for mite damage rates, although the authors again included dorsal dimples in their analyses. When the authors removed dorsal dimples, heritability values drastically improved—albeit to marginal levels but not low enough to dismiss. Overall, it is possible that using damaged mites as an indicator of grooming behaviour may be suitable in some cases but may also not recognize the full suite of behaviours that can lead to the successful grooming of mites and distinguish among the many factors that can result in mite damage.

Mite Mortality Rate as a Measure of Grooming

Another indirect measure of grooming behaviour is to measure the mite mortality rate. This rate compares the number of mites falling onto sticky papers placed onto bottom boards of

hives, or the bottom drawers of small cages, relative to the overall infestation level in a colony or the known or estimated total number of mites present in the colony or cage. For example, in a study comparing different varroa-resistance strategies of bees previously selected for high and low varroa mite population growth, Arechavaleta-Velasco and Guzman-Novoa (2001) showed that bees from colonies with low varroa mite population growth had significantly higher mite mortality rates in cage studies where 30 bees were artificially inoculated with 10 varroa mites. Many studies have also used the mite mortality rate as a means of assessing grooming behaviour in the absence of brood (Andino & Hunt, 2011; Arechavaleta-Velasco & Guzman-Novoa, 2001; Bahreini & Currie, 2015a; Currie & Tahmasbi, 2008). Andino and Hunt (2011) also showed a significant negative correlation between the percentage of mites removed in cage assays and the overall mite infestation in source colonies. In another study looking at the effect of temperature and humidity on levels of grooming behaviour, Currie and Tahmasbi (2008) first screened colonies for high- or low-grooming behaviour by means of collecting fallen mites in small cages of bees where initial infestations were known and calculated a mite mortality rate for the 6 days that bees were in cages. Colonies were then further screened for grooming performance at different temperatures and humidity and the mite mortality rates were again used as a grooming performance metric. The sole use of mite mortality rates as a measure of grooming is confounded by natural mite mortality when brood is absent and mite mortality is related to varroa-sensitive hygiene or hygienic brood removal when brood is present in a colony.

Individual Bee Assays to Assess Grooming Behaviour

Much of the recent research on grooming behaviour has focused on analyzing the grooming responses of individual bees directly in laboratory studies when challenged with live varroa mites (Aumeier, 2001; Bak & Wilde, 2015; Guzman-Novoa et al., 2012; Hamiduzzaman et al., 2017; Invernizzi et al., 2015). Guzman-Novoa et al. (2012), for example, found that individual bees from varroa “resistant” colonies performed significantly more instances of intense grooming behaviour and successfully removed significantly more mites compared to bees from varroa “susceptible” colonies. Other recent studies have also shown that small particles of chalk dust (Land & Seeley, 2004) or wheat flour (Morfin et al., 2020) can be used in place of varroa mites to stimulate grooming responses in *A. mellifera*. Morfin et al. (2020) found that individual Africanized bees stimulated with either varroa or wheat flour placed on the thorax

initiated grooming responses quicker than presumed varroa-susceptible European (Carniolan) bees.

Heritability

Grooming behaviour has been investigated for its use in selective breeding programs. In such programs, it is important to consider how heritable certain traits are. Heritability is a measure of how much influence genetic factors have over a trait, as opposed to environmental factors, and is expressed as a heritability index value from 0 to 1 (Guzman-Novoa, 2007). Heritability is considered high for a trait if its heritability index value is above 0.5 and is considered medium if it is between 0.25 and 0.5; values lower than 0.25 are considered low (Guzman-Novoa, 2007). The heritability of grooming behaviour has been assessed by multiple studies, but a wide range of values have been reported. Moretto et al. (1993) reported a high heritability value of 0.71, while Ehrhardt et al. (2007) found a value less than 0.15. Stanimirovic et al. (2010) found moderate to low values (0.49, 0.18, and 0.16) and concluded that grooming may be a polygenic trait that requires a combination of genes to be successful. More recent research has looked at the heritability of grooming behaviour using genetic markers (Arechavaleta-Velasco et al., 2012). Hamiduzzaman et al. (2017) linked the intensity of grooming behaviour in laboratory petri dish assays with the expression of certain genes. They found several genes associated with more intense grooming and that the expression of one gene, *Neurexin-1*, a gene associated with autism in humans and self-grooming behaviour in mice, could be particularly useful as a biomarker for in honey bees. Similarly, our research group at the University of Manitoba is currently involved in Genome Canada-funded work aimed at developing genomic and proteomic markers for several honey bee traits, including grooming behaviour, and my project is a direct offshoot of this larger study.

Other Factors Influencing Grooming

The range of heritability index values for grooming behaviour may be partially due to genetic variability; however, it may also be partly explained by several other factors that can influence levels of grooming behaviour, including environmental factors, the presence of queen pheromone, the age and reproductive status of mites, the existence of grooming specialists, and the impact of other pathogens.

Environmental Factors

Environmental factors can have a large influence on the degree to which bees will groom mites. For example, Büchler (1993) found a strong seasonal effect on the number of damaged fallen mites in colonies, ranging from 10% in March to 40% in June, which could relate to seasonal changes in brood rearing periods. Currie and Tahmasbi (2008) showed significant differences in grooming at 25°C and 34°C between bees selected for high grooming behaviour and unselected bees, whereas differences were not apparent at 10°C. As well, they found that under low humidity conditions, the high grooming bees successfully removed significantly more mites, compared to conditions of medium and high humidity. It is believed that the susceptibility of mites to desiccation enhances grooming effectiveness at lower humidity because more of the mites that are groomed die and thus cannot reacquire host bees.

Queen Pheromone

The presence or absence of queen pheromone within the hive can also play a role in the level of grooming in a colony. In a study using small colonies maintained under darkness at 5°C, Bahreini and Currie (2015a) found that the addition of either a caged mated queen, or a synthetic queen mandibular pheromone lure increased mite removal from the cluster, relative to queenless colonies.

Age and Reproductive Status of Mites

The age and reproductive status of mites has been shown to influence grooming behaviour as well. Kirrane et al. (2012), for example, conducted a study where caged cohorts of bees were inoculated with either gravid mites (i.e., egg-laying mother mites), phoretic foundress mites, a combination of gravid and phoretic foundress mites, or phoretic daughter mites. They found that phoretic daughter mites were groomed at a significantly higher rate than phoretic foundress mites, suggesting that daughter mites either stimulate grooming behaviour more frequently or are more susceptible to being groomed than foundress mites.

Grooming Specialists

The age of honey bee workers can also be a factor in successful grooming, particularly in allo-grooming, as it is possible for a very small proportion of young workers in a colony to become grooming specialists (Kolmes, 1989; Moore et al., 1995). Within a colony's temporal

polyethism structure (Winston & Punnett, 1982), there also likely exists a small pool of uncommitted workers that can become mobilized to very specialized tasks when needed, including allo-grooming (Kolmes, 1989). In a study using small numbered plastic discs to mark 200 one-day-old workers, Moore et al. (1995) observed that young house-bees spend about 1.5% of their time on social grooming behaviours. They also found that at day 7, one bee (red 93) was spending about 84% of her time grooming, suggesting that highly infested colonies may be able to mobilize a specialized workforce of allo-grooming bees as a resistance mechanism.

Impact of Other Pathogens

The levels of other pathogens in honey bee colonies can also negatively impact the grooming ability of workers. In a study looking at the grooming success of caged worker bees from colonies selected for high mite mortality rates and low mite mortality rates, the grooming success of the high mite mortality rate bees was significantly reduced when cages were inoculated with *Nosema* spores (a mixture of both *N. apis* and *N. cerena* species) (Bahreini & Currie, 2015b).

Potential Biological Cost Associated with Increased Levels of Grooming Behaviour

In some studies, significantly increased worker bee mortality rates have also been observed in bees selected for high-grooming behaviour vs unselected bees, indicating there may be some biological costs associated with increased levels of grooming (Bahreini & Currie, 2015b; Currie & Tahmasbi, 2008). At low temperatures (10°C), Currie and Tahmasbi (2008) found that bee mortality rates were greater in high-grooming lines than low grooming lines; and Bahreini and Currie (2015b) found that high-grooming lines had higher levels of bee mortality than low-grooming lines when inoculated with high levels of varroa.

Study Objectives

Although much research has already been done to quantify grooming behaviour in honey bees, breeding bees with heightened levels of grooming behaviour remains difficult. Screening bees at the colony level for grooming behaviour using mite mortality rates is complex and requires precise knowledge of how many mites are present in a colony at a given time and is confounded by many external factors. Imperfect sampling methods as well as sampling when

brood is present in a colony also makes calculating the total number of mites in colonies and parsing out the proportion related to grooming very difficult. Assessing grooming by means of categorizing physical damage to varroa mites is extremely tedious and time-consuming and might not be the best overall measure of a colony's level of grooming behaviour. Performing assays with individual bees, such as in the study by Guzman-Novoa et al. (2012) may offer a more precise indicator of the genetic components related to grooming; however, collecting live mites to use in such experiments is extremely labour intensive and depends on the availability of large numbers of mites, which is not always realistic, especially at different times of the year. Thus, using alternative stimuli—as in the study by Morfin et al. (2020)—to screen for bees with heightened levels of grooming may be a good alternative to collecting varroa mites. However, while individual bee assays may be useful, they do not assess the social components of grooming, such as allo-grooming or the mechanisms involved in the recruitment of nestmates during allo-grooming.

The principal objectives of this study were to examine in more detail the overall mechanisms behind honey bee grooming behaviour and to research alternative techniques to screen for bees with heightened grooming levels. One specific aim of this thesis was to assess how high-grooming bees are better able to remove mites, and my hypothesis is that they may simply have heightened tactile or chemosensory abilities to detect mites on themselves or on each other. If high-grooming bees have heightened sensitivity overall to both varroa as well as alternative irritants, this will provide further justification for using standardized stimuli to screen for grooming behaviour in laboratory assays. On the other hand, if high-grooming bees display reactions more specific to varroa, this would lessen the justification for using alternative irritants in favour of using live mites in such assays. Another aim of this thesis was to examine cohorts of high- and low-grooming bees to study the colony-level grooming responses related to the bees' abilities to assess their mite infestation levels. If a colony has greater defensive responses under higher mite loads, it must have some mechanisms for assessing that load. If certain response thresholds are reached, for example, the colony as a unit may react by means of increasing communication in the form of dancing, vibroacoustic signals, or other responses. Since various vibroacoustic signals have been previously identified and studied in the context of *A. mellifera* and *A. cerana* behaviours, it is likely that some of these same signals, or other yet identified

signals, play a role in other *A. mellifera* behaviours or communicative dances—such as in the grooming invitation dance.

The controversy over using damaged mites to select for high-grooming colonies also needs further clarification. Even though studies such as Guzman-Novoa et al. (2012) have recently strengthened the argument for using damaged mites as an indicator of grooming, its importance relative to the suite of behaviours necessary to remove mites remains unclear. Thus, further study may help to strengthen the argument for using damaged mites or add additional metrics that can be included. Figure 1.1 below illustrates a model of the known and predicted mechanisms that affect grooming behaviour in colonies of *A. mellifera* against *V. destructor*.

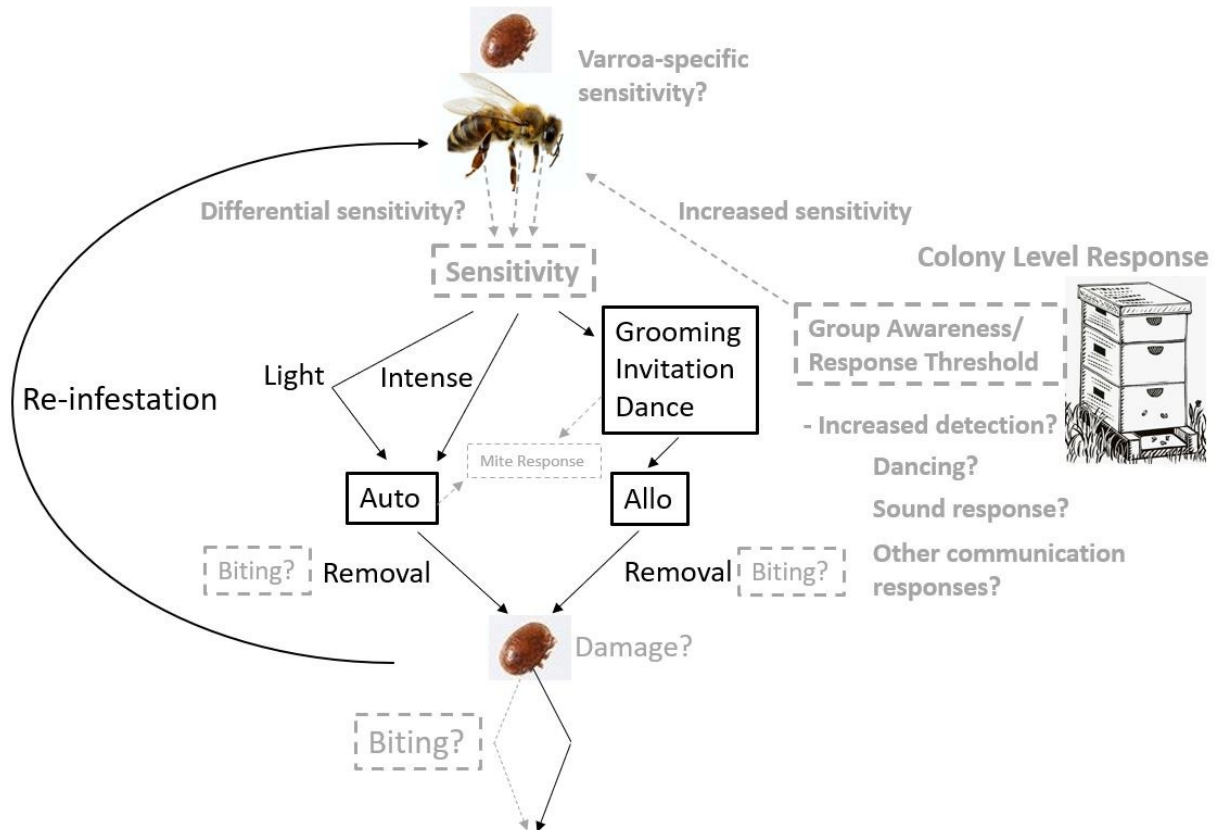


Figure 1.1. A model of the known and predicted mechanisms of grooming behaviour in *A. mellifera* against *V. destructor*. Solid lines and/or black text indicate what is currently known and dashed lines and/or gray text indicate what is currently unknown or poorly understood. I hypothesize that the first step in the act of grooming is for the bee to sense that it has a mite and that there is differential sensitivity over different body regions. A bee that has detected a mite may attempt to remove it via light or intense auto-grooming or may attempt to solicit the help of nestmates by performing the grooming invitation dance. The mite itself may also have responses to grooming that enable it to avoid grooming attempts. Although it has been shown that worker biting of mites plays some role in grooming behaviour, its overall importance is still questionable. Colony level grooming responses are also virtually unknown at this point. I hypothesize that there may be increased dancing, vibroacoustic signals, or other increased communication responses (e.g., pheromones) to mites if certain thresholds are reached and that bees with higher grooming tendencies would have lower thresholds and higher sensitivity to detect these cues.

Preface to Chapter Two

The following chapter is written in manuscript format. It consists of a study where I examined the sensitivities of individual bees from previously chosen high- and low-grooming colonies to a stimulus of either a live varroa mite or a standardized puff of chalk dust applied to the head, thorax, or abdomen. Auto-grooming responses of bees were quantified with a timer and compared. Results are discussed.

Chapter 2. Heightened Sensitivity in Selected High- and Low-grooming Honey Bees (*Apis mellifera*)

Abstract

The parasitic mite *Varroa destructor* has caused the deaths of millions of honey bee colonies worldwide. The grooming behaviour of honey bees is a natural defense mechanism to varroa, however, selective breeding for grooming behaviour has mainly relied on imperfect, indirect measures such as the mite mortality rate or rates of mite damage. The direct observation of the grooming responses of individual bees offers another method of selection but usually involves collecting live mites from heavily infested colonies to use for testing. This study was designed to compare an alternative stimulus of chalk dust against using live mites in assays with individual bees, as well as to gain a better overall understanding of the mechanisms of honey bee grooming behaviour. I hypothesized that individual bees coming from colonies chosen for high-grooming behaviour using a combination of indirect measures (mite mortality and mite damage) would be more sensitive to both stimuli, but that varroa would be a stronger stimulus. Further, I hypothesized that high-grooming bees would have enhanced sensitivity over different body regions. I found that bees from colonies with both high mite mortality rates and high mite damage rates had heightened grooming responses to a stimulus of varroa or a stimulus of chalk dust when applied to the head or thorax, compared to unstimulated bees, while bees from the low-grooming colonies showed no differences among treatment groups. Further, the high-grooming bees selected only on mite mortality showed significant differences across body regions, however no treatments differed from the control. The results of this study indicate that chalk dust may be a useful alternative irritant to use in place of mites in individual bee assays. However, since the high-grooming bees with high levels of mite damage also showed increased sensitivity to varroa compared to bees from colonies exhibiting only high mite mortality, continuing to use live varroa mites in individual bee assays may help select for colonies with more precise sensitivities to varroa.

Introduction

The parasitic mite *Varroa destructor* Anderson and Trueman is considered the single greatest threat to apiculture worldwide (Rosenkranz et al., 2010). Even low infestations of the mite can have significant economic impacts (Currie & Gatién, 2006) and if not regulated by the beekeeper, an untreated honey bee colony will usually perish within two to three years (De Jong, 1996; Korpela et al., 1992). Since honey bees are the most valuable pollinators to agroecosystems worldwide, providing critical pollination services to around one-third of crops (Delaplane et al., 2000; Klein et al., 2007; McGregor, 1976), understanding the mechanisms of resistance against parasites and diseases is of critical importance.

