Chemical ecology and biology of *Varroa destructor* (Anderson and Trueman), a primary pest of western honey bees (*Apis mellifera* L.)

by

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Abstract

Honey bees (*Apis mellifera* L.) are the most agriculturally beneficial eusocial insects for crop pollination. Chemical communication is critical in maintaining colony structure and activity, which may be exploited by parasites. *Varroa destructor* (Anderson and Trueman; Acari: Varroidae) is regarded as one of the biggest threats to apiculture, blamed for annual colony mortalities of over 30% in some regions. The objectives of this thesis were to identify odorants important to *V. destructor* for further development of techniques to disrupt its lifecycles through in-colony treatment. Honey bee colony volatile collections involving *ex-situ* techniques were used to identify individual compounds and odor detection sensitivity of *V. destructor* to these compounds through gas chromatography-mass spectrometry and gas chromatography-linked electrotarsal detection, respectively. Volatile components identified in this and previous research were then tested for concentration-dependent responses using electrophysiology. Electrotarsogram responses indicated significant difference among odorants in eliciting responses, suggesting the potential application of this procedure in screening putative repellents or odors that disrupt host detection (disruptants) to live *V. destructor*. Results from this research can be applied to colony-wide testing of active odorants in developing effective alternative methods for *V. destructor* control as well as developing methods for future research exploring chemical ecology of social insects.

List of abbreviations

ETG: electrotarsogram

ETD: electro-tarsal detection

GC-ETD: gas chromatography electro-tarsal detection

GC-MS: gas chromatography mass spectrometry

GC-FID: gas chromatography flame ionization detection

List of definitions

Apotele: Pretarsus of acarines; a soft appendage allowing attachment to its host.

Arrestment: The amount of time spent arrested (stationary) on a treated surface following first encounter with stimulus.

Disruptant: Something that disrupts response to a semiochemical by altering sensitivity of the receiver or overloading the receiver with particular semiochemicals.

Kairmone: Chemical released from one organism that induces a favourable response by the perceiving individual.

Peritreme: Breathing tubes (movable in *V. destructor*) possibly adapted for low-oxygen environments or to regulate water exchange.

Pheromone: Chemical released from one organism and induces response in another of the same species.

Returns: Quantification of number of subsequent encounters with a treated surface following first encounter.

Semiochemical: A chemical that is conveyed from one organism to another which communicates a message.

Sensilla: Sensory receptor housed within a modified cell or group of cells on the cuticle of an arthropod and some invertebrates.

Valence: The attractiveness or adverseness of a stimulus.

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CHAPTER 1.0 INTRODUCTION

1.1 Background

1.2.1 Pollination and western honey bees *(Apis mellifera)*

Pollination is central to ecosystem function and crop production (Ellis and Delaplane, 2008; Rader et al., 2016). Pollination by insects is estimated to contribute $275 billion to the global economy annually (Gallai et al. 2009). Seventy-five percent of crop species rely to some extent on animal pollination with honey bees and non-bee insects being equally important in pollination in some regions (Klein et al., 2007; Rader et al., 2016). Honey bees are often the most valued generalist pollinator in areas where large scale agriculture is practiced (Morse and Calderone, 2000; Klein et al., 2007; Iwasaki et al., 2015).

1.2.2 Honey bee biology

Honey bees have a eusocial colony structure consisting of a single queen, up to one thousand drones and 50-60 thousand workers, with numbers of workers and drones varying depending on seasonal resource availability, weather, and colony health (Human et al., 2013; Mondet et al., 2016). From egg to emergence, it takes 16 days, 21 days, and 24 days, respectively to produce a queen, a worker, and a drone (Trouiller et al., 1994; Human et al., 2013; Williams et al., 2013). This variation is due in part to the type and amount of food provided to a larva during its lifetime until its cell is capped for pupation (Lercker et al., 1981; Drijfhout et al., 2005; Dietemann et al., 2013). Workers follow a caste-based system with young workers between three to 12 days old having in-hive

duties such as maintaining the brood chamber and feeding bee larvae (nursing) (Pernal et al., 2005; Johnson and Frost, 2012). As workers become older, their tasks within a hive shift, ultimately leading to nectar- and pollen-foraging for a colony, prior to their death at around four to five weeks of age (Johnson and Frost, 2012). Caste progression within a colony does not necessarily follow a linear pattern from nursing to foraging tasks, and depends largely on the needs of the colony, resource availability, and potentially parasite loads (Wahab et al., 2006; Johnson and Frost, 2012).

1.2.3 Honey bee chemical sensing

Honey bees are considered a model organism in chemical ecology due to the wide variety of ants that are important in maintaining colony structure (Naumann et al., 1991; Keeling et al., 2004; Villar and Grozinger, 2017; Ma et al., 2018; Villar et al., 2018). Within a colony, honey bees communicate primarily through both olfactory and contact stimuli (Thom et al., 2007; Plettner et al., 2017); although, recently honey bees were shown to respond to temperature variations (Bauer et al., 2018). Acute odor distinction is essential in detection of foreign invaders and removal of parasitized adults or bee brood to maintain colony resilience (Martin et al., 2001; Falcón et al., 2014; Mondet et al., 2015). Honey bee brood pheromone is important in regulating honey bee activities and influencing caste progression in the colony (Pankiw and Page, 2001; Grozinger et al., 2007; Maisonnasse et al., 2009). Honey bee behavioral responses to floral bouquets are often mediated by few dominant components of complex mixtures, although mixture synergisms can occur (Krofczik, 2008; Reinhard et al., 2010). Only recently the complexity of multi-functional honey bee pheromones in colonies was recognized

(Grozinger et al., 2007; Villar and Grozinger, 2017; Ma et al., 2018; Villar et al., 2018). Factors both internal (e.g. queen status, brood development, parasites) and external (e.g. resource availability) to a honey bee colony are collectively responsible for influencing colony-wide mediated behaviors (Johnson and Frost, 2012; Perry et al., 2015; Simone-Finstrom, 2017; Ma et al., 2018). Honey bee colonies are described as dynamic superorganisms, with individuals collectively responding to changes in semiochemicals (Robinson, 1992; Johnson and Frost, 2012). Similarly, parasites of honey bee colonies can eavesdrop on these semiochemicals to optimize survival within colonies (De Jong et al., 1982; Keeling et al., 2004; Plettner et al., 2017).

1.2.4 Stressors

Honey bees are exposed to a wide range of stressors such as parasites, viruses, apicultural management strategies, environmental conditions, and pesticides (Ellis and Delaplane, 2008; Gisder et al., 2009; Currie et al., 2010; de Miranda and Genersch, 2010; Giacobino et al., 2016; Abbo et al., 2017; Benuszak et al., 2017; Plettner et al., 2017). These stressors, alone or together, can affect managed and feral honey bee colony survival (Ellis and Delaplane, 2008; Gisder et al., 2009; Rosenkranz et al., 2010; Iwasaki et al., 2015; Plettner et al., 2017)

1.2 Varroa destructor

1.3.1 History

Varroa destructor was originally an ectoparasite of *Apis cerana* (Fabricius) and *Apis nigrocincta* (Smith) (Hymenoptera: Apidae), originating from Asia and Indonesia,

respectively (De Guzman et al., 1993; Martin et al., 2002; Rosenkranz et al., 2010; Eliash et al., 2014; Nazzi and Le Conte, 2016). Selective breeding of the honey bee (*A. mellifera*) for desired traits promoted its global use in apiculture, allowing honey bees to share a common distribution with the eastern honey bee in Asia and Russia in the 1950s (Rosenkranz et al., 2010). Presumably around his time, *V. destructor* was able to infest *A. mellifera* colonies for the first time; transportation of honey bees resulted in inadvertent spread of *V. destructor* throughout Europe by the 1970s (Rosenkranz et al., 2010; Le Conte et al., 2015). *Varroa destructor* mites were likely first identified in the U.S. in 1987 in Wisconsin, and later Canada in 1989, leading to a nearly continent-wide distribution by 2002 (De Guzman et al., 1993; Johnson et al., 2009; Currie et al., 2010; Rosenkranz et al., 2010).

In 2000 *V. destructor* became recognized as a separate species from *V. jacobsoni* (Anderson and Trueman). Research prior to 2000 frequently refer to *V. jacobsoni*, although the likely species under review then would have been *V. destructor* (Rosenkranz et al., 2010). References published prior to 2000 cited in this thesis are assumed to be concerning *V. destructor*, although these citations refer to *V. jacobsoni*.

1.3.2 Morphometry

Varroa destructor mites are 1.1-1.6 mm across and dorsoventrally flattened allowing them to fit between the tergites or sternites of adult honey bees, and avoid removal by regular bee grooming (Fries et al., 1996; Rath, 1999; Sammataro et al., 2000; Le Conte et al., 2015). *Varroa destructor* morphometry is comparable to other Acari (Bautz and Coggins 1992, Leonovich 2013, Fig. 1.5.1). The pedipalps and forelegs of *V. destructor*

contain three types of sensilla (wall-pore, non-pore, and gustatory type), arranged similarly to that of ticks (Dillier et al., 2006; Rosenkranz et al., 2010; Eliash et al., 2014), that are used for detecting chemosensory, gustatory, hygro- and thermo-sensory stimuli in a honey bee colony (Endris and Baker, 1993; Dillier et al., 2006; Rosenkranz et al., 2010; Nazzi and Le Conte, 2016). Front tarsi are used primarily for chemosensing, improved through questing the air much like other Acari (Allan, 2010; Leonovich, 2013).

1.3.3 Life history

Varroa destructor has a two-phase life cycle which consist of both a phoretic and reproductive phase (Rosenkranz et al., 2010). *Varroa destructor* are dependent on honey bee hosts for survival throughout their live cycle and lack a free-living life stage (De Jong et al., 1982). Host detection and choice of host are important in both survival and reproduction of *V. destructor* within honey bee colonies (Nazzi and Le Conte, 2016). *Varroa destructor* life span ranges from 2-5 months within the honey bee colony and depends on traits of both host and parasite (De Jong et al., 1982). The variability of *V. destructor* lifespan is due to seasonality in temperate regions where honey bee colonies experience brood-less periods, with limited research exploring differences in *V. destructor* physiology during these periods (De Jong et al., 1982; Fries and Perez-Escala, 2001).

Within the phoretic phase *V. destructor* adult females use adult nurse honey bees to travel within the hive (Rosenkranz et al., 2010). *Varroa destructor* identify suitable phoretic hosts using the odor profile of adult worker honey bees, demonstrating a preference for honey bees of the nurse caste over those of the forager caste (Kraus, 1994;

DelPiccolo et al., 2010; Appendix A4). *Varroa destructor* feed on fat deposits during the phoretic phase, presumably more frequent in regions where colonies experience a brood-less period (overwintering), but the significance and frequency of feeding on adult honey bees is unknown (Fries and Perez-Escala, 2001; Rosenkranz et al., 2010; Eliash et al., 2014; Piou et al., 2016; Ramsey et al., 2019). Although *V. destructor* is frequently described as having a phoretic stage, feeding during this phase of its lifecycle indicates a parasitic behavior.

Nurse bees frequently attend to honey bee larvae before cell-capping, providing a variety of potential cells for mites to invade (Sammataro et al., 2000; Rosenkranz et al., 2010; Plettner et al., 2017). *Varroa destructor* are attracted to fifth instar honey bee larvae, coinciding with peak concentrations of volatile compounds of the larval cuticle (Le Conte et al., 1989, 1990a; Trouiller et al., 1992; Rickli et al., 1994; Appendix A1). At the beginning of a mite reproductive cycle, host choice and cell invasion by female *V. destructor* occurs relatively close to the surface of the brood cell. This behavior is assumed to reduce chances of detection by other adult honey bees (Boot et al., 1994; Donzé and Guerin, 1997; Rickli et al., 1994; Fig. 1.5.2). During the reproductive phase of *V. destructor* life cycles, invading females are referred to as foundresses (Kather et al., 2015; Plettner et al., 2017).

Once in a cell, foundress *V. destructor* quickly move to the bottom and immerse within larval food, using tracheal tubes (peritremes) to breathe (Donzé and Guerin, 1997; Nazzi et al., 2006; Sammataro et al., 2000; Trouiller et al., 1992; Fig. 1.5.1). This behavior further reduces detection by nurse bees while the cell is being capped (Donzé and Guerin, 1997; Rosenkranz et al., 2010). Following cell-capping, honey bee larvae

consume the remaining larval food, freeing the foundress from the larval food (Rosenkranz et al., 2010; Frey et al., 2013). Each foundress then creates a feeding site on a larva, and continues to feed during larval cocoon development (Sammataro et al. 2000, Salvy et al. 2001, Martin et al. 2002, Rosenkranz et al. 2010). A fecal accumulation site is created by foundress close to the feeding site, typically found near the bottom of the cell (Donzé and Guerin, 1997; Rosenkranz et al., 2010). This fecal accumulation site is used by the progeny of *V. destructor* to locate the feeding site and functions as an aggregation site for immature mites, later serving to improve reproductive success (Sammataro et al., 2000; Yoder and Sammataro, 2003).

Foundress egg development (oogenesis) is triggered by changes in honey bee larval odor concentrations indicative of the larval stage (Rosenkranz et al., 1993; Frey et al., 2013). Changes in larval odor profiles indicates optimal periods for foundress mites to begin reproduction (Trouiller and Milani, 1999; Garrido and Rosenkranz, 2003, 2004). Approximately 70 h after cell-capping, a foundress *V. destructor* produces her first egg, which is unfertilized and develops into a haploid male (Ifantidis, 1983; Sammataro et al., 2000; Garrido and Rosenkranz, 2003; Nazzi and Le Conte, 2016). Only one haploid egg is produced during the foundress reproductive cycle. Failure of male maturation can result in infertility of females subsequently produced by foundress *V. destructor* (Martin, 1995; Donzé and Guerin, 1997; Garrido and Rosenkranz, 2003; Rosenkranz et al., 2010).

Following the first haploid egg, foundress *V. destructor* continually deposit diploid eggs at 30-hour intervals; up to six eggs are typically produced (Ifantidis, 1983; Rehm and Ritter, 1989; Trouiller and Milani, 1999). Development time for female *V. destructor* from egg to adult is 6.5-6.9 d, and 5.5-6.6 d for males (Ifantidis, 1983). Immature *V.*

destructor development is divided into two main stages before maturity: protonymph and deutonymph. Between these stages are moult cycles in which immature mites shed their outer layer of cuticle (Ifantidis, 1983; Rosenkranz et al., 2010, Fig. 1.5.3).

The final moult of immature female *V. destructor* produces a mixture of pheromones that are sexually attractive to male *V. destructor* (Ziegelmann et al. 2013a; Fig. 1.5.4; Appendix Table A1). Attraction is specific to freshly moulted female *V. destructor*, ensuring that all reproductive effort is focused on those that are sexually mature (Ziegelmann and Rosenkranz 2014, Nazzi and Le Conte 2016). Mating takes place on the fecal accumulation site, where male *V. destructor* exchange spermatophores repeatedly with the newest freshly moulted female (Donzé and Guerin, 1997; Ziegelmann et al., 2013b). Odor released post-moulting diminishes over a short period of time. This ensures that subsequent freshly moulted sexually mature females also become mated in sequence of mite development ensuring that male reproductive effort is evenly distributed among sexually mature female *V. destructor* (Donzé et al., 1996). The foundress *V. destructor* distances herself from the fecal accumulation site during mating. This behavior may serve to improve reproductive effort by minimizing mating attempts made between foundress and male *V. destructor* (Donzé and Guerin, 1997).