One natural defense against varroa is grooming behaviour, a form of social immunity where bees use their legs and mandibles to remove mites from themselves (auto-grooming) or from nestmates in the colony (allo-grooming) (Peng et al., 1987). Allo-grooming may be solicited through the grooming invitation dance (Haydak, 1945; Land & Seeley, 2004). Grooming behaviour has previously been shown to be able to restrain the growth of *V. destructor*, particularly in colonies of the Asian honey bee, *Apis cerana* Fabricius, and to a lesser degree in colonies of the European honey bee, *Apis mellifera* Linnaeus (Buchler et al., 1992; Peng et al., 1987). Further studies have found higher levels of grooming across different subspecies of *A. mellifera*, including Africanized bees (descendants of *A. mellifera scutellata*) (Aumeier, 2001; Guzman-Novoa et al., 2012), bees from the Primorsky Krai region of Russia (Guzman-Novoa et al., 2012; Rinderer et al., 2001), and *Apis mellifera carnica* (Ruttner & Hänel, 1992; Thakur et al., 1997). Grooming behaviour has also been found to be a key component of tracheal mite (*Acarapis woodi*) resistance in *A. mellifera* (Pettis & Pankiw, 1998) and serves a general function in the removal of pollen, dust, or other irritants that bees regularly encounter when foraging (Winston, 1987).

The overall mechanisms of grooming behaviour that result in individual bees being able to detect and remove irritants or mites from their bodies remains poorly understood but undoubtedly involves multiple steps. For example, to auto-groom, an individual worker must first “recognize” a stimulus and then respond in a generalized or specific way to remove the irritant. Hamiduzzaman et al. (2017) suggested that high-grooming bees may have a heightened ability to perceive stimuli in their environment either through tactile or chemosensory means.

Morfin et al. (2020) looked at stimulating the grooming responses of presumed varroa-resistant Africanized bees and presumed varroa-susceptible European (Carniolan) bees with either varroa or an alternate irritant of wheat flour placed onto the thorax. They found that Africanized bees responded significantly faster than Europeans when stimulated with either varroa or flour, concluding that irritants other than varroa may be used to differentiate between genotypes of bees with different levels of grooming behaviour. This research suggests that bees with higher levels of grooming may have heightened tactile senses that enable them to detect and take subsequent steps to remove varroa mites.

However, further research is needed to clarify the specific mechanisms by which high-grooming bees are better able to detect and remove varroa. For example, bees that are effective groomers may exhibit a generalized response with heightened overall levels of sensory perception to any stimulus or may have more sophisticated sensory capabilities that allow them to target varroa more directly. It also remains unclear how the sensitivities of different body regions compare in bees that have enhanced grooming capability. Understanding these mechanisms is important in helping us understand precisely how bees defend against varroa, how varroa might evade grooming, and assist in developing new behavioural bioassays and molecular markers to select for grooming behaviour. If grooming is a polygenic trait that requires a combination of genes to be successful, as some suggest (Stanimirovic et al., 2010), then identification of the specific steps involved in this complex behaviour will inform the development of markers.

Enhanced grooming is a heritable trait (Arechavaleta-Velasco et al., 2012; Hamiduzzaman et al., 2017; Moretto et al., 1993; Stanimirovic et al., 2010) of interest to breeding programs for varroa resistance behaviours. However, many methods of assessing grooming behaviour are indirect and labour intensive. Usually, they involve collecting and counting all mites falling onto sticky papers placed beneath hives and calculating the mite mortality rate (Currie & Tahmasbi, 2008), or microscopic identification of physical damage to collected mites and calculating the percentage of damaged mites (Guzman-Novoa et al., 2012; Hunt et al., 2016). Both methods are imperfect because overall mite fall may be attributed to other varroa-resistance behaviours, such as varroa-sensitive hygiene (Rinderer et al., 2010), and

damage to fallen mites may also occur as a result of other factors unrelated to direct effects of grooming such as natural mortality (Boecking & Spivak, 1999).

Observing the grooming responses of individual bees is another method of analyzing grooming behaviour (Aumeier, 2001; Guzman-Novoa et al., 2012; Hamiduzzaman et al., 2017) and provides direct evidence of the behaviour of individual bees taken from source colonies. Small particles of chalk dust, for example, have been used previously to stimulate grooming responses in *A. mellifera* held in observation hives (Land & Seeley, 2004). Guzman-Novoa et al. (2012) linked the intensity of grooming responses and the successful removal of mites from individual bees with the proportion of damaged mites found on source colony sticky papers as well as the overall colony mite infestation level, providing additional evidence for using these direct methods of observation in addition to or in place of indirect methods.

The overall goal of this research was to characterize the different mechanisms of grooming behaviour that allow individual bees to better detect and remove mites. An experiment was designed to simultaneously test three separate hypotheses: 1) Bees from colonies chosen for exhibiting high-grooming behaviour (using a combination of two selection metrics) have heightened sensitivity to stimulus from irritants (both varroa as well as a standardized chalk dust stimulus); 2) Bees selected for varroa resistance behaviour will be more sensitive to a stimulus of varroa than a standardized irritant (chalk dust); and 3) Bees from high-grooming colonies have enhanced sensitivity over different body regions compared to low-grooming bees.

Materials and Methods

Selection of colonies

The experiment was carried out at the University of Manitoba in Winnipeg, Manitoba, Canada (49°54' N, 97°14' W). High- and low-grooming colonies for use in laboratory assays were selected in the spring of 2017 based upon an assessment of mite drop (daily mite mortality rate and proportion of damaged mites) from broodless units from a total of 200 colonies that were part of the Genome Canada Bee'Omics project at the University of Manitoba in 2016. Colonies used in that experiment originated from a variety of sources, including donations from nine different producers throughout the province of Manitoba (n = 83), diverse local stock from the University of Manitoba (n = 75), and imported packages from New Zealand (n = 42). In the

spring of 2016, the queens from each parent colony were first caged and broodless colonies were established by shaking bees from the respective colonies into sealed cardboard hive boxes. Worker populations were standardized at 1 kg of bees, equivalent to approximately 8,000 bees. Caged queens were placed into the hive boxes along with the associated bees from their own colonies and the new broodless colonies held overnight under cool conditions in total darkness in the University of Manitoba's overwintering building before being moved to their new locations. The 200 broodless units were then randomly separated and transferred to four separate apiaries the following morning. Frames of bees were transferred out of the sealed cardboard hive boxes into standard Langstroth 10-frame brood chambers. Each hive was then immediately outfitted with a commercially available "varroa-nator®" screened bottom board (Dimo's Tool & Die, 12 Bangor Ave, Winnipeg, Manitoba) below which were placed Vaseline®-type sticky boards (made from freezer paper) (Calderone & Lin, 2003) to measure mite fall over two consecutive 72-hour periods. Then, to increase acceptance of the queens to their new environments, the queens were slow-released from their cages by removing the corks from the cage entrance and plugging it instead with a mixture of wax and honey to allow the bees to slowly chew the entrance open.

Varroa populations for each broodless unit were standardized to the extent possible. The initial mean abundance of mites (mites per 100 bees) was measured by means of an alcohol wash from the parent colony by averaging two samples of roughly 250 bees each. The target range for initial infestation of colonies was 0.5 to 2.0 mites per 100 bees. No colonies used in the 2016 Manitoba Bee'Omics experiment exceeded the 2% maximum infestation at establishment, however, colonies that had an initial infestation of less than 0.5% were inoculated with live varroa mites collected from highly infested colonies (kept in a separate mite-rearing yard at the University of Manitoba) using a modified CO₂ shake method (Currie & Tahmasbi, 2008). On the day of colony establishment, mites were collected to inoculate those colonies needing additional mites. Forty-five mites were placed into petri dishes prepared with round pieces of moistened paper towel which were then stored in Styrofoam® coolers containing Ziploc® bags filled with warm tap water (roughly 30°C). Colonies needing additional mites were inoculated later that day. The paper towels containing the mites were placed under the colony lids and onto the top bars of frames to allow the mites to crawl into the hives and onto the bees. Any mites remaining in the

dishes were transferred to the paper using a fine-tipped paint brush. The total number of mites present in each starting broodless colony was then estimated by taking the % infestation from the parent colony samples multiplied by the estimated total number of bees (8,000) and adding 45 mites to that if extra inoculation occurred.

The daily mite mortality rate (DMMR) (i.e., the proportion of total mites falling to bottom boards each day) was then assessed for each colony by means of collecting the two (72 hour) rounds of sticky papers from the colonies. During this time, any mite fall would have been attributed either to natural mite death or the grooming behaviour of bees since no brood (of a stage that varroa would infest) was yet present. All mites that dropped onto sticky papers were carefully removed in the lab, counted, and kept in vials containing 70% ethanol until they were later analyzed for signs of damage. The DMMR for each colony was calculated by taking the total number of mites that dropped from each colony over the six days (144 hours) divided by the estimated total number of mites in each colony at establishment, divided by six.

Each collected mite was viewed under a dissection microscope at 40x magnification for signs of damage to legs, mouthparts, or ventral shields (mites with dented idiosoma were not categorized as damaged). The level of damage was further classified as mild, moderate, or heavy. Mild damage was when mites had parts of one leg missing, or partial damage to mouthparts or ventral shield; moderate damage was when mites had a whole leg, or whole mouthparts missing; and heavy damage was when mites had multiple legs missing or had multiple damages. The proportion of damaged mites (PDM) for each colony was calculated by dividing the total number of mites with *any* signs of damage by the total number of mites collected off sticky papers. PDM calculations and rankings for the high-grooming group were based on a cut-off of at least 5 mites analyzed, and of those colonies selected, the minimum 6-day mite-drop was 7 mites.

A combination of the DMMR and the PDM was then used to choose presumed high-grooming colonies for subsequent experiments, however only the DMMR was used to choose low-grooming colonies (see Figure 2). DMMR's for the selected high-grooming colonies were all considered high but were further subdivided based on damage rates as described below. Of the selected high-grooming colonies, DMMR's ranged from 0.026 to 0.17 (proportion of total mites dropping per day) and PDM's ranged from 0.17 to 0.71 (proportion of collected mites with any damage). The PDM values were subdivided into moderate and high subcategories, using

0.30 as a cut-off where damage equal to or between 0.17 and 0.30 was categorized as moderate and that above 0.30 was categorized as high.

Prior to any laboratory assays in 2017, the colonies were overwintered indoors in the University of Manitoba Bee Lab's wintering building over the 2016–2017 winter at a temperature of 5°C and held under constant darkness with standard ventilation (Gruszka & Currie, 1998). All colonies used in assays were treated with three rounds of 65% liquid formic acid in Spring 2017 to equalize varroa infestation levels at or close to 0% prior to performing bioassays.

Eleven high- and eleven low-grooming colonies were chosen for testing and were moved on 14 June 2017 to an apiary at 'The Point' at the University of Manitoba, positioned at least 50 m away from any other colonies. Colonies were arranged randomly in a row spaced roughly 1 metre apart and entrances were angled to discourage drifting of bees between colonies. A variety of different coloured boxes were also used for hive bodies to provide orientation cues and further discourage drifting. Colonies were managed throughout the season with an attempt to prevent swarming and queen supersedure by clipping queen cells and providing extra space and ventilation as required. In cases where colonies eventually swarmed or superseded, they were assayed before the population turned over, to avoid testing progeny from the new queen (i.e., new genes).

Individual grooming behaviour trials

Individual bee trials began on 20 June 2017 and ran until 18 August 2017. Two colonies were tested at a time (one high-grooming and one low-grooming) to control for any seasonal variation in grooming. Each morning, about 100 bees from a brood frame (capped or open) from each colony being tested were collected and split into two 150 ml collection vials (i.e., ~50 bees per vial) with one screened end and a sliding lid that allowed for individual bees to be removed one at a time. A sugar cube was placed into each collection vial as a temporary food source for the duration of the trials. Live varroa mites were collected each morning using the same CO₂ shaking method referenced above. About 30 mites were collected each morning and divided into two petri dishes containing around 15 mites each. A moist paper towel was placed in each dish to prevent desiccation of mites, as well as 4-5 worker pupae that had been removed from a brood

frame that morning to serve as a food source for the duration of the trial. Petri dishes with mites were again kept warm by placing them into a small Styrofoam® cooler with a Ziploc® bag full of warm water (~30°C) and were brought to the lab along with the bees.

Experimental trials took place in the flight room in the honey bee research laboratory (see Pernal and Currie (2002) for details). The flight room was maintained at a constant temperature of 25°C for the duration of the trials with full spectrum lighting in the room. To begin each trial, five bees were transferred from the collection vial into a 50 ml Falcon® tube by opening the sliding lid and letting them crawl out. To assist with applying the stimulus treatments, the bees were then briefly anesthetized with CO₂ by means of a small hose attached to the lid of the Falcon tube. A flow meter was used to control the flow of CO₂ to 2 litres/min and the gas was turned on for a count of 8 seconds, which provided just enough CO₂ to gently anesthetize the bees for the time needed to transfer them to petri dishes and apply treatments. The five bees were transferred into five new petri dishes on a table outfitted with white bench liner which provided contrast to help analyze the behaviour of the bees. Treatments were then randomly assigned to each dish using five plastic discs, each representing one of the five treatments. Once the bees were in the dishes, a video camera (iPhone 5 attached to a ring clamp stand positioned above the dishes) was started that captured video of all five dishes in the trial. The treatments were then administered to the bees when they were beginning to move around again. In cases where the bees did not move soon after the anesthetization, the bee was replaced with another (following the same procedure).

The five treatments were: (1) *Control*: a soft touch with a small paintbrush (Heinz Jordan white taklon series 970 size 10/0) to the thorax; (2) *Mite*: one live varroa mite placed onto the bee (usually the thorax) using a small paintbrush; (3) *Head*: a puff of chalk dust applied to the head; (4) *Thorax*: a puff of chalk dust applied to the thorax; and (5) *Abdomen*: a puff of chalk dust applied to the abdomen. For all chalk dust treatments, Mastercraft® marking chalk was used and was administered via a Gilson® P100 adjustable pipette with a disposable tip loaded with chalk dust. The pipette had been calibrated so that one depression of the plunger released approximately 0.3 mg of chalk dust, roughly equal to the weight of one mite, based on weighing multiple varroa mites with a CAHN 25 automatic electrobalance (Model 5725). After the stimuli were applied, the dishes were covered with modified petri dish lids that had the plastic top cut

out and replaced with Mylar® film with about 20-30 holes punched in it with a small nail to allow for airflow (lids were cleaned after each trial with 70% ethanol and reused for subsequent trials, however, new petri dish bottoms were used for each trial).

Immediately after the last stimulus application, a 3-minute timer was started and when time was up video recording was stopped and the trial was ended. Since two colonies were being tested on the same day, trials for each colony were alternated to avoid any effects of the bees sitting in the room waiting to be tested (e.g., trial #1 for colony 1, followed by trial #1 for colony 2, trial #2 for colony 1, trial #2 for colony 2, and so on). Twenty-five trials were completed for each colony. This usually took two or (rarely) three days to complete, however, new bees and mites were used each day. At the end of each day, videos were saved to be analyzed later for grooming responses.

Video Analysis:

I quantified the following components of grooming behaviour: time to start grooming, time spent light grooming, time spent intense grooming, total grooming time (light + intense grooming), and the number of grooming ‘bouts.’ Light and Intense grooming responses were categorized using the same methods as Guzman-Novoa et al. (2012)—i.e., light grooming consisted of slower swipes with just one or two legs at most; and intense grooming consisted of more vigorous swiping using a minimum of two legs. Any mite removals were also recorded, and if so, the bee was watched for an additional one minute to record whether the bee interacted with the mite in the dish. With that data, I calculated the proportion of time spent light grooming, the proportion of time spent intense grooming, the total proportion of time spent grooming, and the average length of each grooming ‘bout.’

Video clips for each trial were analyzed using PowerDVD 16 Media Player (CyberLink Corp, 2016), which allowed the video observer to adjust both the zoom and playback speed to accurately observe and quantify the grooming responses of each individual bee. Thus, video clips for each trial were watched five times, once for each bee in each stimulus treatment.

Three observers watched and analyzed video to collect data. However, to minimize subjectivity, observers were trained carefully, and care was taken to calibrate each observers’ observations by analyzing 25 bees together before each observer analyzed recordings

independently. Observers knew the colony numbers for the videos they analyzed, however, observers were kept blind to which group the colony was in (e.g., high- or low-grooming) to avoid biasing the results.

Grooming responses were quantified using an iPhone and a customized grooming timer ‘app’ programmed by Josh Usiskin (*dev.usiskin.ca:51947/#!/trial*). For each bee, the observer would start the grooming timer to coincide with the point at which the stimulus was applied to the bee in the video, or in cases where the bee had not yet fully recovered from the effect of the CO₂, the grooming timer was started when the bee was standing on all legs and appeared to have recovered from the anesthetic. The grooming timer was programmed such that it ran for three minutes, and the observer could record how long the bee groomed itself by holding down one finger for instances of light grooming or two fingers for instances of intense grooming. When the three-minute grooming timer ended, grooming times were displayed in a spreadsheet in the app.