Following mating, adult *V. destructor* regularly feed from a single feeding site located on the developing honey bee pupa (Martin, 1995; Donzé and Guerin, 1997; Salvy et al., 2001; Rosenkranz et al., 2010). Approximately 12-15 days after cell-capping, the honey bee emerges along with the foundress *V. destructor* and her mature female progeny. Remaining immature female and the male *V. destructor* mites die within the cell following uncapping (Garrido and Rosenkranz, 2003; Dietemann et al., 2013). Nurse

bees attend the newly emerged honey bee, providing an opportunity for female *V. destructor* progeny and foundresses to disperse presumably through close contact of nurse honey bees with newly emerged honey bees (Pernal et al., 2005; Mondet et al., 2016). Newly emerging honey have undefined odor profiles (Breed et al., 2004). It is assumed that *V. destructor* dispersal onto nurse honey bees shortly following emergence with a newly emerged honey bee is an behavior to seek hosts with defined cuticle profiles and avoid detection by other honey bees (Kuenen and Calderone, 1997). Following dispersal, these female *V. destructor* begin their phoretic life stage (Rosenkranz et al., 2010; Ziegelmann et al., 2013a, 2013b). *Varroa destructor* females undergo 2-3 reproductive cycles during their lifetime, producing an average of 1.3-3.9 mature mated offspring each cycle depending on the sex of the brood cell infested (Ifantidis, 1983; Martin and Kemp, 1997).

1.3.6 Brood preference

Varroa destructor prefer drone over worker larvae (Koeniger and Veith, 1983; Boot et al., 1994; Dillier et al., 2003; Rosenkranz et al., 2010). This preference occurs in both *A. mellifera* and *A. cerana* colonies (Tewarson et al., 1992; Rosenkranz et al., 2010). Sex bias in *V. destructor* host selection is presumed to be a result of the difference in ratios of drone larval cuticle s, when compared to worker larvae of a similar age, because larger drone brood may have greater surface area for volatilization of brood pheromone (Le Conte et al., 1989; Trouiller et al., 1992; Boot et al., 1995; Appendix Table A1). In addition, drone larval cells protrude from the wax comb and have a greater diameter than those of worker cells, which may enhance the formers' attractiveness to *V. destructor*

(Boot et al., 1995; Donzé et al., 1998). Larva brood pheromones have been widely explored in research, and still offer a possibility for managing *V. destructor* populations (Koeniger and Veith, 1983; Rickli et al., 1992; Plettner et al., 2017).

Despite this preference for drone brood, *V. destructor* can reproduce effectively on honey bee worker brood. This contrasts to its native *A. cerana* host where *V. destructor* reproduction on worker brood can lead to larval mite death, nurse bee detection of infested cells, or incomplete maturation of progeny before worker emergence due to shorter pupation times of worker brood (Rath, 1999; Pernal et al., 2005; Rosenkranz et al., 2010). Reproductive potential of *V. destructor* is limited by pupal development time in both *A. mellifera* and *A. cerana* (Trouiller et al., 1994; Nazzi et al., 2009; Fig. 1.5.5). Infesting foundress *V. destructor* will continually deposit eggs through pupa maturation even though not all of these offspring will reach maturity before honey bee emergence (Rosenkranz et al., 2010).

1.3.7 Factors affecting reproductive success

Cell invasions can occur by more than one foundress *V. destructor*, and in instances where this happens, it is believed to be a key mechanism contributing to genetic exchange (Fuchs and Langenbach, 1989; Martin, 1995; Donzé et al., 1996). As the number of foundress *V. destructor* increases in a single cell, the number of eggs laid decreases, but multiple males increases the number of sperm transfers to unrelated females (Fuchs and Langenbach, 1989; Martin, 1995). Differences in number of progeny produced per foundress in cells infested with more than one foundress suggests that additional semiochemicals relating to honey bee larval and/or *V. destructor* development

may be involved (Fuchs and Langenbach, 1989; Donzé et al., 1996). This reproductive restraint is not observed in cells with only one foundress (Martin and Cook, 1996).

1.3 Management and Semiochemistry

Varroa destructor treatment encompasses a wide variety of compounds commonly referred to as miticides. These can vary by mode of action (Tarpy and Summers, 2000; Rosenkranz et al., 2010; Dulin et al., 2014). Despite the effectiveness of synthetic miticides (e.g tau-fluvalinate, coumaphos), *V. destructor* populations can recover relatively quickly following treatment, creating a need for multiple treatments in some climates (Wilkinson and Smith, 2002; Gatien and Currie, 2003; DeGrandi-Hoffman et al., 2017). *Varroa destructor* resistance to synthetic miticides rapidly developed in countries where annual mite treatment is required. Resistance is attributed to sub-lethal dosage exposure to *V. destructor* as a result of the persistence of some miticides within wax for up to five years following initial treatment (Milani, 1999; Milani and Della Vedova, 2002; Johnson et al., 2009; Rinderer et al., 2010).

Varroa destructor treatments using natural formulations such as organic acids and essential oils derived from plant extracts are collectively termed “soft treatment methods” (Koeniger and Veith, 1983; Kraus and Berg, 1994; Umpiérrez et al., 2011; Plettner et al., 2017; Stanimirović et al., 2017). Soft treatments typically are less effective in removing mites than synthetic miticides on a non-resistant *V. destructor* population (Kraus and Berg, 1994; Calderone, 1999; Underwood and Currie, 2003). In addition, several of these treatment alternatives result in the loss of some of the worker population and queen mortality in some cases, requiring additional monitoring of honey bee colony

productivity during application periods (Kraus and Berg, 1994; Mondet et al., 2011). For large-scale apiaries, limited effectiveness of soft treatments can present logistical implications in colony management through re-applications of soft treatments (Kraus and Berg, 1994; Calderone, 1999; Melathopoulos et al., 2000; Underwood and Currie, 2005).

In addition to miticides, beekeepers also use cultural management (Imdorf et al., 2003; Rosenkranz et al., 2010). One example involves physical removal of infected honey bee brood (Imdorf et al., 2003; Plettner et al., 2017). Provided that brood-rearing is timed properly, a significant proportion (50 – 70 %) of *V. destructor* populations can be removed, especially when a gap in the brood cycle is created by caging the queen (Dietemann et al., 2013; Plettner et al., 2017). The downfall of this method is that it can be labour-intensive and can also put additional stress on honey bee colonies, reducing production of honey (Plettner et al., 2017).

Several areas of research are currently being explored to develop *V. destructor* management alternatives including: natural selection of *V. destructor*-resistant honey bees, mite-specific fungal treatments, use of mite predators, developing new essential oil treatments, and life cycle disruption using either synthetic or naturally occurring volatile compounds (Shaw et al., 2002; Donovan and Paul, 2005; Hussein et al., 2016; Bixby et al., 2017; Plettner et al., 2017). Semiochemical disruption of *V. destructor* life cycle is identified by some as the most achievable long-term solution (Ziegelmann and Rosenkranz, 2014).

Varroa destructor interception of honey bee s at specific times through brood development is critical in ensuring the former's successful reproduction (Foster and Harris, 1997; Frey et al., 2013; Ziegelmann et al., 2013b; Nazzi and Le Conte, 2016).

Varroa destructor reproductive cycle offers potential for management of the parasite through chemosensory disruption using synthetic or honey bee colony-derived odors (Foster and Harris, 1997; Frey et al., 2013; Eliash et al., 2014; Ziegelmann and Rosenkranz, 2014; Singh et al., 2015; Nazzi and Le Conte, 2016; Pinnelli et al., 2016).

Despite the wealth of research exploring chemical sensing in ticks and mites, there are still several gaps in knowledge associated with ant detection, particularly regarding *V. destructor* (Le Doux et al., 2000; Dillier et al., 2006; Blenau et al., 2012; Dietemann et al., 2012; Del Fabbro and Nazzi, 2013). Uncovering semiochemical aspects of *V. destructor* life cycles and the importance in detection of host volatile cues within honey bee colonies may offer new avenues for pest management (Foster and Harris, 1997; Garrido and Rosenkranz, 2004). Characteristics of *V. destructor* semiochemical detection may provide additional insight into mite chemoreception and the evolution of particular behavioral characteristics of megostigmatid mites that can be applied to other acarine pests (Bissinger and Roe, 2010; Leonovich, 2013).

1.4.1 Varroa destructor attraction to honey bee larvae

Several stages of *V. destructor*'s life cycle were identified as critical for development of management methods involving semiochemical disruption (Fig 1.5.6). Given the apparent importance of honey bee brood age in triggering changes in adult *V. destructor* life stages, researchers have examined components of honey bee brood pheromone (Le Conte et al., 1989, 1990b, Rickli et al., 1992, 1994; Trouiller et al., 1992; Boot, 1994; Appendix Table A1). Further research concluded variable attraction of some odorants, suggesting the importance of additional factors (temperature, proportions of odors

released, additional non-host odors) (Boot, 1994; Bruce, 1997; Donzé et al., 1998; Trouiller and Milani, 1999; Pernal et al., 2005; Nazzi et al., 2006).

The process of honey bee larval pupation produces a layer of cocoon material within the capped brood cell. Analysis of this material revealed a range of hydrocarbons and components of the honey bee brood pheromone. Behavioral trials revealed a range of individual, binary, and quaternary mixtures of these compounds to be behaviorally important to adult female *V. destructor* mites (e.g. octadecanol, eicosanol, eicosanal, docosanal at 3 µg each per 100 µL of dichloromethane) (Donzé et al., 1998).

Components of honey bee larval food and royal jelly elicited behavioral responses from *V. destructor*, with some components identified as attractants and repellents, respectively (Nazzi and Milani, 1994; Milani et al., 2004; Drijfhout et al., 2005; Nazzi et al., 2009; Appendix Table A3). Research has yet to explore the range of volatiles from other colony sources and their relative importance in both reproductive and phoretic life stages of *V. destructor*.

1.4.2 *Varroa destructor* reproductive cycle

Previous studies have examined in detail *V. destructor* reproduction and triggers involved in the initiation of egg generation (oogenesis) as potential avenues for behavioral disruption (Garrido and Rosenkranz, 2003, 2004; Milani et al., 2004; Cabrera Cordon et al., 2013; Frey et al., 2013; Ziegelmann and Rosenkranz, 2014). Initially, it was thought that oogenesis began shortly after *V. destructor* fed on a honey bee larva, and this was a result of a detection of host levels of larval juvenile hormone. It was later determined that oogenesis occurs rapidly, and is most likely due to changes in host larval

odors (Rosenkranz et al., 1993; Rath, 1999; Trouiller and Milani, 1999; Garrido and Rosenkranz, 2004). *Varroa destructor* foundresses are able to stop egg development if the larval host cues change as a result of larval age, suggesting that additional host cues guide progression of mite reproduction (Frey et al., 2013; Garrido and Rosenkranz, 2003; Nazzi and Milani, 1996, Table A7 in Appendix A).

1.4.3 *Varroa destructor* aggregation

Following initial feeding, a foundress *V. destructor* constructs a fecal deposition area close to the bottom of the brood cell (Fig. 1.5.4). The fecal site aids in immature *V. destructor* aggregation and possibly in locating established feeding site located on developing honey bee larva (Donzé and Guerin, 1997; Yoder and Sammataro, 2003). Fecal deposits of mature non-reproductively active *V. destructor* were composed of up to 95% guanine, and this is similar to other mites that use fecal sites as points of aggregation (McEnroe, 1961; Grenacher et al., 2001; Martin et al., 2002; Yoder and Sammataro, 2003; Allan, 2010; Carr and Roe, 2016). 8-azaguanine, a microbial degradative component of guanine, elicited stronger electrophysiological responses compared to guanine from *Ixodes ricinus* ticks. This suggests that additional *V. destructor* faecal components may be present within capped honey bee brood cells containing foundress mites and could influence mite behavior within the context of reproduction (Grenacher et al., 2001).

1.4.4 *Varroa destructor* mating pheromone

Short-term mating pheromones associated with sexually mature freshly moulted *V. destructor* females have also been identified (Appendix Table A1). This allows male *V. destructor* to mate with several females during honey bee larval pupation (Ziegelmann et al., 2013a, 2013b). Mating disruption offers another potential avenue to manage *V. destructor* populations, given the importance of this pheromone in ensuring fertilization of *V. destructor* offspring (Nazzi and Milani, 1996; Nazzi et al., 2002; Ziegelmann and Rosenkranz, 2014). One study identified the in-colony application of 2 µg dosage of oleic acid on honey bee colony frames containing fifth instar larvae resulted in 20% reduction of number of female daughter mites containing spermatozoa, suggesting possibility in disrupting ability of males to successfully mate with fertile female daughter mites (Ziegelmann and Rosenkranz, 2014).

1.4.5 Volatiles detected by *Varroa destructor*-hygienic bees

Research exploring the difference in volatile profiles of pupae, foundress *V. destructor*, and immature *V. destructor* revealed that foundresses possessed the most dissimilar volatile profile, primarily composed of dimethyl-alkanes (Martin et al., 2002). Some of these components were then electrophysiologically tested on honey bees with or without hygienic behavior. Hygienic honey bees demonstrated acute detection to some odorants (e.g. stearic acid) suggesting importance in potentially triggering uncapping and removal behavior (Martin et al., 2002).

Development of *V. destructor* resistance in different lineages of honey bees could be a result of variability in bee brood pheromone concentrations that are *V. destructor* -

attractive, rather than a result of hygienic honey bee detection and removal of infested pupae (Fuchs, 1994; Aumeier et al., 2002).

1.4.6 Phoretic host choice

During their phoretic stage, *V. destructor* rely on adult honey bee host odor profiles to choose appropriate hosts, with a preference for nurse bees over foragers (Le Conte and Arnold, 1987; Kraus, 1990; Kuenen and Calderone, 1997; Le Doux et al., 2000; DelPiccolo et al., 2010). Phoretic host selection can affect both mite survival and chances of reproduction (Kuenen and Calderone, 1997). *Varroa destructor* mites in the phoretic stage show an 80% preference for nurse honey bees over foragers in choice assays, and was a result of differences in honey bee cuticular hydrocarbon (CHC) profile (Kuenen and Calderone, 1997; Breed et al., 2004; Pernal et al., 2005; Nazzi and Le Conte, 2016). *Varroa destructor* must be able to interpret CHC profiles and mask itself to prevent detection while on its phoretic host; odor-masking by *V. destructor* occurs within three hours following initial direct contact with the host (Kather et al., 2015; Le Conte et al., 2015).

The alarm pheromone, released from several glands located at the tip of the abdomen of worker honey bees, contains s that are repellent to *V. destructor* (Pickett et al. 1980, Kraus 1990, Pernal et al. 2005, Branco et al. 2006, Table A4 in Appendix A). Some of these compounds are found frequently in odor profiles of foraging honey bees, likely relating to *V. destructor* preference for nurse honey bees over foragers (Kraus, 1990; Pernal et al., 2005; DelPiccolo et al., 2010).