Statistical Analysis:

For colony selections, the daily mite mortality rates and proportion of damaged mites were analyzed using a one-way analysis of variance (ANOVA) to compare chosen groups of putative high and low-grooming colonies (PROC MIXED, SAS Institute Inc. 9.4, 2016). Residuals of data were first plotted visually in R (Version 1.3.1056) to examine normality and homogeneity of variance. Since data were non-normal, an arcsine square root transformation was applied to the data after adding a small constant (0.001) to all values of 0 within both the daily mite mortality rate and the proportion of damaged mites variables. Where differences were observed between selected groups, a post-hoc Tukey test was used to compare differences among the three groups. For bioassay experiments, the proportion of time spent intense grooming was chosen as the most appropriate metric to compare among selected groups as intense grooming has previously been identified as one of the most important grooming metrics, and one that is more closely associated with successful mite removals in individual bee assays (Guzman-Novoa et al., 2012; Hamiduzzaman et al., 2017). To analyse the proportion of time spent intense grooming, a split-split-plot ANOVA design was used in which colony was considered the experimental unit and selection-method was a main plot factor, treatment was a subplot factor and trials were the sub-sub plot. Colony(selection-method), colony*treatment(selection-method),

and trial*colony(selection-method) were random effects. Prior to analysis, residuals were plotted visually in R (Version 1.3.1056) to check normality and homogeneity of variance, however, statistical analyses were performed in SAS (SAS Institute Inc. 9.4, 2016). Since data were not normal, an arcsine square root transformation was performed after adding a small constant to each value (0.1), however all data are presented as untransformed means. A variance components covariance structure was selected based on the lowest Akaike Information Criterion and the degrees of freedom calculated using the ddfm = Kenward Rodger option to adjust for poor homogeneity of variance. Interactions between selection-method and treatment were partitioned using the SLICE option in the LSMEANS statement and individual contrasts were used to compare treatment means within each selection group (Protected LSD, PDiff PROC MIXED, SAS Institute Inc. 9.4, 2016). Data for other metrics that were quantified can be found in the University of Manitoba's Dataverse:

<https://dataverse.lib.umanitoba.ca/dataset.xhtml?persistentId=doi%3A10.34990%2FFK2%2FGSOPG6>

Results

Colony Selections:

Mean daily mite mortality rate was significantly higher in the pool of high-grooming colonies selected based on high-DMMR than in the low-grooming colonies ($F_{1,20} = 43.18$, $p < 0.001$) (Fig 2.1A). When the high-grooming colonies were further subdivided into high- and moderate-damage subgroups (Fig 2.1B), there also was a significant effect of selection-method ($F_{2,19} = 27.60$, $p < 0.001$) on DMMR. DMMR's for both the high-DMMR/high-damage subgroup ($p = 0.0001$, Tukey) and the high-DMMR/moderate-damage subgroup ($p < 0.001$, Tukey) were significantly higher than the low-grooming colonies. Similarly, the mean proportion of damaged mites was significantly higher in the selected high-grooming colonies (based upon DMMR) than selected low-grooming colonies ($F_{1,20} = 6.74$, $p = 0.017$) (Fig 2.2A). When high-grooming colonies were categorized based upon high- and moderate-damage, a significant effect of selection on damage was also found ($F_{2,19} = 4.50$, $p = 0.025$) (Fig 2.2B). The high-damage category showed significantly more damage than in the low-grooming colonies ($p = 0.019$,

Tukey). Whereas the moderate-damage subgroup was intermediate and not significantly different from the low-grooming colonies ($p = 0.59$, Tukey) or from the high-damage subgroup ($p = 0.37$, Tukey).

Intense Grooming:

There was no overall effect of selection-method on the proportion of time that individual bees spent intensively grooming when comparing across all five treatment methods ($F_{2,19} = 0.23$, $p = 0.79$). However, the selection-method*treatment interaction was ($F_{8,75} = 2.05$, $p = 0.052$) warranting further exploration (Fig 2.3). Partitioning the interaction by treatment indicated that intense grooming was similar within all five treatment groups (control, mite application, and chalk dust applied to the head, thorax, or abdomen). However, partitioning the data by selection-method showed treatment effects varied significantly depending on the method used to select colonies. There was an effect of treatment on intense grooming within the high-DMMR/high-damage colonies ($F_{4,75} = 4.40$, $p = 0.003$, Slice) as well as within the high-DMMR/moderate-damage colonies ($F_{4,75} = 3.07$, $p = 0.021$, Slice), but not within the low-grooming colonies ($F_{4,75.2} = 0.79$, $p = 0.537$, Slice) (Fig 2.3).

Within the high-DMMR/high-damage colonies, bees exposed to mites exhibited more intense grooming than control bees ($p = 0.0004$) or bees treated on the abdomen with chalk dust ($p = 0.002$). Bees treated with chalk dust on the head ($p = 0.044$) or thorax ($p = 0.021$) showed greater responses than the controls.

Interestingly, within the high-DMMR/moderate-damage colonies, control bees showed less intense grooming than bees with chalk dust applied to the thorax ($p = 0.050$), however, bees exposed to mites did not groom more intensely than any other treatment, including controls ($p > 0.05$). Chalk dust treatment applied to the thorax elicited greater responses than mites ($p = 0.019$) while chalk dust applied to the head elicited only a marginally greater responses than mites ($p = 0.051$). Applications of chalk dust to the head and thorax both showed significantly higher levels of intense grooming than chalk dust applied to the abdomen ($p = 0.020$ and $p = 0.007$, respectively). The exposure of chalk dust on the head showed a trend towards more intense grooming than the controls but was not significant ($p = 0.121$).

Although there were no significant differences in intense grooming responses among selection-methods within each type of treatment as discussed above ($p > 0.05$), single degree of

freedom contrasts showed that high-DMMR/high-damage colonies that were treated with mites had more intense grooming than low-grooming colonies where chalk was applied to the head ($p = 0.047$), thorax ($p = 0.044$), or abdomen ($p = 0.024$) and had more intense grooming than the low-grooming controls ($p = 0.022$).

Discussion

This study analyzed the grooming responses of individual bees from colonies that were previously chosen based upon high- and low-grooming behaviour using mite drop and mite damage as indicators of grooming from broodless units. The findings increase our overall knowledge of the mechanisms of honey bee grooming behaviour and may influence how high-grooming bees are selected for queen breeding. Differences were observed in how chosen groups of colonies responded to the different treatments. For intense grooming responses, the low-grooming group of colonies showed no significant differences among the two types of stimuli applied (mites or chalk dust). While significant differences among treatments were found in both high-grooming categories, the ability to discriminate varroa more than a standard stimulus occurred only in colonies that were chosen based upon both mite mortality and mite damage rates. Differences in the sensitivity of different body regions was also found within both groups of high-grooming colonies but varied depending on the colony level selection metrics used to establish each category.

When stimulated with varroa or chalk dust applied to the head or thorax, bees from the high-DMMR/high-damage colonies groomed intensively for a significantly longer proportion of time compared to the unstimulated control bees. A similar difference was observed within the high-DMMR/moderate-damage colonies when chalk dust was applied to the thorax compared to the control bees. A similar trend was observed for chalk dust applied to the head but was not significant. Nevertheless, the hypothesis that high-grooming bees would show heightened sensitivity to both varroa as well as a secondary stimulus was supported within both selection methods and significant differences among treatment stimuli were not observed within low-grooming selections.

An overall trend in both the high-damage and moderate-damage categories was for less intense grooming responses when chalk dust was applied to the abdomen versus other regions.

While this trend was not significantly lower than chalk applied to the other two body regions in the high-damage colonies, chalk applied to the abdomen was significantly lower than the head and thorax in the moderate-damage colonies. These findings support the second hypothesis that high-grooming bees would exhibit differential sensitivity over different body regions.

Ramsey et al. (2019) showed that varroa has a strong preference for the ventrolateral abdomen, commonly found wedged between the sternites on the third metasomal segment, and with a significant preference for the left side. The authors point out that the preference for the abdominal region is likely due to the larger deposit of fat body tissue, but also posit that the preference for the third segment may be because the mite is largely shielded from grooming by the host bee in this location. My results show that the abdomen is also a location where mites may be less detectable by workers who would be less likely to activate grooming, although it should be noted I did not apply dust to the underside of the abdomen where varroa commonly resides. The fact that there was a significant effect of body region in the moderate-damage colonies, but less evident in the high-damage colonies could be partly because the level of intense grooming when dust was applied to the abdomen was also slightly higher in the high-damage colonies, thus reducing the relative difference over body regions relative to the moderate damage group. This suggests that high-DMMR/high-damage bees may have heightened overall sensitivity to foreign stimuli and that bees selected based upon more than one criterion may have better capacity to recognize and respond to mites through grooming. Since varroa are more commonly found on the abdomen, the high-DMMR/high-damage bees may also have heightened sensitivity to any stimulus on the abdominal region and may initiate grooming responses at a lower threshold of stimulation. However, this difference, if it occurs was not large enough to be detected within my study, perhaps due to the lower sample size in our moderate-damage category.

The generalized grooming responses of honey bees allows for the removal of foreign substances like dust and pollen as well as more harmful ectoparasites (Pettis & Pankiw, 1998; Winston, 1987). One interesting and unexpected difference between the two high-grooming categories was that the high-damage colonies intensely groomed significantly longer than the control treatment with a stimulus of varroa, whereas the moderate-damage colonies showed shorter bouts of intense grooming in response to stimulus from varroa and did not differ from the

control. This indicates that the selected high-DMMR/high-damage colonies likely had heightened varroa-specific sensitivity compared to the moderate-damage colonies. This observation is in conjunction with the lack of treatment effects in the low grooming colonies, supports the hypothesis that high grooming bees can have heightened varroa-specific sensitivity. Thus, my results indicate that including mite damage in addition to mite mortality rate as a selection criterion when selecting for heightened grooming behaviour in colonies may result in bees that are more specifically sensitive to varroa.

It should be noted, however, that in my experiment mites from selected colonies were prevented from re-acquiring hosts as they were trapped using sticky boards. Since un-damaged mites falling onto bottom boards with no sticky trap may be able to re-infest bees, whereas damaged ones likely could not, the combination of traits that produces high mite mortality *and* varroa-specific sensitivity leading to higher levels of damage is also likely to result in greater benefits from grooming as a defence mechanism. I advocate for more studies looking at the comparisons between the two colony level selection metrics and the individual grooming responses to multiple stimuli.

Successful mite removal was extremely rare in my assay, occurring only 9 times out of a total of 550 mite additions, 4 of which were removed by high-grooming bees and 5 by low-grooming bees. Previous studies by Guzman-Novoa et al. (2012) and Hamiduzzaman et al. (2017) have both found intense grooming behaviour to be associated with increased removal of mites in petri dish assays. In the Guzman-Novoa et al. (2012) study, mite removal was particularly high, ranging from 15% to 34.9% in bees from supposed mite-resistant colonies. Both Africanised and Russian stock were used in their experiments which are known to have good levels of mite-resistant traits, and their colonies were specifically selected for mite resistance over multiple years. My study, however, used a set of colonies from diverse sources made up mainly from beekeepers who used locally-adapted stock but did not actively select and breed bees for grooming behaviour or other mechanisms of resistance to varroa. Thus, it is possible that if I had used colonies selected for grooming behaviour over multiple generations, there may have been larger differences between selected groups. My study also did not use a 2–3-minute acclimation time after the bees were introduced into the dishes as did the Guzman-

Novoa et al. (2012) study, so it's also possible I would have seen more mite removal had I also done that.

The high-damage group cut-off rate of more than 30% of the mites being damaged that I used is in line with other studies that have measured mite damage. Guzman-Novoa et al. (2012), for example, found mite damage rates of between 26.2% and 59.5% in colonies with presumed mite-resistance, and rates of 7% and 23.8% in presumed mite-susceptible colonies. Andino and Hunt (2011) found mite damage rates of 7%-44% in their studies that correlated the level of damage found in source colonies with the overall mite mortality rate in caged bees. Hunt et al. (2016) also bred specifically for mite damage over many years, starting with a damage rate of about 3% and selectively breeding up to a damage rate of almost 50% eight years later. However, none of these mite damage rates come close to the 73.8% damage rate noted in the study by Peng (1988) using *A. ceranae*. Thus although *A. ceranae* are clearly better groomers than *A. mellifera*, these studies do provide strong evidence that grooming behaviour may be improved over time with selection.

Recent studies have investigated molecular markers for grooming behaviour to help quicken the pace at which heightened grooming behaviour can be identified and selected. Arechavaleta-Velasco et al. (2012) identified 27 candidate genes using quantitative trait-locus interval mapping that correlated with the time to initiate grooming behaviour. Included were three genes known to be associated with neurodevelopmental and behavioural effects as well as one gene, Neurexin-1, known to be associated with self-grooming behaviour in mice. Interestingly, Hamiduzzaman et al. (2017) also correlated the expression of Neurexin-1 with intense grooming behaviour and found another gene that correlated with intense or light grooming (pUF68), and another correlating with the presence of a mite on a bee (CYP9Q3). Honey bee hygienic behaviour has also been correlated with the expression of certain genetic markers (Lapidge et al., 2002; Tsuruda et al., 2012) and more recently with protein markers (Guarna et al., 2017). The results of this study support the idea that multiple genes are involved in the grooming response and that mechanisms that allow bees to identify and respond to cues produced by mites, as well as behavioural traits that allow recruitment of allo-groomers and effective communication of the presence of mite should be considered when trying to correlate the presence of putative markers with grooming success.

One potential limitation of this study is that the CO₂ that was used to gently anesthetize the bees before placing them into the petri dishes could have influenced their behaviour. This was a trade-off done to slow the bees' activity due to the challenges associated with applying a stimulus to a bee moving around in a petri dish—particularly one vibrating its wings, causing the mite or chalk to blow around before successfully applying it to the bee. The anesthetization of the bees with CO₂ was, in most cases, just enough to get the bees into the dishes and apply the stimuli. By then, the bees were usually awake and just beginning to move about in the dish, and after a few seconds moving around the bees appeared to be back to “normal.” In cases where the bees were not returning to a normal level of activity following anesthetization, the bee was removed and replaced with a new bee, following the same methods. On rare occasions, marginally active bees were not noticed until they were watched more closely during video analysis. If bees were deemed to be unrecovered from the CO₂ during video analysis, they were noted as such and were removed from the data set for any statistical analyses. It is also possible, however, that the CO₂ influenced the bees' ability to groom intensely or may have caused them to begin grooming earlier. On occasion, it was noted that bees that were returning to a normal level of activity began light grooming almost immediately (usually slowly cleaning their antennae) and continued to lightly groom after returning to a ‘normal’ level of activity. However, since all bees in all stimulus treatments (including the controls) were treated the same, the relative differences I documented among treatments should be valid.

One other limitation of this study is that there were multiple observers that assessed the grooming responses of bees in the videos. Thus, some subjectivity inevitably exists within the data. However, the care taken to train and calibrate observations, using recorded video that allowed each observer to watch the bees' responses over again if needed, and the use of the grooming timer ‘app’ all served to reduce subjectivity and made data collection much less subjective than other methods.

Overall, the results of this study clarify how individual bees identify and respond to the presence of irritants through grooming responses. My results reveal that high-grooming bees had heightened sensitivity to both varroa as well as a stimulus of chalk dust compared to low-grooming bees. Additionally, the pattern of heightened intense grooming in the varroa-stimulated bees from high-damage colonies versus those from moderate-damage colonies suggests that

varroa-specific sensitivity is in some way linked with high mite damage rates. Thus, using alternative irritants such as chalk dust or wheat flour, while valuable in scoring generalized grooming responses may not be able to select for bees with all the components needed for successful grooming of varroa at the colony level. My results suggest that colonies with high damage rates in addition to high mite mortality rates may have additional sensory abilities, such as heightened tactile or chemosensory abilities, that allow them to more accurately target varroa. Auto-grooming is also only part of a range of behaviours existing at the colony level and it could be that allo-grooming as well as communication signals used to recruit allo-groomers also play a role in the successful grooming and injuring of mites in high-grooming colonies. Further work should be done to assess the correlations between colony level metrics such as mite damage and the use of alternative irritants to screen bees at the individual level and to ultimately correlate these with molecular and proteomic markers.

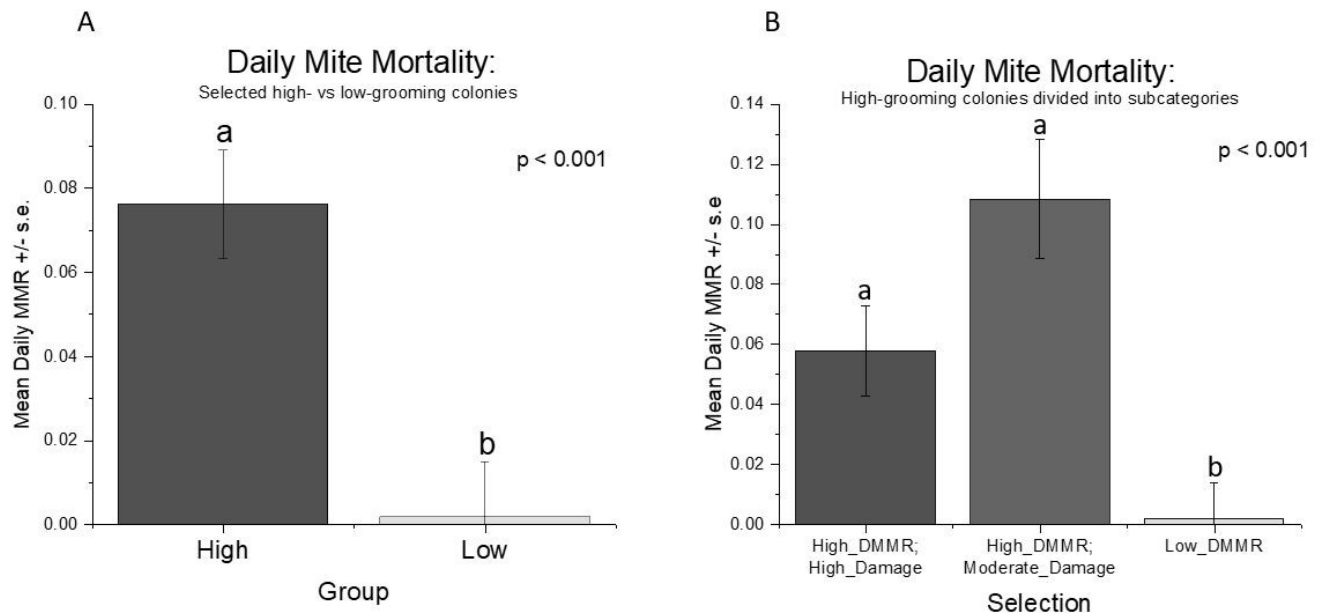


Figure 2.1. Mean daily mite mortality rate (DMMR) +/- standard error (SE) for (A) high- and low-grooming groups of colonies selected based upon DMMR, and (B) high- and low-grooming groups of colonies with the high-grooming colonies further divided into high- and moderate-damage subcategories. Means followed by the same letter are not significantly different ($P < 0.05$, Tukey).