1.4.7 Other possibilities of semiochemical management

Some honey bee volatile components identified as attractive to *V. destructor* are also important in maintaining colony structure and could potentially create difficulty in developing effective semiochemical treatment options without affecting colony dynamics (Plettner et al., 2017; Ma et al., 2018). Investigations of synthetic volatiles which disrupt *V. destructor* host detection seem promising but these compounds still require field testing (Eliash et al., 2014; Singh et al., 2015; Pinnelli et al., 2016).

Currently, a fragmented understanding exists regarding the ability of *V. destructor* mites to differentiate host s and the exact triggers that result in behavioral changes during critical stages of a mite's life cycle (Dietemann et al., 2012; Plettner et al., 2017). Rarer components of honey bee brood pheromone have been a focus in research; however, other hydrocarbons may have equally important behavioral relevance to *V. destructor* but have received little attention (Le Conte et al., 1989; Donzé et al., 1998; Martin et al., 2002). Additional behaviorally important components emitted from honey bee larvae likely exist and remain to be identified (Nazzi et al., 2004). One method to address these gaps would be to develop a means to screen previously unexplored putative attractants, repellents, and odorants. Furthermore, exploration of honey bee colony volatile collection is still in its infancy, with standardized methods for ex-situ volatile collection recently established (Carroll and Duehl, 2012; Torto et al., 2013). Development of an electophysiological approach which allows screening of honey bee colony volatiles with live *V. destructor* mites may offer new insight to mite repertoires and refine the array of possible important semiochemicals through identifying those with strong relative electrophysiological responses from isolated honey bee colony sources. Another gap in

research is *V. destructor* acuity of detection, with studies focusing on honey bee concentration-dependent electrophysiological and behavioral responses to colony semiochemicals (Pankiw and Page, 2001; Reinhard et al., 2010; McAfee et al., 2017). Exploration of *V. destructor* acuity of detection and contrasting to previous honey bee research could lead to the discovery and implementation of *V. destructor*-relevant odorants that minimally affect honey bee colony dynamics.

Objectives of this thesis are to (1) develop a novel protocol to collect electrophysiological responses from the first tarsi of *V. destructor* (electrotarsograms). (2) Develop an adapted approach to perform honey bee colony frame volatile collections using dynamic headspace analysis. (3) Through developed electrotarsogram approaches, research outcomes aim to have a more complete understanding of *V. destructor* repertoire to a variety of honey bee colony odors and their putative importance in host detection through qualitative and quantitative analysis of electrophysiologically-active odors. (4) Using electrotarsograms, this thesis will attempt to elucidate *V. destructor* detection sensitivity towards a range of attractants and repellents through characterization of concentration-dependent electrophysiological responses. (5) Furthermore, efficacy of electrotarsograms will be explored in differentiating pituitative attractants, repellents based on *V. destructor* relative responses towards those of solvent controls.

The following Chapters 2 and 3 in this thesis are formatted for publication as separate manuscripts in The Journal of Experimental and Applied Acarology. Some information in Chapter 1 may be repeated within the following chapters.

1.5 Figures

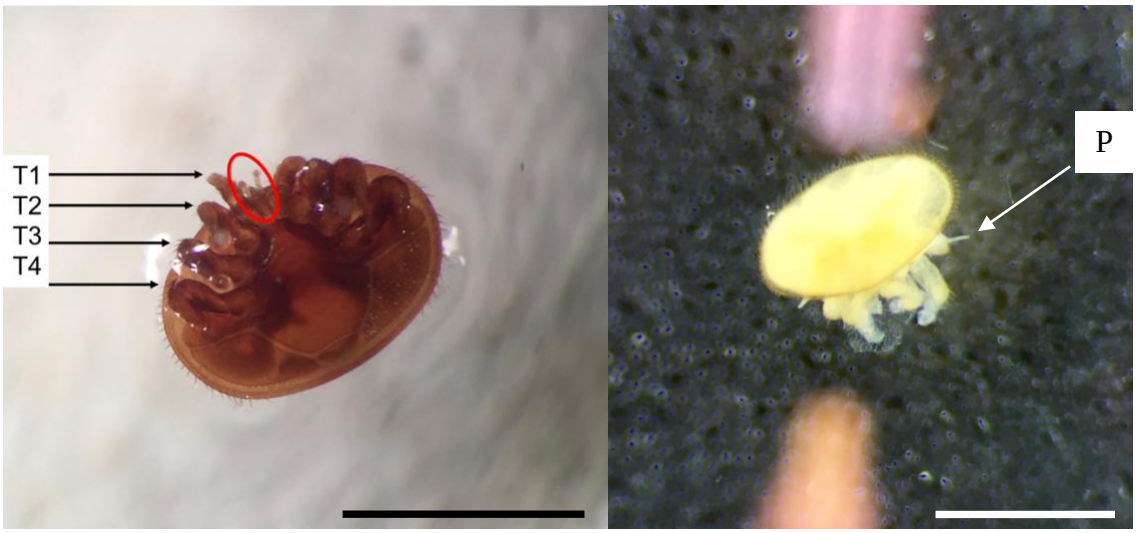

Fig. 1.5.1 Left – adult female *Varroa destructor*; right – immature female final molt *V. destructor*, preserved in 70% ethanol, gnathosoma is circled red, T1 to T4 indicate sets of tarsi, and P indicates left peritreme, scale bars are 1 mm.

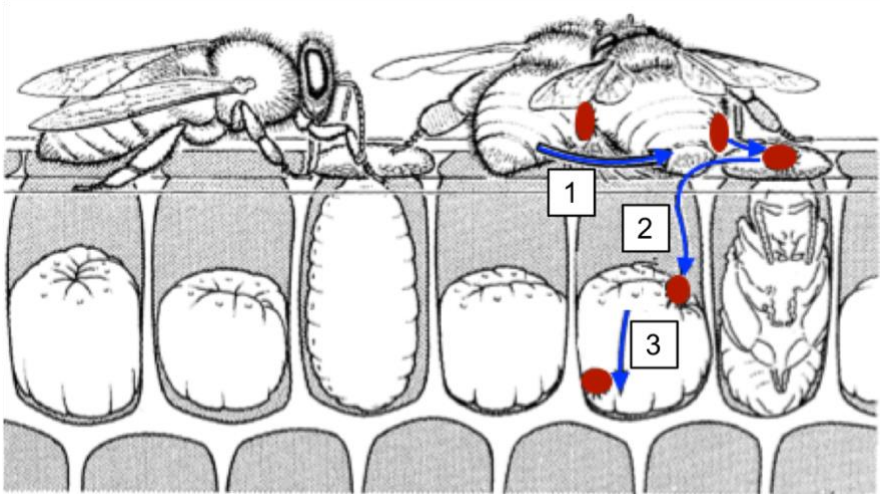

Fig. 1.5.2 *Varroa destructor* cell invasion process: 1 – interception of fifth instar brood volatiles and associated cues, 2 – rapid movement from adult host honey bee to larvae cell, 3 – movement past larvae to bottom of cell; figure adapted from Boot et al. 1990.