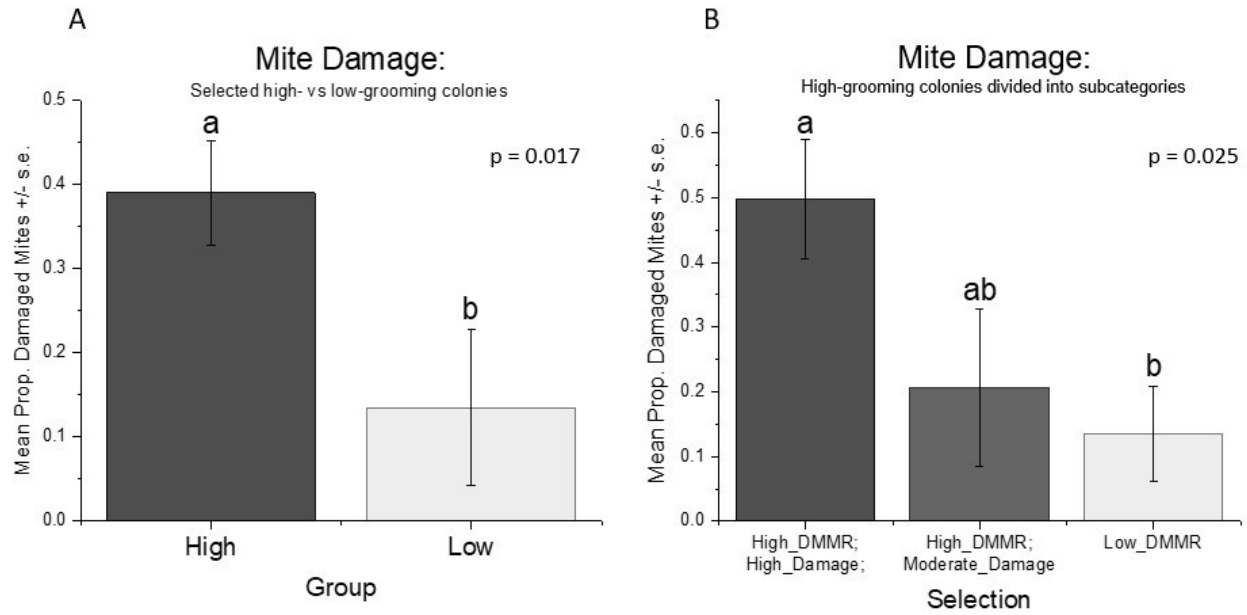


Figure 2.2. Mean proportion of damaged mites (PDM)+/- standard error (SE) for (A) high- and low-grooming groups of colonies selected based on DMMR; and (B) high- and low-grooming groups of colonies with the high-grooming colonies further divided into high- and moderate-damage subcategories. Means represented by bars followed by the same letter are not significantly different ($P < 0.05$, Tukey).

Intense Grooming by Selection & Treatment

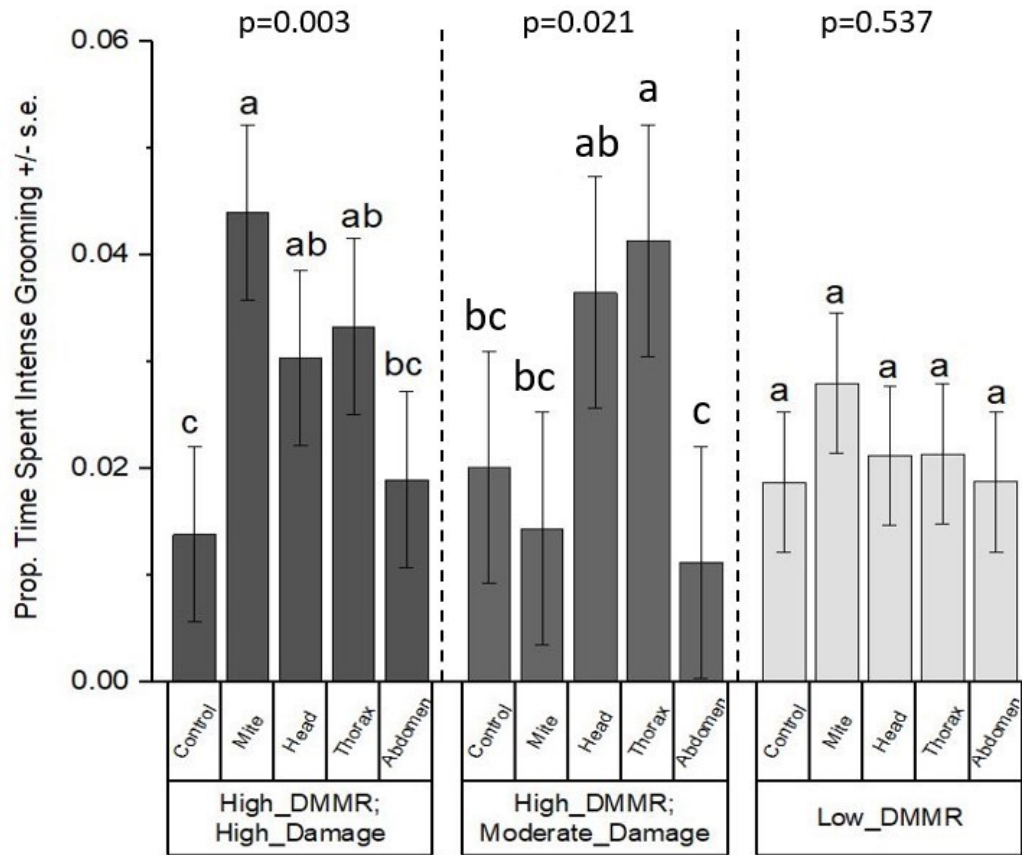


Figure 2.3. Proportion of time spent intense grooming +/- standard error (SE) by selection & treatment for high-DMMR/high-damage colonies, high-DMMR/moderate-damage colonies, and low-DMMR colonies. A marginally significant selection-method*treatment interaction was found (see text), warranting further exploration. Slicing the data by selection-method found a significant effect of treatment within the high-DMMR/high-damage and high-DMMR/moderate-damage subgroups, but not within the low-DMMR subgroup. Means represented by bars followed by the same letter within groups are not significantly different ($p \leq 0.05$, Protected LSD).

Preface to Chapter Three

In Chapter Two, I studied the sensitivities of individual bees from previously chosen high- and low-grooming colonies to a stimulus of varroa or a puff of chalk dust applied to the head, thorax, or abdomen. I found that the high-grooming bees had heightened sensitivities to both varroa as well as chalk dust on the head or thorax, compared to control treatments, while the low-grooming bees showed no differences among treatment groups. Further, I found varroa-specific sensitivity in a sub-group of high-grooming colonies that had been chosen based on high levels of mite damage. However, a hive of honey bees is made up of tens of thousands of bees. Therefore, in Chapter Three I studied cohorts of bees from high- and low-grooming colonies in cages to gain a better understanding of the mechanisms of honey bee grooming behaviour in the hive environment and inter-bee communication in general. I also wanted to study the bees' responses to different levels of stimuli added to the cages to gain insight into the bees' abilities to assess mite infestation levels and their thresholds for responding.

Chapter 3. Quantification of Grooming and Communication Responses in Cohorts of Selected High- and Low-grooming Honey Bees (*Apis mellifera*)

Abstract

The European honey bee (*Apis mellifera*) has been subject to increased losses in recent decades. A major culprit for these losses is the parasitic mite *Varroa destructor*. Though many chemical and physical control methods have been developed, lesser established methods include breeding for resistance through selecting for natural social immunity traits. One such trait is grooming behaviour, whereby bees can remove mites from themselves or from their nestmates in the colony. While many studies have explored grooming behaviour, many questions remain regarding the overall mechanisms of how bees perceive and communicate threats such as varroa within the larger context of the hive. The objective of this study was to characterize different components of grooming behaviour and elucidate potential signals through which bees might communicate the presence of mites to nestmates. I used small cohorts of bees in cages and quantified their responses before and after inoculation with either live varroa mites or a standardized stimulus of chalk dust. Colonies were categorized as high- or low-grooming using a combination of mite mortality rate and the proportion of damaged mites collected off sticky papers. Bees from high-grooming colonies exhibited more auto-grooming overall, increased allo-grooming at low levels of either stimulus, increased grooming invitation dancing behaviour at low levels of a varroa stimulus and produced more worker piping noises than low-grooming colonies. The results of this study contribute valuable information to the wider body of knowledge on honey bee grooming behaviour and offer novel avenues for further research.

Introduction

Honey bees (*Apis mellifera* L.) have suffered considerable losses in recent decades, due to a combination of stressors including pests, pathogens, parasites, poor nutrition, inclement weather, and pesticide exposure (Currie et al., 2010; Pernal & Clay, 2013; Steinhauer et al., 2018). Of these, the ectoparasitic mite, *Varroa destructor* Anderson & Trueman (hereon referred

to as varroa), is commonly cited as the most damaging and the greatest threat to apiculture worldwide (Guzmán-Novoa et al., 2010; Rosenkranz et al., 2010). Elucidating the mechanisms of resistance to this parasite to provide alternative methods to control varroa infestations is increasingly important.

Honey bees use various forms of ‘social immunity’ to fend off pests, pathogens, and parasites. Some examples of social immunity strategies include the collection of antimicrobial resins (propolis) by workers, and the collective generation of heat to create “social fever” that helps regulate pathogens (Simone-Finstrom, 2017; Simone-Finstrom & Spivak, 2012). Other social immunity behaviours that can impact varroa include hygienic brood removal (Spivak, 1996), varroa-sensitive hygiene (Danka et al., 2011) and grooming behaviour (Boecking & Spivak, 1999). Hygienic removal and the associated varroa-sensitive hygiene behaviours involve the detection and removal of diseased, dead, or in some cases varroa-infested capped brood by worker bees and thus can result in the removal or death of varroa. Grooming behaviour is a generalized response against a variety of potential contaminants and involves bees using a combination of their mouthparts and legs to remove dust, pollen, or ectoparasites from their own bodies (auto-grooming) or from those of their nestmates (allo-grooming) (Boecking & Spivak, 1999). Honey bees live in large, complex societies and communication within the colony is achieved through an array of different pheromones, dances, shaking/vibrational signals, sounds, and trophallaxis (food sharing) behaviours (Kirchner, 1993a; Winston, 1987). Allo-grooming for example, can be solicited from nestmates through the grooming invitation dance (Haydak, 1945; Land & Seeley, 2004), which is a relatively short—often lasting only a few seconds—tremble-like dance, where a bee “stands stationary and vibrates her whole body from side-to-side at a frequency of 4.2 ± 0.2 Hz” (Land & Seeley, 2004).

In varroa’s original host, the Asian honey bee, *A. cerana* Fabr., grooming by adult workers is one of the primary mechanisms to regulate varroa. Peng (1988) found that the grooming behaviour of *A. cerana* bees results in the removal of more than 99% of mites in just two hours, with over 70% having visible physical injuries. They characterized grooming of varroa as a series of actions that include auto-grooming, dancing, allo-grooming, and group cleaning. In the same study, however, fewer than 17% of *A. mellifera* workers even attempted to engage in auto- or allo-grooming actions and less than one percent of mites were removed.

Levels of grooming may vary with the genetic source of bees in both *A. cerana* and *A. mellifera*. Compared to Peng (1988), other studies have found lower levels of grooming against varroa in *A. ceranae* and higher levels of grooming in *A. mellifera*. Buchler et al. (1992), for example, found successful removal of 75% of varroa mites in *A. cerana* versus 48% in *A. mellifera*. Other studies have demonstrated a range of grooming effectiveness and intensity across different *A. mellifera* subspecies. Africanized subspecies, for example, have repeatedly exhibited more efficient grooming of varroa than European subspecies (Aumeier, 2001; Guzman-Novoa et al., 2012; Moretto et al., 1993; Morfin et al., 2020). Similarly, Rinderer et al. (2001) showed that bees originating from the Primorsky region of Russia engaged in more grooming activity against varroa than bees from Louisiana, USA. Similarly, Thakur et al. (1997) showed video evidence of grooming behaviour in *A. mellifera carnica* Pollman, including the bees detecting, grabbing, and biting free-moving mites. These studies show that there is a range of genetic diversity in *A. mellifera* through which selection could be used to improve this trait in bee stock.

Grooming behaviour has also been found to be an effective mechanism of control of tracheal mites (*Acarapis woodi* Rennie) in *A. mellifera*. Pettis and Pankiw (1998) found that allo-grooming and grooming invitation dance levels increased significantly when tracheal mites were present, and that young worker bees 1–3 days old exhibiting higher levels of grooming dances acquired the lowest number of mites.

Since grooming has been established as a heritable trait (Arechavaleta-Velasco et al., 2012; Hamiduzzaman et al., 2017; Moretto et al., 1993; Stanimirovic et al., 2010), it is of interest for breeding programs aimed at selecting for bees with varroa-resistance behaviours and many studies have focused on analyzing different selection methods for grooming behaviour. Several have focused on indirect methods including collecting and counting all mites falling onto sticky papers placed beneath hives and calculating the mite mortality rate (Andino & Hunt, 2011; Currie & Tahmasbi, 2008), or microscopic identification of physical damage to collected mites and calculating the proportion of damaged mites (Guzman-Novoa et al., 2012; Hunt et al., 2016; Moosbeckhofer, 1992; Ruttner & Hänel, 1992). Other recent research has focused on analyzing the grooming responses of individual bees directly when challenged with live varroa mites collected from highly infested colonies (Aumeier, 2001; Bak & Wilde, 2015; Guzman-Novoa et

al., 2012; Hamiduzzaman et al., 2017; Invernizzi et al., 2015). Others have shown that small particles of chalk dust (Land & Seeley, 2004) or wheat flour (Morfin et al., 2020) can be used to stimulate grooming responses in *A. mellifera* bees in place of mites in individual grooming assays.

While these individual bee assays may offer alternative ways to screen for heightened levels of grooming behaviour, many questions remain regarding the overall mechanisms of grooming resistance to varroa mites in *A. mellifera* at the colony level. Some studies have attempted to directly observe and quantify the social components of grooming behaviour such as allo-grooming and grooming invitation dances using multiple bees or cohorts in observation hives or cages (Aumeier, 2001; Bak & Wilde, 2015; Bozic & Valentincic, 1995; Buchler et al., 1992; Fries et al., 1996; Kolmes, 1989; Land & Seeley, 2004; Moore et al., 1995; Peng, 1988; Pettis & Pankiw, 1998; Thakur et al., 1997); although, fewer have successfully shown direct evidence of such behaviours in *A. mellifera* (Buchler et al., 1992; Kolmes, 1989; Land & Seeley, 2004; Moore et al., 1995; Pettis & Pankiw, 1998; Thakur et al., 1997).

Using observation hives, grooming invitation dances have been directly witnessed and quantified by Pettis & Pankiw (1998) and Land & Seeley (2004). Allo-grooming was directly witnessed by Buchler (1992) in 8% of artificially varroa-infested bees within 5 minutes, although no mite removal was observed. Thakur (1997) recorded video evidence of grooming invitation dances and allo-grooming behaviour, however, again no direct mite removal was witnessed. Moretto (1993) also witnessed allo-grooming in Italian *A. mellifera* and found a small portion (1.6%) of bees had mites removed by nestmates within 5 minutes of infestation. Kolmes (1989) & Moore (1995) witnessed allo-grooming as well and found that a small portion of workers may become “grooming specialists,” performing few other activities than grooming nestmates. These studies show that these behaviours can be directly observed and quantified using cohorts of bees, however, the relative importance of these components in controlling varroa in *A. mellifera* remains questionable.

It is also unclear if *A. mellifera* bees have specific responses that allow them to better detect varroa compared to alternative irritants like dust that have been used previously. Further, if *A. mellifera* bees have increased sensitivity to varroa versus other irritants (Chapter 2), it remains unclear whether this information can be used to recruit the help of allo-groomers

through communication mechanisms such as the grooming invitation dance, or perhaps other unknown mechanisms.

In addition to dancing, it is likely that other communication signals are involved at the colony level to communicate the need for allo-grooming. For example, vibroacoustic communication signals, such as worker piping and beeping sounds, have previously been identified in *A. mellifera* as communication signals during swarming and foraging activity (Schlegel et al., 2012; Seeley et al., 2012), and in anti-predator signalling in *A. cerana* (Mattila et al., 2021). However, these acoustic signals have not previously been studied in the context of grooming and it is possible that these, or other signals, also help communicate a need for social grooming.

The overall objective of this study was to clarify the behavioural mechanisms by which the bees in high-grooming colonies of *A. mellifera* are better able to detect and remove mites from themselves and from other bees in the colony than they are for a standardized irritant such as chalk dust. Specifically, I hypothesized that: (1) bees from colonies chosen for high-grooming behaviour would engage in increased levels of auto-grooming and allo-grooming relative to colonies chosen for low-grooming behaviour (i.e. varroa susceptible) after a stimulus of either varroa or chalk dust; (2) high-grooming bees would exhibit increased communication in the form of dances, vibroacoustic signals, or trophallaxis after a stimulus of either varroa or a standardized chalk dust stimulus; and (3) compared to low-grooming bees, high-grooming bees would exhibit increased allo-grooming or communication responses at lower levels of stimulation (i.e. number of mites or puffs of chalk dust added to cages).

Materials and Methods

Selection of colonies:

The experiment was carried out at the University of Manitoba in Winnipeg, Manitoba, Canada (49°54' N, 97°14' W). The high- and low-grooming categories were assigned using the same metrics as in chapter 2 of this thesis (i.e., daily mite mortality rate and proportion of damaged mites). Most of the same colonies as described in chapter 2 were assayed, except fewer colonies were used overall, and some replacement colonies were used when colonies from experiment 1 had either died or become too weak to test reliably. Nine high- and nine low-

grooming colonies were tested. Of the selected high-grooming colonies included in this experiment, daily mite mortality rates (DMMR's) ranged from 0.03 to 0.17 (proportion of total mites falling each day) and the proportion of all collected mites with any damage (PDM's) ranged from 0.14 to 0.71.

Cage Trials:

Trials to examine group grooming responses in high- and low-grooming bees stimulated with either chalk dust or varroa mites began on 6 September 2017 and ran until 22 September 2017. Typically, one high- and one low-grooming colony were chosen arbitrarily to be tested each day except for occasional days when only one colony was able to be tested due to unforeseen interruptions. For each tested colony, two experimental cages (Currie & Tahmasbi, 2008) were filled with approximately one hundred and twenty-five bees collected from the brood nest using a 160 ml sample container calibrated to indicate the level required to obtain 125 bees. Ventilation screen (#8 hardware cloth) was present at the top, bottom, and sides of the cages and the back of the cage was outfitted with a piece of artificial plastic comb. Before bees were added to the cages, a “TempQueen” queen pheromone strip (Intko Supply Ltd., Suite 604, 3345 Kingsway, Vancouver, BC, V5R 0A7, Canada) was poked into the top of the cage via the ventilation screen and affixed with a staple. Fifteen ml of 2:1 sucrose sugar syrup (by volume) was also injected into the plastic comb using a 30 ml syringe to feed the bees while they were in the cage. A transparent window cover was fabricated for each cage consisting of a thin plastic frame (3 mm puckboard) with clear Mylar® film affixed to it with scotch tape. A small window flap (1 cm x 2 cm) was cut in the Mylar® film in the centre of the cage to serve as an access point for applying stimuli (varroa or chalk dust) to bees in the cage. The flap was taped shut with a small piece of transparent cellophane tape to prevent bees from escaping.

Cages were brought up to the experimental “flight room” (Pernal & Currie, 2002) and left for a minimum of one hour to settle and acclimate to the room. During acclimation, live varroa mites were collected using the same modified CO₂ shaking method described in Chapter 2. Cages were randomly assigned to one of two treatments—chalk dust application or introduction of varroa—via the flip of a coin. Two Apple iPhones (one iPhone 5s and one iPhone 6s), held in place with test tube clamps attached to ring stands, were used to simultaneously record video

from each cage. Audio from each cage was also recorded using two identical AKG C1000 condenser microphones that were positioned against the ventilation screen at the top of each cage and held in place with microphone stands (Fig 3.1). Microphones were run through an M-Audio Fast Track C600 Digital Audio Interface connected to a MacBook laptop. ProTools for MacOS was used to record the full duration of each trial (see next paragraph) and audio files were saved to an external hard drive immediately following the trials' completion.

Trials for both treatment cages were run in unison. To obtain a baseline (control) level of cage activity, audio and video was recorded for 5 minutes before any stimuli were added to the cages. Stimuli were then added through the small window flap cut in the Mylar® film. For the chalk dust stimulus cage, sequential applications of chalk dust puffs—spaced 2 minutes apart—were applied onto 20 haphazardly selected bees in the cage. Chalk was targeted for application to the thorax of each selected bee and was achieved most of the time, however, the quickly moving bees in the cage made it difficult to control which body region the chalk was puffed onto and therefore chalk was puffed onto any region of the bee that could be accessed. The body region (head, thorax, or abdomen) onto which the chalk dust was puffed was recorded during video analysis. Similarly, for the varroa-stimulated cage, sequential introductions of varroa mites—spaced two minutes apart—were applied to 20 bees. To keep better track of which bee the mites were added to, the mites were marked white with a queen marking pen (Uni Posca water-based paint pen) just before adding them to the bees. Two minutes after the 20th stimulus had been added to each cage, video and audio recording was stopped and the recordings were saved for later analyses.

The number of individual hives tested on a given day varied from one to three. In cases where two hives were tested on the same day, the four cages for both hives were brought into the flight room at the same time. The two cages for one hive were tested first followed by the two cages for the second hive. Thus, the cages for the second hive tested had a longer acclimation time, however the order in which they were tested was random. On the one day that three hives were tested, cages for the first two hives were brought up and tested as normal, then returned, and the cages for the third hive were brought up later to better standardize acclimation time before testing.

Video Analysis:

Video for each cage was analyzed using PowerDVD 16 Media Player (CyberLink Corp, 2016), which allowed for adjustments to both the zoom and playback speed. This allowed for more accurate quantification of the grooming and communication responses of each bee. For each of the twenty stimulated bees, the zoom function was used to identify which bee had been stimulated, and the bee was then followed around the cage for two minutes. At times, it was difficult to follow the bee as it was often lost for sections of time underneath other bees or in pockets of the cage not visible to the camera. Watching the bees in slow-motion or, at times, frame-by-frame, greatly helped to keep track of the bees; however, in cases where the stimulated bee was lost completely, it was recorded as such, and the amount of time the bee was observed was recorded. More than 85% of all bees were watched for the full two minutes

Behaviours that were observed and quantified were: auto-grooming (self grooming), allo-grooming (group grooming), grooming invitation dances, and trophallaxis (food exchange). For auto-grooming, the length of time a bee groomed itself was estimated, and an auto-grooming score was given to each bee on a scale of 0–4: A score of 0 was given for no grooming; 1 for low levels of grooming totalling less than 8 seconds; 2 for moderate levels of grooming between 8 and 25 seconds; 3 for high levels of grooming longer than 25 seconds; and 4 for very high levels of grooming at least 90% of the time, or 108 seconds. For allo-grooming, grooming invitation dances, and trophallaxis responses, the number of “light” or “intense” responses were recorded. “Light” events were defined as those lasting less than two seconds, and “intense” events as those longer than two seconds. A score was attributed to each event, where “light” events were given a score of 0.1 and “intense” events a score of 1.0. Total overall scores were then calculated for allo-grooming, grooming invitation dancing, and trophallaxis.

Pre-treatment observations as described above, were also recorded in untreated bees by randomly selecting cages and observing 20 bees within a cage during the 5-minute pre-treatment period. Ten bees were then randomly assigned as pre-treatment controls for each experimental unit (odd-numbered bees assigned to varroa cages and even-numbered to chalk dust cages).

Audio Analysis:

Audio recordings were processed using *Kaleidoscope Pro* (Wildlife Acoustics, 2019, version 5.1.9) to quantify the number of worker piping noises in each of the audio clips. Prior to analysis, piping noises were compared to those supplied in the supplemental material by Schlegel et al. (2012). An “advanced classifier” was trained to pick out piping noises and all audio clips were then run through the advanced classifier to quantify the number of noises. For each cage, the number of piping noises for the full audio clip was broken into piping noises “pre-stimulus” (i.e., control period) and piping noises “post-stimulus” (i.e., stimulus period).

Statistical Analysis:

Analysis of mean DMMR’s and mean PDM’s in source colonies selected as high-grooming or low-grooming groups of colonies were analyzed with analysis of variance (ANOVA) (PROC MIXED, SAS Institute Inc. 2020). Prior to analysis, normality and homogeneity of variance were first checked visually using R statistical software (Version 1.3.1056) to view histograms and Q-Q plots. Since data were non-normal, an arcsine square root transformation was applied to the data after a small constant was added to all values of 0.

For each observed behaviour (auto-grooming, allo-grooming, grooming invitation dances, trophallaxis), data were analyzed using a repeated measures ANOVA (PROC MIXED, SAS Institute Inc. 2020) with a compound symmetry covariance structure and with group (high- or low-grooming) and stimulus-type (varroa or chalk) as fixed factors. Colony(group), stimulus-type*colony(group), stimulus-level*colony(group), stimulus-level*stimulus-type*colony(group) were random factors. Stimulus-level was treated as a repeated measure with the subject assigned as bees within cages (group*stimulus-type*colony). Stimulus-level was categorized into 5 levels of stimuli applied to the cage (i.e., stimulus-level = 0 (pre-treatment), 1–5, 6–10, 11–15, or 16–20 applications of either mites or chalk dust). For all observed behaviours, normality and homogeneity of variance were first checked using R statistical software (Version 1.3.1056) to view histograms and Q-Q plots. Data for all dependent variables were then log-transformed to better approximate a normal distribution prior to analysis; however, all data are presented as untransformed means. Where significant interactions were observed, data were then partitioned using the slice option in LSMeans to analyze differences within separate factors.

For vibroacoustic analysis, the number of worker piping noises was analyzed using a repeated measures ANOVA (PROC MIXED, SAS Institute Inc. 2020) using a compound symmetry covariance structure, with group and stimulus-type as fixed factors, colony(group) as a random factor, and time (i.e., pre-stimulus or post-stimulus) as a repeated measure. Prior to analysis, data were plotted and tested for normality and homogeneity of variance in Origin (Version b9.4.0.220, 2017). One outlier point was noted when visually inspecting the plots and identified as a significant outlier using Grubbs Test ($g = 4.16$, $p < 0.001$) and was removed prior to analyses. Levene's test also indicated that variances for the number of piping noises were not equal ($F_{3,67} = 46.49$, $p < 0.0001$), therefore degrees of freedom were calculated using the $ddfm = SATTERTHWAITE$ option to adjust for poor homogeneity of variance. Tests for outliers, normality, and homogeneity of variance were considered non-normal or outliers where $p \leq 0.05$.

Results

Colony selections:

Mean daily mite mortality rate (DMMR) of the selected high-grooming colonies was higher than the selected low-grooming colonies ($F_{1,16} = 39.35$, $p < 0.0001$) (Fig. 3.2). The proportion of damaged mites (PDM's) for high-grooming colonies did not differ from low-grooming colonies ($F_{1,16} = 0.01$, $p = 0.93$) (Fig 3.3). Mite damage level was not a significant covariate affecting other analyses of other variables so only two categories based upon mite mortality rates were used for subsequent analyses.

Auto-grooming:

For auto-grooming scores (Fig 3.4), there was a significant effect of group ($F_{1, 16.6} = 5.79$; $p = 0.028$) with high-DMMR colonies showing more auto-grooming than low-DMMR colonies. There was no overall effect of stimulus-type (varroa or chalk) ($F_{1,17.6} = 1.63$; $p = 0.22$), however, stimulus-level ($F_{4,67.3} = 12.63$; $p < 0.0001$) did influence auto-grooming. A post-hoc Tukey test for stimulus-level found that all levels of stimulus increased the amount of auto-grooming relative to unstimulated "pre-treatment" bees.

Allo-grooming:

There was no overall effect of group ($F_{1,16} = 0.04$, $p = 0.84$) or stimulus-type ($F_{1,16} = 3.08$, $p = 0.099$) on allo-grooming. However, there was a significant overall effect of stimulus-level ($F_{4,60} = 3.14$, $p = 0.021$) as well as a significant group*stimulus-level interaction ($F_{4,60} = 2.79$, $p = 0.034$) (Fig 3.5). Slicing the data by stimulus-level revealed the high-DMMR group had greater levels of allo-grooming than the low-DMMR group within the lowest level of infestation when '1–5' mites or puffs or chalk dust were applied ($F_{1,60} = 4.48$, $p = 0.038$). At higher levels of stimulation (within the '6–10' level), there was a trend towards greater levels of allo-grooming in the low-DMMR colonies than in the high-DMMR colonies which was marginally significant ($F_{1,60} = 2.98$, $p = 0.09$). Slicing the data by group found a significant effect of stimulus-level within both the high-DMMR colonies ($F_{4,60} = 2.84$, $p = 0.032$) as well as within the low-DMMR colonies ($F_{4,60} = 3.10$, $p = 0.022$). Within the high-DMMR colonies, allo-grooming at the lowest stimulus level ('1–5') was greater than in no stimulus and most of the higher stimulus levels except for levels of 11–15 which was also lower but not statistically different. In contrast, allo-grooming within the low-DMMR colonies differed from no stimulus at only the 6–10 applications level, and lower or higher levels of stimulus did not differ from zero or each other.

Dancing:

A three-way group*stimulus-type*stimulus-level interaction was found for grooming invitation dance scores (Fig 3.6) ($F_{4,63.4} = 2.55$, $p = 0.048$). Slicing the data by group*stimulus-type found a significant effect of stimulus-level for the high-DMMR colonies within the varroa stimulus ($F_{4,124} = 4.62$, $p = 0.0017$). Individual contrasts found that stimulus-levels of 1–5 ($p < 0.0001$), 11–15 ($p = 0.042$), and 16–20 ($p = 0.013$) mites produced significantly more dancing responses than no stimulus, and that a level of 1–5 mites also produced significantly more dancing than a level of 6–10 mites ($p = 0.033$). Further, a slice by stimulus-type*stimulus-level found that within the '1–5' level of the varroa stimulus, the high-DMMR colonies performed significantly more grooming dances than the low-DMMR colonies ($F_{1,125} = 9.10$, $p = 0.003$).

Trophallaxis:

Trophallaxis scores (Fig 3.7) did not vary with group ($F_{1,16} = 0.21$, $p = 0.65$) or stimulus-type ($F_{2,16} = 0.8$, $p = 0.78$). However, stimulus-level ($F_{4,60} = 4.37$, $p = 0.004$) did affect the amount of trophallaxis with more food exchange among bees when no stimulus was applied than at all other levels of stimulus with varroa or chalk dust.

Vibroacoustics (worker piping):

There was an overall effect of group ($F_{1,16.1} = 6.08$, $p = 0.025$) on worker piping sounds, which were greater in high-DMMR colonies than in low-DMMR colonies. However, there was also a significant group*time interaction ($F_{1,47.4} = 5.15$, $p = 0.028$) showing this occurred only in the presence of the stimuli that were introduced (Fig 3.8). Slicing the data by time revealed a significant effect of group with the high-DMMR colonies exhibiting greater numbers of piping signals than low-DMMR colonies within the post-stimulus period but not in the pre-treatment period. There was no effect of stimulus-type ($F_{1,47.4} = 1.18$, $p = 0.28$) on worker piping.

Discussion

This study analyzed auto-grooming responses, allo-grooming responses, and communication responses of cohorts of bees from colonies that had previously been chosen for high- or low-grooming behaviour. I selected colonies using a combination of colony-level selection metrics and then exposed selected groups of caged bees to different levels of either a standardized irritant stimulus or varroa mites. I found that high-grooming bees engaged in significantly more auto-grooming overall; significantly more dancing and allo-grooming behaviour at low levels of stimulus (i.e., they were quicker to respond to a stimulus); and workers produced significantly more piping noises during the stimulation period. Additionally, both the high- and low-grooming bees engaged in significantly more trophallaxis interactions with no stimulus, compared to any level of stimulation with either stimulus.

Bees from high-grooming colonies auto-groomed significantly longer when exposed to either stimulus (varroa or chalk dust) than the low-grooming colonies, supporting my original hypothesis that high-grooming bees would engage in increased levels of auto-grooming behaviour. This finding is in line with previous studies that have directly observed and quantified

differing levels of auto-grooming behaviour in different sub-species of *A. mellifera* (Aumeier, 2001; Bak & Wilde, 2015; Guzman-Novoa et al., 2012; Invernizzi et al., 2015; Morfin et al., 2020), or in *A. mellifera* bees selected for low varroa-mite population growth (Guzman-Novoa et al., 2012) or specifically for heightened grooming behaviour (Chapter 2). In this study, responses to mites were similar to those of chalk dust and it did not appear that bees in cages were able to discriminate between these stimuli as they have been reported to do in single bee studies (Chapter 2, Figure 4)—even when the high-grooming colonies were split into high-damage and moderate-damage selections as was done in Chapter 2. The auto-grooming responses of individuals—both high- and low-grooming bees—was similar at differing levels of stimuli added to cages. However, since auto-grooming by workers relies on a response to a direct stimulus and I monitored only the bee that was stimulated, I cannot evaluate whether auto-grooming was enhanced throughout the cage.

Allo-grooming also differed between high- and low-grooming colonies but the extent of the group response depended upon the level of infestation. Both the high- and low-grooming groups of colonies exhibited increased allo-grooming responses compared to their respective unstimulated control bees regardless of the type of stimulus (varroa or chalk dust), however, the high-grooming colonies showed increased allo-grooming responses at lower levels of stimulation than the low-grooming bees. This finding indicates that groups of workers from honey bee colonies with heightened levels of grooming behaviour may have reduced thresholds for initiating allo-grooming. It is possible that increased levels of auto-grooming do not result in successful mite removal or that mites that are removed acquire alternate hosts and are not killed as a result of grooming. Thus, inducing allo-grooming responses earlier when mite levels are increasing in a colony would potentially confer an advantage for these colonies resulting in more successful control of mite populations. However, this needs to be confirmed in experiments at the cage or colony level where mite removal success from the group is monitored which I did not do in my study.

No previous studies that I am aware of have directly observed and compared the levels of allo-grooming behaviour at differing levels of stimulation, however, my findings are in line with previous studies that have observed allo-grooming behaviour at low levels in *A. mellifera* (Buchler et al., 1992; Peng, 1988). Buchler (1992) quantified allo-grooming in *A. cerana* versus

A. mellifera and found that 33% of *A. cerana* bees allo-groomed after artificial inoculation with varroa within 5 minutes, versus only 8% of *A. mellifera* bees. While I only analyzed bees for two minutes, allo-grooming was observed in 5 to 10% of stimulated bees, suggesting a slightly higher response than that found by Buchler. Peng (1988) noted 17% of *A. mellifera* bees engaged in either auto- or allo-grooming but it is unclear exactly how much allo-grooming occurred in *A. mellifera*. However, Peng (1988) did document that most mites are removed by *A. cerana* within 15 minutes of which 74% are injured and 60% are removed by nestmates, so at least in *A. cerana*, a clear link exists between allo-grooming and grooming success.