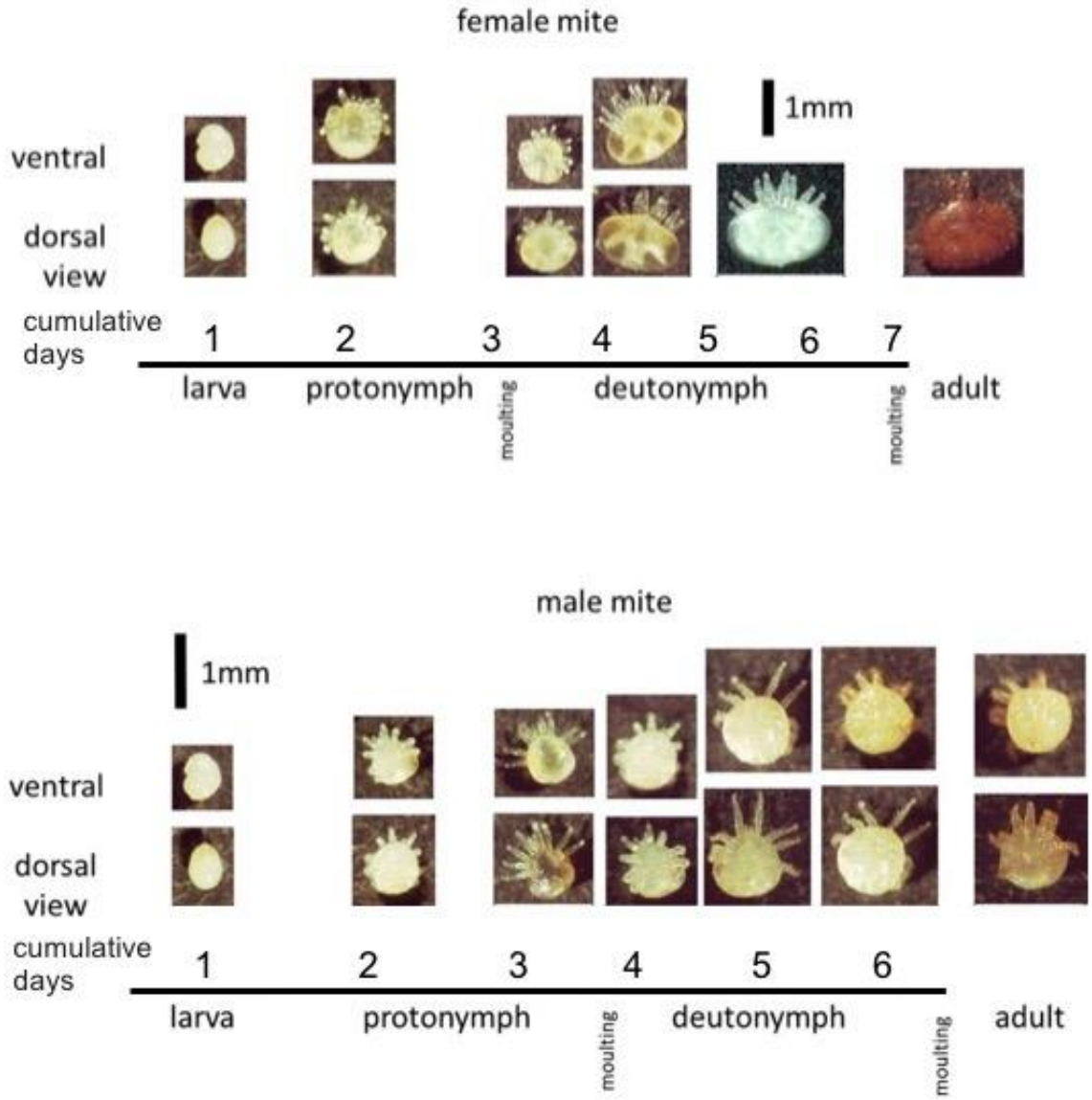

Fig. 1.5.3 Female *Varroa destructor* progression from egg to adult featuring the two primary stages: protonymph and deutonymph, taken from Dietemann et al. (2013).

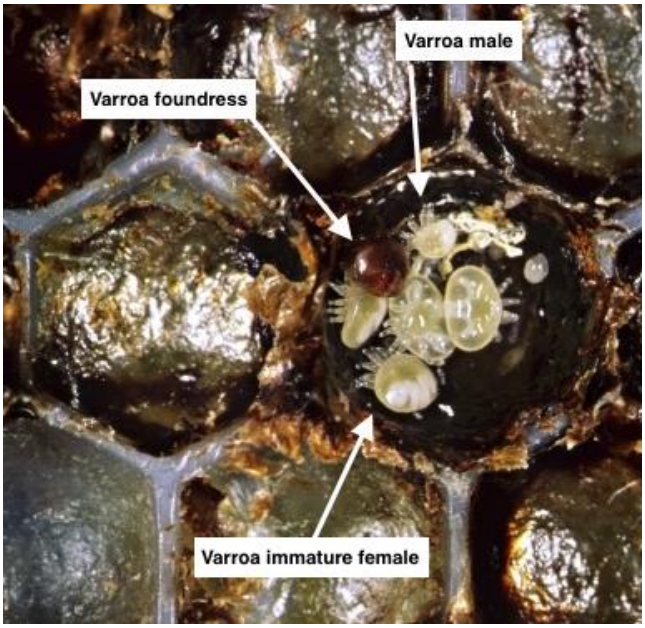

Fig. 1.5.4 *Varroa destructor* foundress with progeny (arrows), adapted from D. Anderson, CSIRO via Wikimedia Commons.

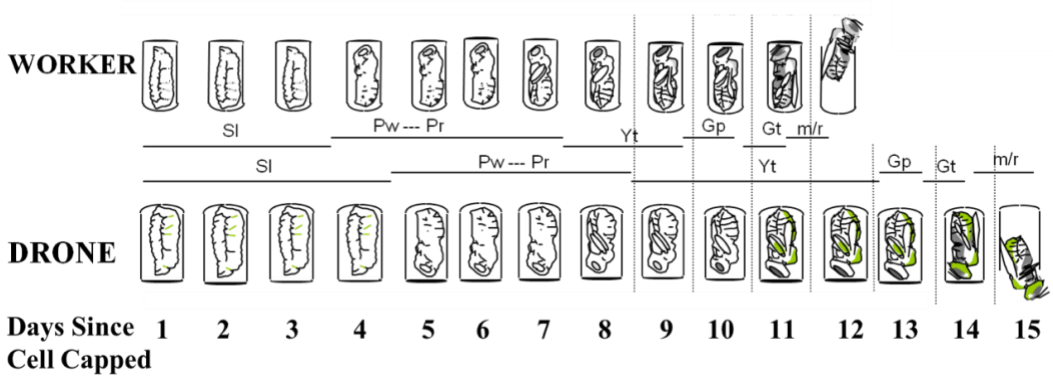

Fig. 1.5.5 Comparison of *Apis mellifera* worker and drone brood developmental times from cell capping to emergence. Abbreviations follows: stretched larvae (Sl), white-eyed pupae (Pw), red-eyed pupae (Pr), yellow thorax (Yt), grey pads (Gp), grey thorax (Gt), moulting or resting (m/r), adapted from Dietemann et al. 2013.

1.6 References

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CHAPTER 2.0 ELECTROTARSOGRAM RESPONSES OF *VARROA DESTRUCTOR* TO BEHAVIORALLY RELEVANT ODORANTS

2.1 Abstract

The parasitic mite *Varroa destructor* is globally the most important pest of honey bees (*Apis mellifera*). Repeated evolution of miticide resistance has stimulated exploration of new methods to manage *V. destructor*. One promising approach involves exploiting honey bee colony volatile cues that are important in *V. destructor*'s life cycle. I refined techniques to mount mites and conduct electrophysiological recordings (electrotarsograms) of their responses to odorant stimuli. Results of 271 electrotarsogram recordings from *V. destructor* revealed significant differences between 10 odorants versus solvent controls. For some odorants (methyl palmitate, ethyl palmitate, 2-heptanol), electrotarsogram response at the weakest concentration (10^1 ng) elicited the strongest response, suggesting *V. destructor* may have acute sensitivity to odorants of low concentration that are directly related to its host. Results suggest that odor origin (e.g. honey bee larvae or adult alarm pheromone) within a honey bee colony can influence threshold sensitivity at 10^4 ng stimulus concentration (e.g. methyl oleate, geraniol, respectively). *Varroa destructor* responses to attractants were not significantly different from solvent control responses, whereas responses to repellents were less than those from solvent controls. Electrotarsograms offer potential for screening odorants to determine their putative importance in *V. destructor* host detection; this could allow future research to screen odorants with unknown *V. destructor* valence and cross-reference to known attractants and repellents.