Several studies have found relationships between reductions in mite infestation and mite damage rates (Arechavaleta-Velasco & Guzman-Novoa, 2001; Moosbeckhofer, 1997), however, it is difficult to attribute mite damage to either auto- or allo-grooming since damage can be inflicted after the mites are removed from bees if mites die of natural causes, or if they are removed through other resistance mechanisms such as hygienic behaviour. There is some evidence to support linkages between damage and grooming success. Guzman-Novoa et al. (2012) for example, found that colonies selected for low mite population growth also had higher proportions of damaged mites at the colony level, increased intensity of auto-grooming responses in individual bees, and more mite removal due to auto-grooming at the individual level. While their study only directly observed auto-grooming responses, it is possible that bees from those colonies would have also exhibited elevated levels of allo-grooming, which could have partly contributed to their low mite infestations and higher damage rates of mites at the colony level.

My study did not collect mites removed from bees in the experimental cages, however, mite removal via allo-grooming was directly observed twice within the 2-minute observation periods. Interestingly, these two observations were from the same high-grooming colony that had the highest daily mite mortality and mite damage rates at the colony level, as well as the highest number of allo-grooming instances in the cage trials. Only one other study that I am aware of has reported direct observation mite removal via allo-grooming in *A. mellifera*. (Moretto et al., 1993).

If high-grooming bees have decreased thresholds for initiating allo-grooming in the colony as my data suggest, mechanisms to both assess the level of infestation and communicate the need for grooming would undoubtedly play an important role in initiating allo-grooming.

Grooming invitation dances are one mechanism for communicating the level of infestation in groups of bees. In my study, grooming invitation dance scores did differ between the high- and low-grooming groups of colonies but depended on the type of stimulus (varroa or chalk dust) and the level of stimulation (number of mites or chalk dust puffs). Overall, for both the high- and low-grooming groups of bees, varroa was a much stronger stimulus in producing grooming invitation dances, and chalk dust did not stimulate grooming dance responses relative to the unstimulated control. Within the varroa stimulus treatment, the high-grooming bees performed significantly more grooming dances than the unstimulated control at almost all levels of stimulus, while low-grooming colonies did not respond to either stimulus by increasing their level of dancing. Further, at the lowest (1–5) level of varroa infestation, the high-grooming bees danced more than the low-grooming bees. These results show that groups of bees respond to varroa differently than a generalized irritant, and further that groups of bees selected for high-grooming behaviour may have a heightened ability to quickly assess their level of varroa infestation and communicate a need for allo-grooming compared to low-grooming bees. This result agrees with the results of Chapter 2, showing that some groups of bees selected for heightened grooming may have varroa-specific mechanisms of sensitivity, allowing them to target varroa more directly and effectively.

The similarities in the trends witnessed for allo-grooming (with either stimulus) and for grooming dance responses (with a varroa stimulus) suggest that increases in grooming dances may have successfully solicited increased allo-grooming responses in bees inoculated with mites. However, this is unconfirmed in my study as direct observation of grooming invitation dances followed by allo-grooming responses was rarely witnessed. Of the 60 bees that performed grooming invitation dances, only eight bees (four high- & four low-grooming) also received any level of allo-grooming during the two-minute observation period, and only four bees (two high-grooming and two low-grooming) received allo-grooming immediately after dancing. Still, for these few bees that received allo-grooming immediately after dancing, the relationship seemed to be clear. For example, during one instance of mite removal via allo-grooming mentioned above, the bee with the mite was observed to be auto-grooming intensively, apparently trying to remove the mite, while also mixing in short dancing vibrations. The groomer bee that removed the second mite was an adjacent bee that was observed responding to the bee that was intensively

grooming and shaking. The allo-groomer first antennated the bee with the mite and then proceeded to groom, at first slowly checking parts of the abdomen before quickly snatching the mite off the far side of the abdomen. The groomer bee then appeared to chew the mite with its mandibles, and even attracted the help of another neighbouring bee that appeared to be helping to chew the mite. It was also noted that once the allo-groomer initiated grooming, the bee receiving grooming stood still with her wings slightly spread, which is very similar to the “social grooming display” described by Bozic and Valentincic (1995).

In one other instance of mite removal via auto-grooming, the bee was also noted to be biting the mite, providing direct evidence that mites may be bitten and damaged through auto- or allo-grooming behaviour. Peng (1988) noted that out of the 59.6% of mites removed by *A. cerana*, 10.5% of them were removed by group cleaning involving more than one groomer bee, however, no evidence of group cleaning was noted in my video analyses.

For trophallaxis behaviour, while no significant differences were found between high- and low-grooming groups, nor between varroa and chalk dust stimuli, a significant effect of stimulus-level was found. Trophallaxis interactions were highest in unstimulated control bees, and all levels of stimulus resulted in significantly reduced trophallaxis behaviour. This finding agrees with a previous study (Geffre et al., 2020) which found that experimental inoculation with Israeli Acute Paralysis Virus also significantly reduced the number of trophallaxis interactions between bees in observation hives. The authors conclude that this may be an adaptive social immune response by bees to reduce virus transmission. My finding of significantly decreased trophallaxis behaviour after the addition of a varroa or chalk dust stimulus indicates that this pattern of decreased social interaction may extend more broadly to other stressors, such as varroa or even benign irritants like small dust particles. However, I did not analyse viruses and the time frame of my experiment would not have allowed assessment of their impact. For my study, it seems more likely that the observed reduction in trophallaxis may be a result of a displacement to higher priority activities such as self-grooming or grooming dances that are used to recruit nestmates.

Auditory signals are another mechanism through which bees could communicate and respond to differences in colony level infestations of mites. Worker piping signals have previously been described and linked to different colony functions. Descriptions of these signals

have ranged from shorter, lower frequency vibrations that serve as stop signals that curb recruitment to less than ideal foraging locations or nest sites (Seeley et al., 2012; Thom et al., 2003), to longer, higher frequency vibrations that function as excitation signals, serving to mobilize quietly resting bees to ready themselves to leave the colony during swarming (Schlegel et al., 2012). Antipredator piping signals have also been described in *A. cerana* during hornet attacks (Mattila et al., 2021). The piping noises in my study were identified using the description by Schlegel et al. (2012), who define worker piping as vibrational signals having a “longer mean duration and...a slightly higher and upward sweeping fundamental frequency.” The worker piping noises in my experiment were identified by first listening to some of the high-pitched vibrational noises in my recordings and then comparing those sounds against the examples of worker piping provided in the supplementary material by Schlegel et al. (2012). Using these initial positively identified pipes, an “advanced classifier” was trained using *Kaleidoscope Pro* software which was then used to pick out and quantify other similar sounds in the other audio recordings. I found that bees from high-grooming colonies produced significantly more piping noises in response to stimuli, than those from low-grooming colonies.

To my knowledge, worker piping noises have not been previously correlated with grooming behaviour or grooming invitation dances. My results suggest that these auditory signals may serve a broader “excitation” purpose, serving to mobilize other bees in the colony to increase their activity levels and begin performing colony tasks as needed. A direct link between piping signals and increased levels of auto- or allo-grooming behaviour is not shown here, however, as I was not able to confidently identify the specific bees creating these piping noises within the cage to be able to correlate a specific behaviour with the audible piping noises I recorded. Still, it is reasonable that the observed difference in piping noises produced by the high- and low-grooming bees was a direct result of the irritation of the stimulus of varroa or chalk dust added to the bees. Increased levels of piping in high-grooming bees may be one mechanism used to recruit allo-groomers by direct signalling of a need for grooming assistance or by a more generalized mechanism by creating a higher response stimulus that in turn stimulates resting bees to increase their activity level to help assist with things like social grooming. More study in this area is suggested to help confirm this hypothesis.

The findings of this experiment add to my overall knowledge of the mechanisms of grooming behaviour within *A. mellifera*. In my study, bees collected from colonies selected for grooming behaviour showed increased levels of auto-grooming. Further, my high-grooming bees were quicker than low-grooming bees to engage in allo-grooming behaviour when stimulated with either varroa or a standardized stimulus, indicating high-grooming bees may have decreased thresholds for detecting and initiating grooming in response to varroa or other irritants. My high-groomers were quicker than low-groomers to perform grooming invitation dances when stimulated with varroa, suggesting a heightened ability to perceive varroa specifically as a threat and to perform dances to solicit the help of nestmates. I have also provided evidence for diminished trophallaxis behaviour after stimulation (irritation) of my cages, which may be due to a displacement to other higher priority activities. Additionally, my high-grooming bees also produced more worker piping noise than my low-grooming bees during the post-stimulation period, which may serve as an excitation signal for other bees in the colony to increase their activity level, and in turn, lead to increased grooming activity. I recommend more research by done into acoustic signals in the context of grooming behaviour and that all these behaviours be considered in future work to develop genomic and proteomic markers associated with resistance and breeding bees resistant to varroa.

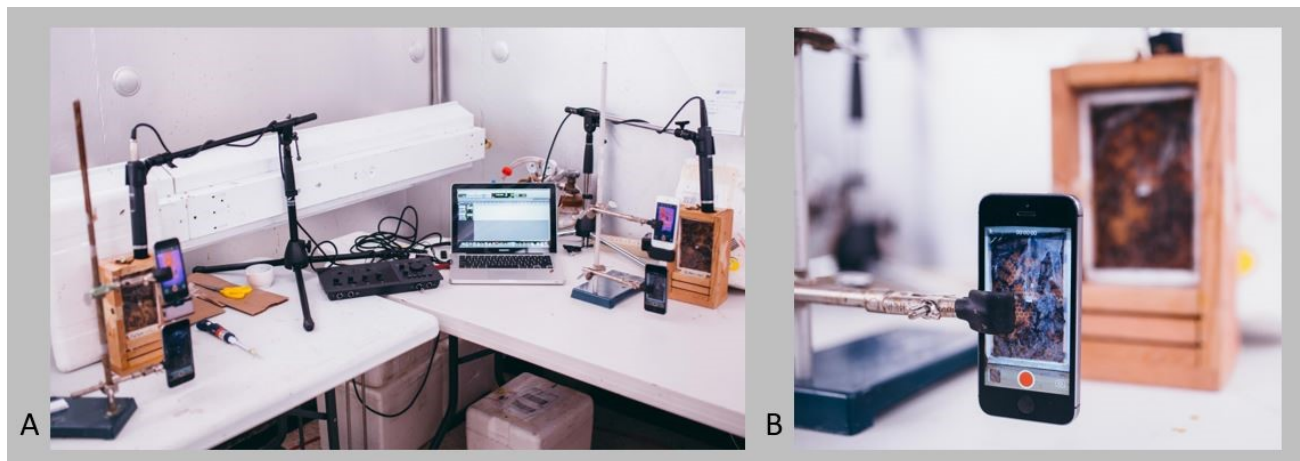


Figure 3.1. (A) Experimental setup showing the ring stands holding the iPhones used to record video, as well as the condenser microphones held in place by the mic stands—positioned to record audio from the ventilation holes in the top of the cages. (B) A zoomed in photo of an iPhone recording video of a cage of bees.

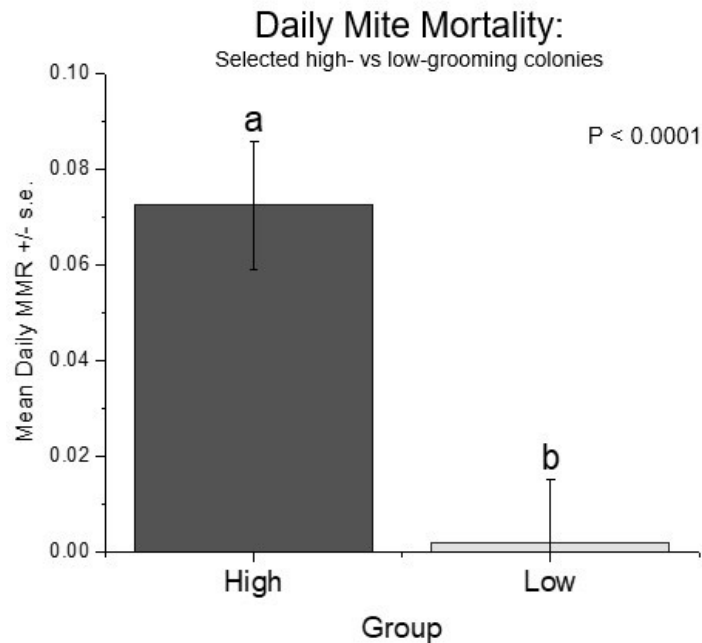


Figure 3.2. Mean daily mite mortality rates (DMMR) +/- standard error (SE) for selected high- and low-DMMR groups of colonies. The mean DMMR's differed significantly between selected high- and low-DMMR groups ($F_{1,16} = 39.35$, $p < 0.0001$). Bars followed by the same letter are not significantly different (F-test).

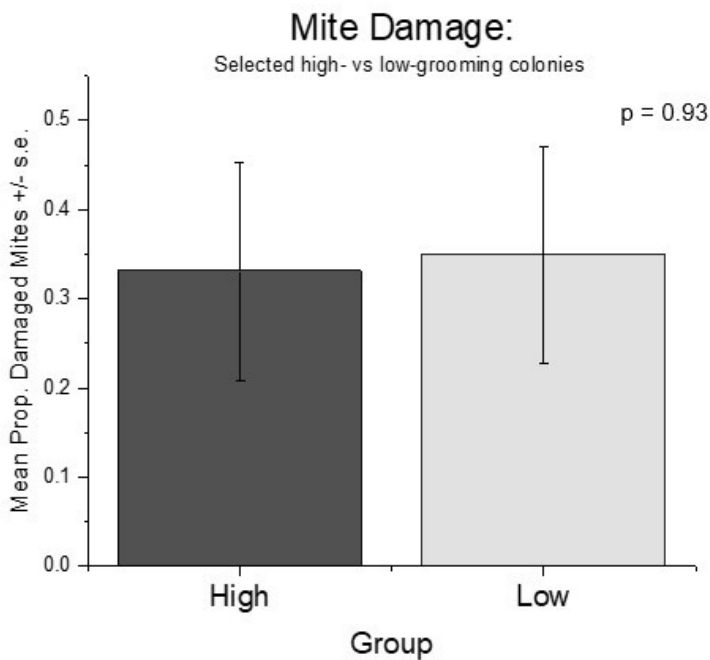


Figure 3.3. Mean proportion of damaged mites (PDM) +/- standard error (SE) for high- and low-DMMR groups of colonies. No difference between groups was observed ($F_{1,16} = 0.01$, $p = 0.93$).

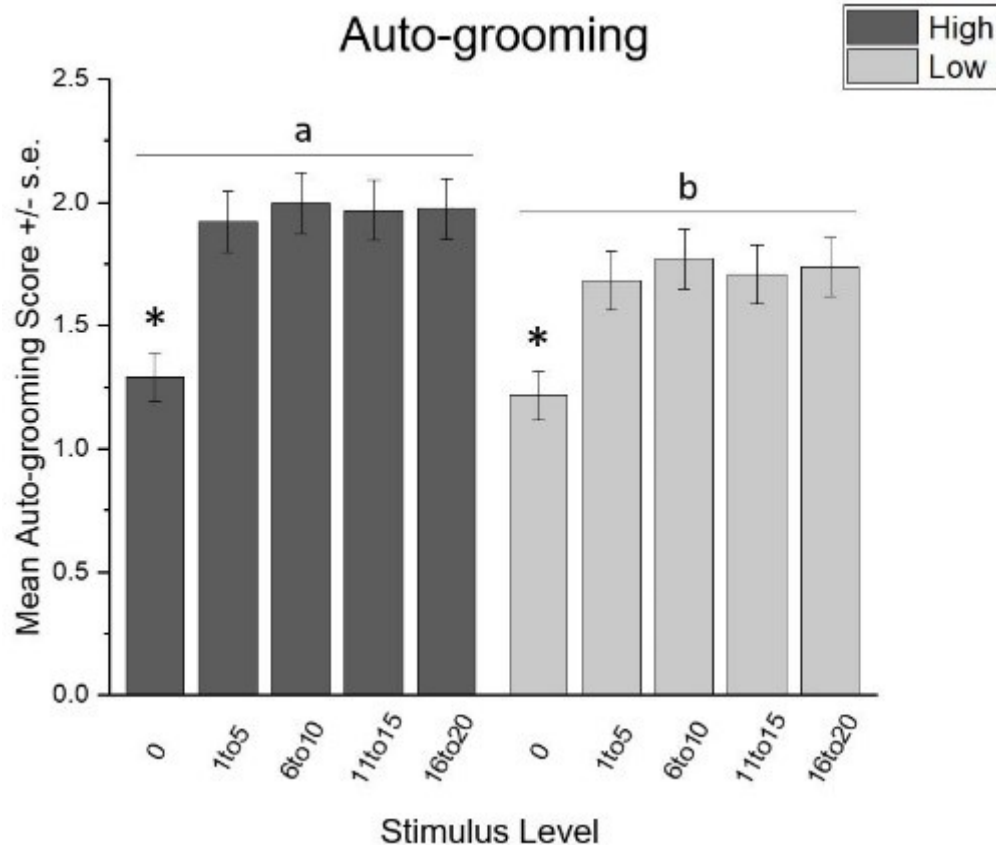


Figure 3.4. Mean auto-grooming scores +/- standard error (SE) for high-DMMR colonies (dark grey) and low-DMMR colonies (light grey) by stimulus-level (with varroa and chalk dust stimuli pooled). A significant effect of group was found ($F_{1,16.6} = 5.79$, $p = 0.028$) as well as a significant effect of stimulus-level ($F_{4,67.3} = 12.63$, $p < 0.0001$). Numbers on x-axis represent number of stimuli (mites or chalk dust puffs applied). Letters over horizontal lines represent overall test of grooming group (F-test). * represents a stimulus level of 0 was significantly different from other levels ($P < 0.0002$, Tukey). High = high-DMMR colonies and Low = low-DMMR colonies.

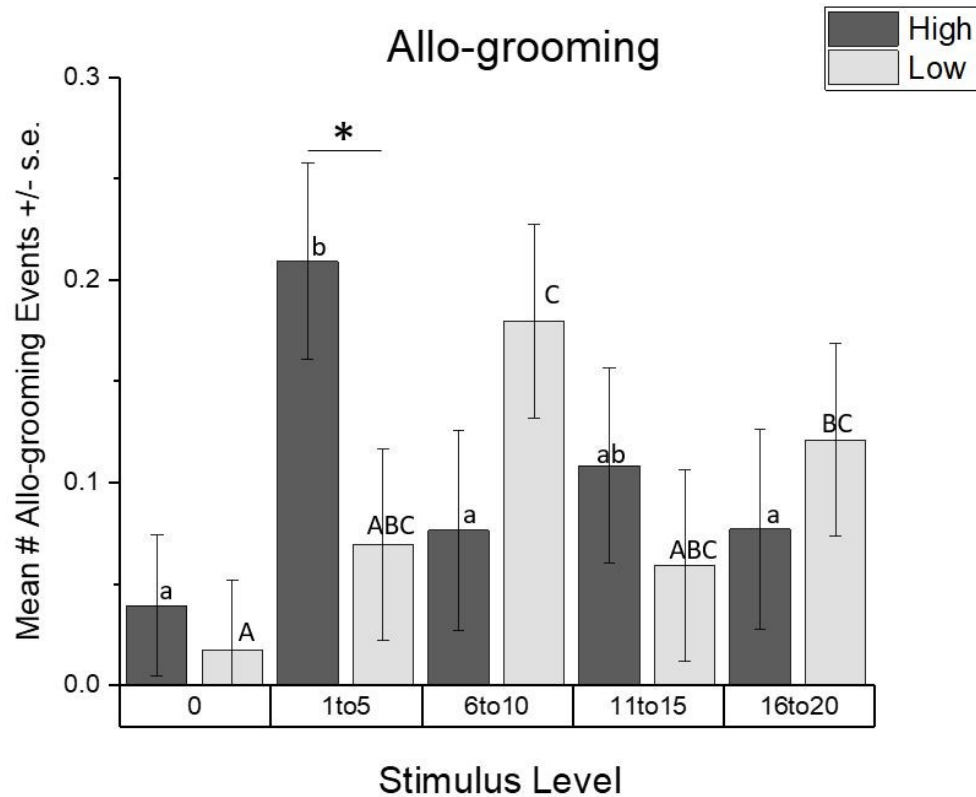


Figure 3.5. Mean number of allo-grooming events +/- standard error (SE) by stimulus-level for high- and low-DMMR colonies (stimulus-type pooled). A significant interaction between group*stimulus-level was found ($F_{4,60} = 2.79$, $p < 0.034$). Slicing the data by stimulus-level revealed a significant difference between the high- and low-DMMR groups within the '1to5' level (asterisk*). Slicing the data by group found a significant effect of stimulus-level within the high-DMMR colonies ($F_{4,60} = 2.84$, $p = 0.032$) as well as within the low-DMMR colonies ($F_{4,60} = 3.10$, $p = 0.022$). Within groups, bars with similar lower-case or upper-case letters are not significantly different ($P > 0.03$, protected LSD). High = high-DMMR colonies and Low = low-DMMR colonies.

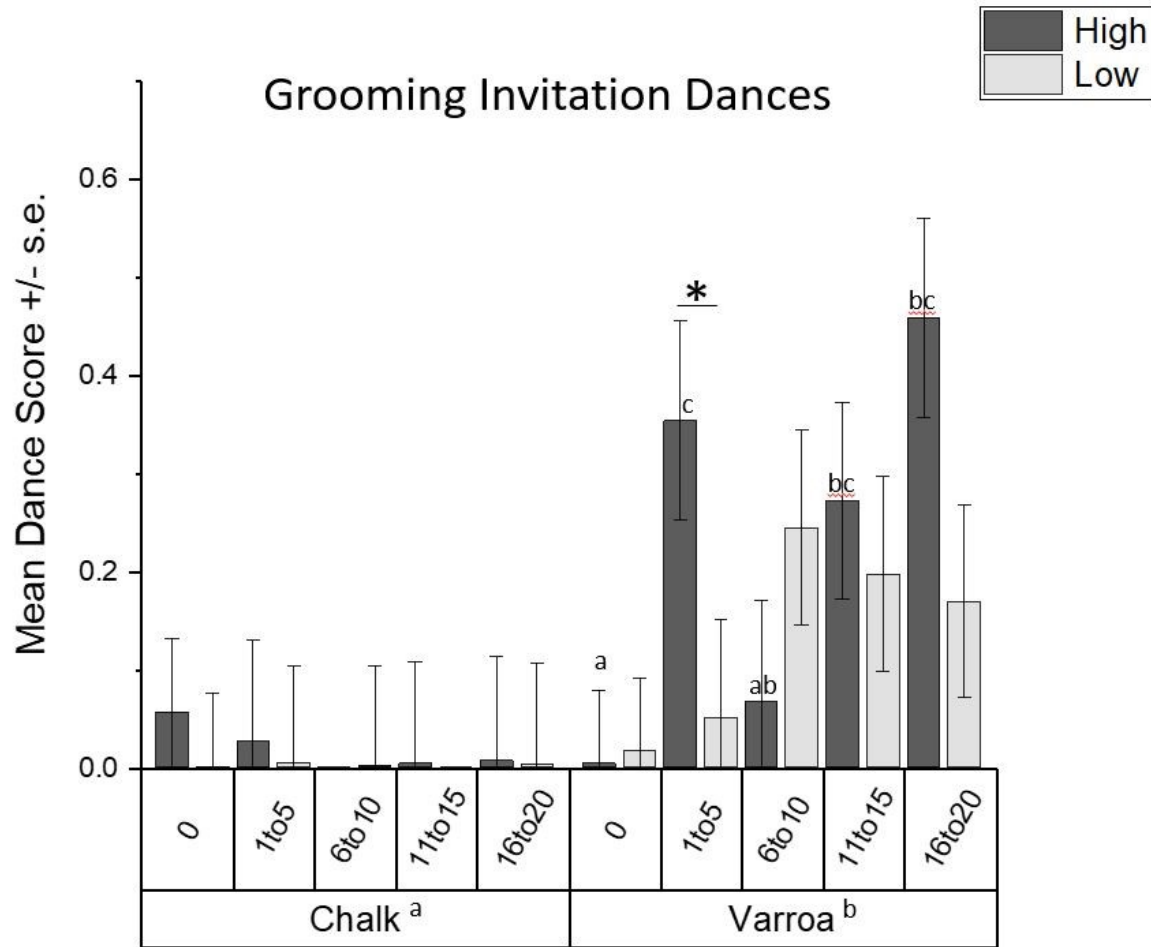


Figure 3.6. Mean dance score +/- standard error (SE) by stimulus-type and stimulus-level for high-DMMR (dark grey) and low-DMMR (light grey) colonies. A significant effect of stimulus-type ($F_{1,17.4} = 15.24$, $p = 0.001$) and a significant 3-way group*stimulus-type*stimulus-level interaction ($F_{4,63.4} = 2.55$, $p = 0.048$) were found. Slicing the data by group*stimulus-type found a significant effect of stimulus-level within the high-DMMR colonies stimulated with varroa ($F_{4,124} = 4.62$, $p = 0.0017$) but no effect of stimulus-level within the low-DMMR colonies stimulated with varroa ($F_{4,119} = 0.48$, $p = 0.749$). High-grooming bars followed by the same letter are not significantly different ($P > 0.05$, protected LSD). Slicing the data by stimulus-type*stimulus-level revealed a significant difference between the high- and low-DMMR groups within the '1to5' level of varroa ($F_{1,125} = 9.10$, $p = 0.003$) (*asterisk). High = high-DMMR colonies and Low = low-DMMR colonies.

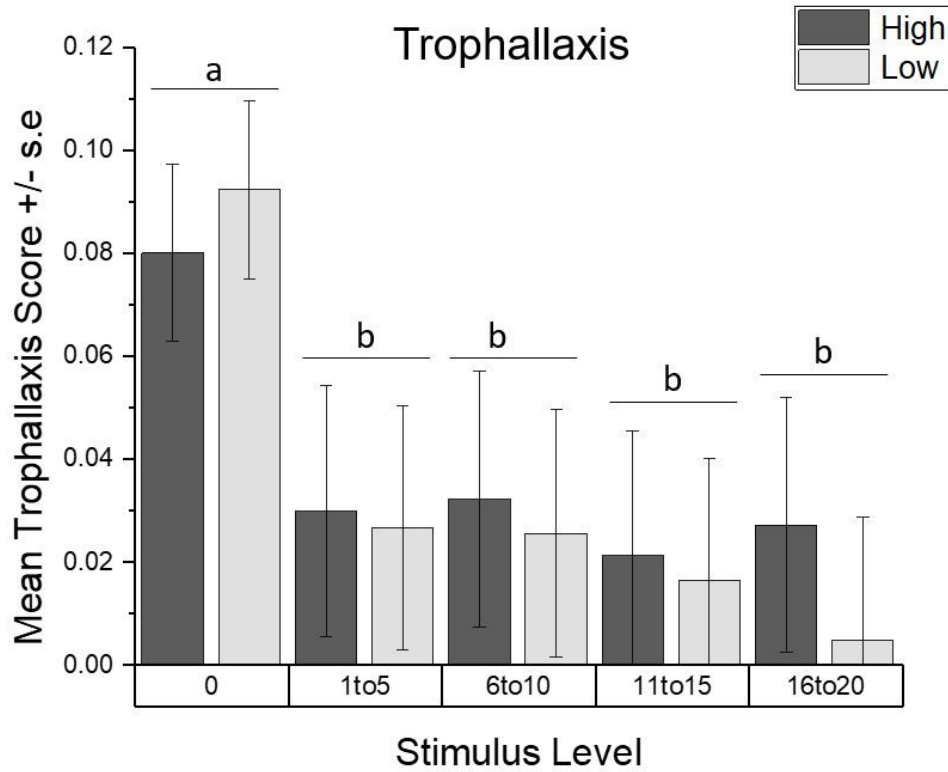


Figure 3.7. Mean trophallaxis score +/- standard error (SE) by stimulus-level for each group. A significant overall effect of stimulus-level was found ($F_{4,60} = 4.37$, $p = 0.004$). Horizontal lines above bars represent pooled comparisons among stimulus levels. Lines followed by the same letter are not significantly different ($P > 0.01$, protected LSD). Numbers on x-axis represent number of stimuli applied (varroa mites or chalk dust puffs). High = high-DMMR colonies and Low = low-DMMR colonies.

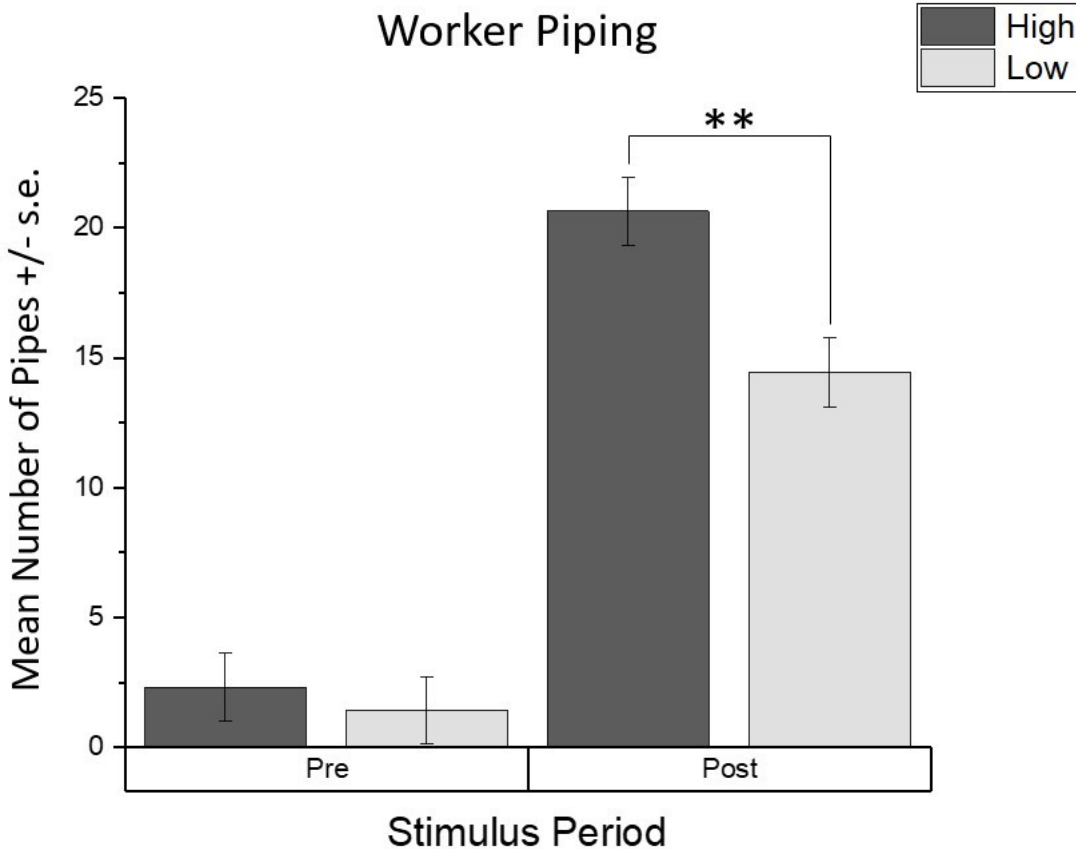


Figure 3.8. Mean number of worker piping noises by stimulus period +/- standard error (SE) for high- and low-DMMR colonies. A significant effect of group was found ($F_{1,16.1} = 6.08$, $p = 0.025$) as well as a significant group*time interaction ($F_{1,47.4} = 5.15$, $p = 0.028$). Slicing the data by time revealed a significant effect of group within the post-stimulus period ($F_{1,38.9} = 11.05$, $p = 0.002$), indicated by **. There was no difference among groups within the Pre stimulus period ($P > 0.05$, slice). Pre = period of 5 minutes prior to introduction of stimuli and Post = period after sequential application of varroa or chalk dust. High = high-DMMR colonies and Low = low-DMMR colonies.

Chapter 4. General Discussion

The invasive ectoparasite *Varroa destructor* has caused the deaths of millions of colonies of honey bees worldwide and has resulted in billions of dollars in economic losses through reduced honey production and the pollination of crops (Rinderer et al., 2010; Sanford, 2001). The behavioural mechanisms of one natural defense against the mite, honey bee grooming behaviour, were examined in my thesis.

In my first experiment (Chapter 2), I examined the sensitivities of individual bees coming from colonies previously chosen based upon high-grooming or low-grooming behaviour using a combination of colony-level selection metrics. I showed that individual bees from high-grooming colonies had significantly increased grooming responses to both a stimulus of varroa as well as chalk dust applied to either the head or thorax compared to unstimulated control bees, while bees from low-grooming colonies did not show elevated sensitivity to varroa or to chalk dust applied to any body region. In my second research experiment (Chapter 3), I studied the behavioural and communication responses of small cohorts of bees in cages coming from selected high- or low-grooming colonies. I found that high-grooming bees in groups still engaged in significantly more auto-grooming than low-grooming bees but were also quicker to initiate allo-grooming at low levels of stimulation with either a varroa or chalk dust stimulus, quicker to perform grooming invitation dances at low levels of varroa stimulation, and produced more worker piping noises.

In Chapter 2, the enhanced sensitivity observed in the high-grooming colonies varied with respect to the colony-level selection metrics used to categorize them. The high-grooming bees from colonies also exhibiting high levels of mite damage displayed higher varroa-specific sensitivity in my experimental trials. This result suggests that continuing to use varroa as a stimulus in individual bee assays to screen for grooming behaviour may be useful to help pick out colonies with higher varroa-specific sensitivity that are more capable of inflicting damage to mites at the colony level. Since undamaged mites falling to bottom boards without sticky traps may be able to re-infest bees, inflicting higher levels of damage to mites would provide better control by precluding that outcome. These results agree with Guzman-Novoa et al. (2012) who also found correlations between the level of mite damage at the colony level and the level of intense grooming behaviour and mite removal in individual bee assays. Mite removal in my petri dish assays, however, was quite rare.

Both sub-categories of high-grooming bees (i.e., those selected based on high DMMR's and high damage and those selected based on high DMMR's only) showed a general trend toward increased sensitivity (compared to the unstimulated control bees) when stimulated with chalk dust on the head or thorax, with one exception being chalk dust on the head in the colonies selected based on high DMMR's only. Interestingly, those colonies categorized with high mite drop rates (high DMMR's) and only moderate damage rates also showed significantly different sensitivities across body regions when stimulated with chalk dust. Overall, these results suggest that bees coming from colonies with high mite drop rates have higher general sensitivity to irritants, which apparently also enables them to knock mites off at the colony level using generalized grooming responses. Thus, assays with chalk dust may be useful to select for bees with heightened general sensitivity but this approach would not capture all the traits associated with resistance behaviour. Since the bees coming from colonies with high mite damage rates also displayed heightened varroa-specific sensitivity at the individual level, my results suggest that continuing to use varroa mites in selection programs where dish assays are used to screen for grooming behaviour may be the best way to select for the full suite of behaviours to control varroa in the colony. Assays using only alternative irritants, such as chalk dust—or wheat flour, as suggested by Morfin et al. (2020)—may miss out on the varroa-specific sensitivity that maximizes grooming effectiveness. Still, in times when varroa mites are scarce, or when time does not permit varroa mites to be collected and used in such assays, I agree with Morfin et al. (2020) that wheat flour (or chalk dust) assays may still provide useful information, but that they should be used in combination with additional colony evaluations, such as the proportion of damaged mites collected off bottom boards or overall mite population growth.

Hamiduzzaman et al. (2017) indicated that multiple genes are likely involved in regulating grooming behaviour. My results show that there are also differences in varroa-specific sensitivity among “high-grooming” bees with respect to the level of damage they inflict on varroa mites. Thus, studies aimed at developing proteomic or molecular biomarkers should keep in mind not only that grooming is likely a polygenic trait, but that grooming also involves several behavioural components, which would undoubtedly require their own set of biomarkers to successfully select for those components of behaviour.