2.2 Introduction

Western honey bees (*Apis mellifera* L.; Hymenoptera: Apidae; hereafter honey bees) are the most widely used pollinators of agricultural crops (Morse and Calderone, 2000; Klein et al., 2007). *Varroa destructor* (Anderson and Trueman, 2000; Acari: Varroidae) is an invasive parasitic mite of honey bees originally from southeast Asia. It is considered the most economically important and challenging threat to apiculture today (Currie et al., 2010; Rosenkranz et al., 2010; Nazzi and Le Conte, 2016; Ferland et al., 2017). For example, in 2007, up to 85% of overwintering colony mortalities in some parts of Canada were ascribed to *V. destructor* infestations (Currie et al., 2010).

Current *V. destructor* management often involves using synthetic miticides, organic acids, essential oils, or non-chemical methods (Calderone, 1999; Imdorf et al., 2003; Currie et al., 2010; Ferland et al., 2017). However, miticide-resistant *V. destructor* have repeatedly evolved (Rosenkranz et al., 2010; van der Zee et al., 2012), and 'soft' treatments or non-chemical methods can have variable efficacy or cause honey bee worker and queen mortality (Melathopoulos et al., 2000; Underwood and Currie, 2003). A possible management alternative involves manipulation of *V. destructor* behavior using in-colony volatiles that are important in the mite's life cycle (Yoder and Sammataro, 2003; Pernal et al., 2005; Plettner et al., 2017).

Acute differentiation of host odor profiles is critical in *V. destructor* lifecycles (Martin et al., 2001; Frey and Rosenkranz, 2014; Nazzi and Le Conte, 2016). Primary chemosensory organs of *V. destructor* are on the first tarsi, situated in a cluster of sensilla similar in structure to Haller's organ in ticks (Dillier et al., 2003, 2006), and have been a focus in previous research (Rickli et al., 1992, 1994). There have been only a few

attempts to identify electrophysiological responses of these sensory organs to attractants or repellents (Endris and Baker 1993; Dillier et al. 2003). Other cues involved in *V. destructor* host detection (temperature, humidity, and vibrations) have also not been widely studied (Kirchner, 1993; Bruce, 1997; Dillier et al., 2006).

Varroa destructor alternates between phoretic stages on worker bees and reproductive life stages within brood cells (Boot et al., 1994; Kather et al., 2015). *Varroa destructor* reproductive cycles closely coincide with those of honey bee brood (Boot et al., 1994; Plettner et al., 2017), and honey bee brood odors play a crucial role in *V. destructor* host choice and physiology during the latter's reproduction (Trouiller et al., 1992; Pernal et al., 2005; Frey et al., 2013; Singh et al., 2016). Semiochemicals originating from adult honey bees and brood have been studied because they are likely cues determining host suitability (Trouiller et al., 1992; Rickli et al., 1994; Foster and Harris, 1997; Aumeier et al., 2002; Pernal et al., 2005; Nazzi and Le Conte, 2016). It may be possible to manage *V. destructor* through manipulation of in-hive honey bee semiochemicals (Donzé et al., 1998; Yoder and Sammataro, 2003; Plettner et al., 2017).

To date, approximately 60 different chemicals have been identified that modify *V. destructor* behavior (Appendix A). Although experiments have identified many of these chemicals from different colony sources, solvent extractions in those experiments typically did not provide colony-relevant concentrations (Boot, 1994; Rickli et al., 1994; Donzé et al., 1998; Pernal et al., 2005).

Quantification of sensory acuity through concentration-responses may provide a better interpretation of how *V. destructor* intercepts host odors in honey bee colony environments compared to previous solvent extraction research (DelPiccolo et al., 2010).

Specificity of particular odorants could be quantified by comparing concentration-responses for an array of compounds previously identified in honey bee colonies that evoke behavioral responses from *V. destructor* (Martin et al., 2002). Concentration-dependent responses can then be tested with odorant mixtures to identify possible mixture synergisms (Pernal et al., 2005; Plettner et al., 2017). Furthermore, responses to putative attractants and repellents, as well as compound structure, can be explored to identify possible relationships among structurally similar compounds and electrophysiological responses. Relationships between odor molecular structure and electrophysiological response could provide a means to screen for similar compounds that share similar biological activity from *V. destructor*, and may lead to development of novel pest management techniques through synthetic mixtures of these compounds (Del Fabbro and Nazzi, 2013).

I tested *V. destructor* sensitivity to concentration series of several known attractants and repellents using an electrotarsogram protocol. Odorant concentrations were selected based upon honey bee and larval cuticle extractions or volatile collections identified in previous research (Martin et al., 2002; Gilley et al., 2006; Schmitt et al., 2007; Thom et al., 2007; DelPiccolo et al., 2010; Carroll and Duehl, 2012).

2.3 Methods

2.3.1 *Varroa destructor* collection

From June through August 2017 and 2018, six Langstroth bee hives provided by two local beekeepers located in Wolfville and Berwick, Nova Scotia (NS) (45.0452° N, 64.7347° W), Canada were used to rear drone brood via queen-trapping. Collection and maintenance of *V. destructor* followed protocols described by Dietemann et al. (2013). Drone frames containing brood were transferred from donor colonies to an untreated *V. destructor*-infested colony in Coldbrook, NS (45.0585° N, 64.5925° W). After drone frames were capped, they were collected and transferred to environmentally controlled chambers (32°C and 65 % relative humidity; Conviron - Controlled Environments Ltd.; Model E-16, Winnipeg, Manitoba, Canada) at Acadia University. All frames were placed on wooden racks that had similar dimensions to a honey bee nucleus box (53 × 21 × 24 cm), all bees and frames on wooden racks were kept inside nylon insect-rearing tents (90 × 60 × 60 cm) within environmental chambers. Live adult worker honey bees, at a ratio of 2:1 for every drone, were used to maintain colony structure and in prolonging *V. destructor* lifespan within environmental chambers by providing suitable phoretic hosts. Queen mandibular pheromone (Intko Supply Ltd, Vancouver, British Columbia, Canada) was applied to a glass coverslip at a concentration of 0.1 queen equivalents (42.2 ng in 10 µL of 2-propenol) and allowed to evaporate for 5 min under a fume hood. Treated coverslips were then placed within insect-rearing tents and replaced every 48 h to promote honey bee health and longevity (Grozinger et al., 2007). Adult worker and drone

honey bees were transferred in groups of 10 to 20 into wooden hoarding cages (17 × 12 × 13 cm) using a vacuum modified into a bee aspirator (Dewalt DCV517B; Baltimore, MD, USA) (Rogers and Williams, pers. comm.). Transferred bees were then individually picked up and examined for phoretic *V. destructor* using nitrile gloves. *Varroa destructor* were transferred in groups of five using both a moistened paintbrush and aspirator to 50-mL falcon tubes (Thermo Fisher Scientific; NY, USA) containing 2 × 4 mm moistened filter paper. All electrotarsogram experiments were performed on *V. destructor* within 5 h following collection.