The colonies characterized with high mite damage rates in this study had slightly lower overall mite mortality rates. While this was not a significant difference, the observed trend where colonies with high numbers of damaged mites had lower overall mite drop hints at a possible trade-off to having higher varroa-specific sensitivity. It is possible that selecting for higher varroa-specific sensitivity leads to less overall grooming even though the fitness of the mites that do fall is reduced. However, in Chapter 2, bees coming from the colonies with high mite damage rates also had high general sensitivity to chalk dust applied to the head or thorax, so the colony-level differences I observed may also be attributable to random chance and this “trade-off” hypothesis needs further study.

Selecting solely based upon heightened mite mortality may still be useful if sticky traps or screened bottom boards are also used at the colony level as a management technique as in those situations mites are prevented from re-infesting bees. It should be noted that my study used colonies chosen based on maximizing genetic diversity of stock representative of that found in western Canada and did not rear queens over multiple generations to help enrich and separate these traits. Interesting future work would be to rear queens over multiple generations based on each of the selection metrics (mite mortality and mite damage) separately and then repeat the studies to compare the behaviour of bees selected on each metric. I hypothesize there would be even greater behavioural differences, particularly for varroa-specific sensitivity, if bees were selected for mite damage over multiple generations.

In Chapter 3, no differences were observed between the sub-groups of the two sub-categories of high-grooming bees (chosen based on mite mortality vs mite damage). Therefore, the data were pooled and were presented as “high-DMMR bees” only, though the group also consisted of some colonies with high mite damage rates. These high-DMMR colonies showed similar behaviour related to auto-grooming as in Chapter 2, but also showed differences related to aspects of allo-grooming and communication when compared to the low-DMMR bees. First, when both stimuli (varroa and chalk dust) were pooled, it was found that the high-DMMR colonies auto-groomed significantly longer than low-grooming bees overall. The varroa and chalk dust stimuli were pooled because no significant differences were observed between the two stimuli. Since it was nearly impossible to time the grooming responses of bees in cages when watching the video recordings, the grooming timer used in Chapter 2 was not used, and a

categorical scoring system was created to quantify the length of time that the individual bees auto-groomed. This scoring system, however, was based on the results of Chapter 2. Of the 2750 bees analyzed in that experiment, roughly one third of them groomed for less than 7% of the total time; another one third groomed for 7-22% of time; and the remaining one third groomed longer than 22% of the time. Thus, these percentages of time spent grooming were used to define low, medium, and high levels of auto-grooming for this experiment. A fourth category (very high) was added to account for bees that groomed almost the entire time after a stimulus was applied. The bees in this experiment were watched very carefully—often examining video frame by frame to help keep track of each stimulated bee moving about the cage—and this careful observation was used to estimate what percentage of time each bee spent grooming. Each bee was then given a score on a scale of 0 to 4 to account for bees that did not groom at all, or bees that groomed at low, medium, high, or very high levels. Due to the video analysis constraints of my study, unlike in Chapter 2, I also did not differentiate between light or intense auto-grooming behaviour, and instead just estimated the total time spent grooming. Although the results are not directly comparable to what would have been observed if I had divided grooming into light and intense categories as I did in Chapter 2, the results of the cage experiment largely agree with those of Chapter 2, in that the bees selected for high-grooming behaviour at the colony level showed increased auto-grooming responses, relative to the low-grooming bees. Further, compared to the individual bee assay in Chapter 2, the bees in the cages seemed to groom for similar lengths of time and it did not appear that being in a cage of bees had an impact on the amount of auto-grooming. For the high-grooming bees, roughly 5% of bees did not groom, 44% of bees groomed at a low level, 25% at a medium level, 25% at a high level, and 1% at a very high level. For the low-grooming bees, roughly 8% did not groom, 49% groomed at a low level, 23% at a medium level, 18% at a high level, and 1% at a very high level. Thus, while this scoring system was not perfect, it seems that, if anything, the bees in cages auto-groomed slightly less than the individual bees in petri dishes. It is important to note that since I only observed the behaviour of the bees that were stimulated, it is not known what the impact of increased numbers of stimuli added to the cages was on the other unstimulated bees in the cage. I hypothesize that with increased stimulation added to a cage, even unstimulated bees may begin to auto-groom at

increased levels, and that these increased levels of auto-grooming would likely be greater in colonies selected for high grooming behaviour. This aspect needs further study.

The scoring system for allo-grooming, grooming invitation dances, and trophallaxis in this experiment was slightly different than that for auto-grooming. For these responses, the number of light or intense responses was recorded and a weighted score was given to instances of each behaviour. This was included because it was noted that many short instances of each behaviour were occurring after a stimulus, but instances longer than two seconds were less common. Therefore, a higher score was given to those rarer longer instances. A similar system of data collection was used by Aumeier (2001) in individual petri dish assays involving 3 bees that quantified different components of auto-grooming or allo-grooming. However, Aumeier's study classified intense auto- and allo-grooming responses as those lasting longer than 5 seconds and allo-grooming was very rarely witnessed in their experiments. Further, only the number of specific instances were recorded and higher scores for intense observations were not awarded. For my study, a different weighted score was awarded to each light or intense response and summed to obtain a final score for each response category. The weighted scores were decided on before any analyses were complete and were not altered at any time.

Compared to the low-grooming bees, the high-grooming bees were quicker to allo-groom in response to either stimulus and were quicker to perform grooming invitation dances when stimulated with varroa. While these experiments were done on small cohorts of bees removed from the natural colony environment, these novel findings suggest that bees selected for heightened grooming behaviour have reduced thresholds for responding to colony threats like varroa and/or foreign irritants entering the colony. It also indicates that when it comes to varroa, further steps are taken by the colony to communicate the perceived threat and to recruit the attention of other bees to remove mites from the colony.

While it was rare to witness allo-grooming immediately following a grooming dance, the fact that there was more allo-grooming during the same periods as increased grooming dances suggests that dancing may have played a role in increasing the level of allo-grooming, as has been found in previous experiments (Land & Seeley, 2004). Interestingly, however, Land and Seeley (2004) witnessed grooming invitation dance responses in 50% of bees that were puffed with chalk dust, while in my study, those bees puffed with chalk dust very rarely danced.

However, their study used 0.5 mg of chalk dust applied to the base of the wings, while my study used only 0.3 mg (simulating the weight of a varroa mite) applied to whichever body region could be accessed, so for multiple reasons their puff of chalk dust was likely a stronger stimulus. Perhaps more importantly, they followed bees for 10 minutes, whereas I only followed bees for 2 minutes, so they undoubtedly would have witnessed more dancing with an extra 8 minutes of observation.

These results agree with other studies that have found selected bees to be quicker than unselected bees at engaging in grooming responses when stimulated. For example, Morfin et al. (2020) found that supposed varroa-resistant Africanized bees were faster to engage in auto-grooming behaviour than supposed varroa-susceptible European (Carniolan) bees after being stimulated with either wheat flour or varroa. My results suggest that their findings for auto-grooming responses may also extend to allo-grooming behaviour, since my high-grooming bees also responded quicker to a stimulus of varroa or chalk dust by allo-grooming. When it comes to dancing responses, however, the findings seem to extend only to a stimulus of varroa, as chalk dust produced very few grooming invitation dances—in both high- and low-grooming bees. Since allo-grooming behaviour is likely of greater importance in inflicting damage to mites than auto-grooming, I advocate more studies be completed on allo-grooming behaviour.

The trophallaxis results found in Chapter 3 were different than expected. It was hypothesized that trophallaxis responses would increase after a stimulus as another mechanism of heightened communication. However, it was observed that trophallaxis responses significantly decreased in both high-grooming and low-grooming bees. As discussed in Chapter 3, decreasing trophallaxis behaviour may be an adaptive social immunity trait in response to colony stressors, as suggested by Geffre et al. (2020) in the context of honey bee viruses, or that it may more simply be due to the bees diverting their energy to more important tasks, such as increased grooming activity. As far as I am aware, this is the first study to report significantly reduced trophallaxis behaviour after a stimulus of varroa or chalk dust.

The worker piping results found in Chapter 3 offer a novel avenue for future study. While a direct link was not made between the significantly increased piping noises and grooming behaviours witnessed in my video recordings, my study provides evidence for a possible link between the two observations, and further research is recommended in this area. Since previous

studies have found increased worker piping to be associated with heightened bee activity, notably in swarming (Schlegel et al., 2012) and alarm-signalling/anti-predator behaviour (Mattila et al., 2021), it is plausible that increased worker piping may also be associated with grooming activity, and that worker piping may be a broader communication mechanism to increase the activity of resting bees and their recruitment to activities like allo-grooming.

As described in Chapter 3, the automated call recognizer software *Kaleidoscope Pro* was used to quantify worker piping noises by training an advanced classifier to detect the sounds of interest in the audio recordings. Generally, the use of automated call recognizers is in its infancy when it comes to using them with insects (Chesmore & Ohya, 2004), but is much more common in other fields, especially with birds and studies involving bird call identification (Priyadarshani et al., 2018; Terry & McGregor, 2002). In such studies, properly calibrated automated call recognizers can save substantial time of manually processing audio recordings and may also reduce observer bias and general error as they can provide a more objective method of data collection (Priyadarshani et al., 2018). Moreover, automated call recognizers may sometimes provide better accuracy than human ears at detecting certain sounds, for example the echolocation signals of bats (Jennings et al., 2008), or correctly detecting sounds in recordings with substantial background noise (Brumm et al., 2017). However, automated call recognizers require substantial expert time to calibrate the classification software, and if not properly done so may result in substantial false-negatives or false-positives (Glorioso, 2009).

More recently, automated detection algorithms have been used in research on honey bees to quantify recordings of “whooping” signals (Ramsey et al., 2017), however, not without criticism (Bell et al., 2019), due largely to the way the authors trained their automated detection algorithm and how they learned to differentiate between whooping versus non-whooping sounds. In my study, I first learned to identify worker piping noises by listening to those provided in the supplementary material by (Schlegel et al., 2012), and then compared those to the piping noises in my recordings to make sure what I was hearing were legitimate worker piping noises before training the advanced classifier to run against my audio recordings. In some of the clips where positive piping noises were identified by the advanced classifier, piping noises could not be heard with my ears upon listening to the selected clips. In all such cases, I took the classifier to

be correct as it was assumed that the classifier was better than my ears at identifying such noises against background buzz of the bees in cage.

Other studies, such as the one by Mattila et al. (2021), have also used automated call recognizers to analyze audio data collected from within hives. In their study, the authors also generated spectrograms for each audio clip which they viewed and analyzed separately, and this served to validate the analysis of the audio clips alone. I did not do this for all clips that were processed, however, I did view some spectrogram images for the specific clips that were picked out by *Kaleidoscope Pro* to serve as an initial justification that the program was correctly identifying piping noises. One suggestion for future studies is to follow the example of Mattila et al. (2021) and create visual spectrograms for each positively identified clip to serve as a secondary validation of the data.

Another suggestion for improving the collection of behavioural data for individual bees in studies such as the one in Chapter 3, or in similar cage assays, would be to improve the system of quantifying behavioural responses. Geffre et al. (2020), for example, affixed barcodes to the thoraxes of selected bees in the cages before beginning the experiments and then used an automated monitoring system to quantify behavioural interactions. A similar automated system may be very useful in subsequent grooming trials such as these.

Summary & Conclusions

The results of the two studies in this thesis increase our overall understanding of the mechanisms of honey bee grooming behaviour. As such, Figure 4.1, below, has been updated with new information linkages in bold. I have demonstrated that individual bees coming from colonies chosen for heightened grooming behaviour, using colony-level selection metrics, display significant differences in their sensitivities to varroa as well as chalk dust, thus lending some support to the use of alternate irritants to screen for heightened grooming behaviour. However, in contrast to the use of chalk dust alone, bees coming from colonies with higher mite damage rates also had heightened varroa-specific sensitivity. Thus, continuing to use live varroa mites in such screening assays may help to select for bees with enhanced mite sensitivities and overall defence mechanisms. This finding also lends more support to the use of damaged mites as an indicator of grooming. I also showed that high-grooming bees differed in terms of their

sensitivity across body regions and were found to have enhanced sensitivity to chalk dust on the thorax and head, compared to the abdomen.

When groups of bees from high- and low-grooming colonies were studied, high-grooming bees were found to exhibit increased communication responses in the form of worker piping noises, as well as increased grooming invitation dances at low levels of varroa stimulation, which may be indicative of increased varroa sensitivity and a lower response threshold to a perceived threat. Increases in allo-grooming behaviour followed the same trend as the increase in grooming invitation dances, however a direct connection between the two behaviours was rarely observed. Thus, at the colony level, there appears to be an increased ability to detect varroa and a quicker impulse to communicate a need for social grooming. Interesting future studies could look further at the connections between allo-grooming, grooming dances, worker piping noises, and mite damage rates—and all such behaviours should be considered in the future development of genomic and proteomic markers aimed at breeding for varroa resistance. For example, multiple biomarkers (i.e., not just one “grooming” biomarker) may exist for each of the different components of grooming, including sensitivity, intense auto-grooming, allo-grooming, mite biting, and different communication responses (e.g., dancing, piping). Simply using a single metric such as mite mortality or mite damage rates are unlikely to capture the full suite of behaviours required for a robust grooming response.

In closing, grooming behaviour in honey bees is an important natural resistance behaviour that has the potential to be developed through selective breeding. However, the use of grooming behaviour in the fight against varroa mites should be seen as an integrated pest management tool, rather than a behaviour to be relied upon solely for mite control. To keep healthy honey bee colonies, it is recommended that beekeepers continue to follow integrated pest management strategies, which also involve continuous monitoring of mite levels, knowledge of economic thresholds, rotation of different synthetic and/or organic chemical treatments, and other physical and cultural controls.

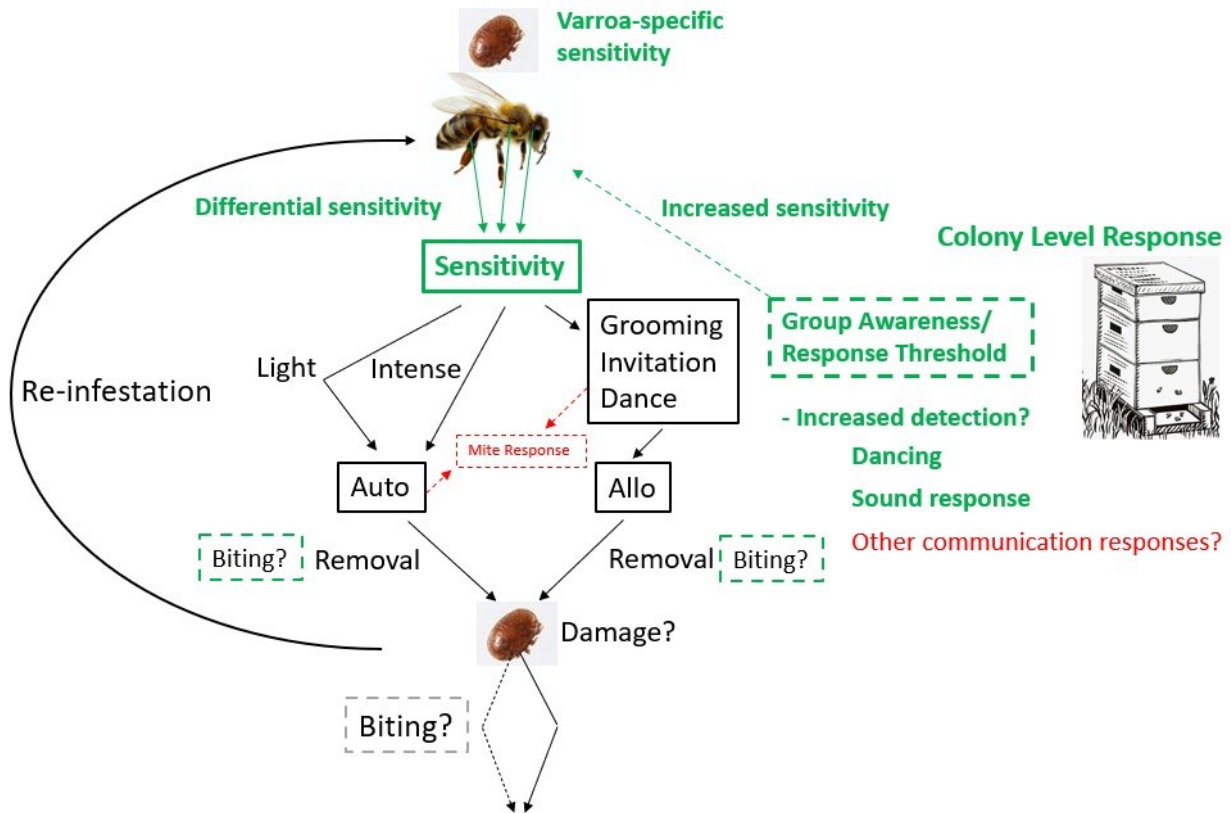


Figure 4.1. An updated model of the known and predicted mechanisms of grooming behaviour in *A. mellifera* against *V. destructor*. Dashed lines and/or red text indicate what is still unknown; solid lines indicate what is currently known; and green text indicates what information has been added from this thesis. The results of this thesis have shown that individual bees have differential sensitivity to stimuli in different body regions as well as varroa specific sensitivity. High-grooming bees in cages were also witnessed to have significantly increased group responses by eliciting more worker piping sounds after stimulation and performing more grooming invitation dances at low levels of varroa stimulation. Thus, high-grooming bees may have a lower response threshold to stimuli, particularly to varroa, however, more work should be completed to study response thresholds closer. Future work could also look closer at possible increased sensitivity of individual bees that were not directly stimulated but that are responding to the increased communication signals of other bees. Other communication mechanisms such as pheromones or heat signals could also be studied. While instances of mite biting were directly witness by auto- and allo-grooming bees, it is still poorly understood how important mite biting is in the grand scheme of grooming behaviour and varroa control. The responses of mites to attempts of grooming is also still very much unknown.

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