2.3.2 Odorant stimuli preparation

Electrotarsograms (ETGs) were conducted using a range of single odorants previously identified as evoking behavioral responses in *V. destructor* mites and several with an unknown attractiveness or adverseness (valence) (Table 2.6.1). A subset of these odorants was diluted in decadic series of increasing concentrations (10^0, 10^1, 10^2, and 10^3 ng μL^{-1}) using HPLC grade hexane solvent (Sigma-Aldrich, St. Louis, MO, USA) (Pinnelli et al., 2016; Singh et al., 2016). These concentrations were selected to capture the range of concentrations *V. destructor* would likely encounter in colony environments (Martin et al. 2002; Del Piccolo et al. 2010). The concentrations tested are reflective of honey bee headspace (volatile profile) collection (Martin et al., 2002; Schmitt et al., 2007; Torto et al., 2007; McAfee et al., 2017) and cuticle solvent extractions (Le Conte et al., 1990; Kraus, 1990; Rickli et al., 1994; Donzé et al., 1998; Calderone and Lin, 2001).

All compounds were analyzed through gas chromatography mass spectrometry (GCMS) to ensure samples were free of contaminants before developing stimulus

cartridges. All serial dilutions were made in 2-mL glass vials sealed with Teflon tape and thereafter stored at -20 °C.

Stimulus cartridges were prepared by pipetting diluted compounds onto ethanol-washed filter paper strips cut into 1 × 3 cm pieces (Fisherbrand P8, 90-mm diameter) (Fisher Scientific Company, Ottawa, Ontario, Canada). Ten µL of each dilution of an odorant were loaded onto filter papers, individually inserted into disposable borosilicate glass pipettes (Fisherbrand 14.6 cm) and capped with 1-mL plastic pipette tips. A stimulus cartridge was prepared for each compound at 10^1-, 10^2-, 10^3-, and 10^4-ng loadings using prepared stepwise concentrations. Serial concentrations of each odorant were grouped together and wrapped in aluminum foil. All grouped stimuli were placed in freezer bags and stored at -20 °C until use. Prior to electrotarsogram experiments, all odor stimulus cartridges were brought to 25 °C before use. Each odorant series was used on a maximum of four separate *V. destructor* preparations before being replaced with new treated filter paper. Odorants followed an increasing concentration series (10^1 – to 10^4-ng) with a solvent control preceding and following a series to account for changes in *V. destructor* responses through time. Each mite (*V. destructor* preparation) consisted of 50 individual stimulus recordings. The sequence of each odorant concentration series was randomized to account for possible interactions between stimuli and changes in responses over time due to preparation degradation.

2.3.3 Electrotarsography

Varroa destructor were chilled for 2-3 s and then mounted on a microscope slide coated in clear dental wax (Electron Microscopy Sciences, Hatfield, PA, USA). A single

V. destructor was placed on its dorsum without pressing into the dental wax and held in place with two parallel, horizontally-positioned minuten pins (ENTO SPHINX, Černá za Bory, Czech Republic) to restrict movement (Fig. 2.6.1).

ETG recordings were performed through electrotarsography using a design adapted from single sensilla recordings (Dillier et al., 2003; Hanes, 2015). Changes in electrical potential were measured from either the left or right foretarsus using tungsten recording electrodes. The ETG signal was collected and amplified (Low Cut-off: 0.05 Hz, Offset: 0, Ext amp: 10) by Intelligent Data Acquisition Controller-2 (IDAC-2) (Ockenfels SYNTECH GmbH - Buchenbach, Germany). Before recording, *V. destructor* preparations were positioned in front of humidified airflow (0.5 L min^{-1}) (Endris and Baker, 1993). Small amounts of electrode gel (SIGNAGEL, Parker Laboratories Inc. - FAIRFIELD, NJ, USA) were placed on prepared *V. destructor* anal plates and all tarsi except the foretarsi. The ground electrode was inserted into a *V. destructor* anus at the base of the anal plate of the ventrum. The recording electrode was inserted just past the apotele of the foretarsus.

Stimuli were puffed in series of increasing concentration with solvent stimulus preceding and following a series of concentrations of a particular odorant. A single odorant puff lasted 0.3 s controlled by a Syntech stimulus controller CS-55 V2.7 (Ockenfels SYNTECH GmbH - Buchenbach, Germany). Each stimulus cartridge was puffed in intervals of 30 s to allow for preparation recovery (Eliash et al. 2014; Plettner and Soroker pers. comm.). All odorant series were tested in random order for each *V. destructor* preparation. Mite recordings represented an incomplete design due to incomplete recording of some odorant series as a result of preparation degradation in

some cases. Data were later refined to exclude responses less than the solvent control across all *V. destructor* preparations, resulting in an incomplete randomized design (see below for justification).

2.3.5 Statistical analysis

Peak amplitude for each odorant was recorded in mV and collected using GcEad ©2014 software v. 1.2.5 (Ockenfels SYNTECH GmbH - Buchenbach, Germany). Data were analyzed in the R-Studio software package (R Foundation for Statistical Computing 2014). Responses to different odor stimuli were identified as the first of two depolarizations (Endris and Baker 1993, Fig. 2.6.2). Initial electrotarsogram data indicated a large variability among mites. Changes in electrotarsogram signal strength were identified among mite preparations. These differences could result from subtle changes among mite preparations regarding the placement of the recording tungsten electrode on or near chemosensory sensilla as well as changes in equipment sensitivity. A second source of variability in electrotarsogram responses arises from a gradual depreciation of signal quality within a mite preparation as a result of drying out of electrical connections. For longer electrophysiological recordings where a depreciation in signal quality is observed through time, a linear interpolated value of responses towards solvent controls is used (Martin et al., 2002). To account for both of these variabilities, data were normalized to linear interpolated solvent control (hexane) responses using an equation derived from Eliash et al. (2014).

Mites that indicated no linear trend in responses to solvent controls due to large inconsistencies among responses (variability >20 mV among solvent control stimuli) and

with ETG amplitudes in which differentiation among mechanical and odorant responses could not be made were removed from the analysis (n = 12). An inconsistency in responses to the control stimulus through time suggests poor electrical connection or motor activity from the preparation, thereby affecting the consistency in *V. destructor* responses to odorants and concentrations within a particular mite preparation.

I examined both normalized and filtered responses that were greater than the solvent control baseline to account for possible bias in removal of data where responses were weaker than to the solvent control. Normalization and removal of negative responses accounted for differences in among-preparation signal sensitivity compared to examining raw amplitude data. Following filtering electrophysiological responses, data represented an unbalanced and randomized design due to removal of responses less than solvent control.

Additional factors (year of data collection, outdoor temperature, and outdoor relative humidity) were modelled using both raw V. destructor electrotarsogram responses and normalized data. Data analysis included all mite preparations to capture possible influence of outdoor temperature, outdoor relative humidity, or year of study on quality of mite preparations.

General linear mixed-effects models were used to identify differences among normalized responses in relation to odorants, concentrations, year of study, temperature, relative humidity, and their interactions using the ordered quantile normalization transformation (R packages: lme4, emmeans, ggplot2, ggpubr, bestNormalize). Each individual mite was treated as a random effect. Interaction terms (odor, concentration, year, outdoor temperature, outdoor relative humidity, interactions of odor × concentration

× year and of odor × concentration × outdoor temperature × outdoor relative humidity), if not significant ($\alpha = 0.05$), were sequentially removed from models until only main effects remained. Following identification of significance in responses, either post-hoc pairwise comparisons were performed with a Bonferroni correction or post-hoc least squares means was performed using Tukey adjustment.

2.4 Results

I refined a method for collecting ETG responses from *V. destructor* in which preparations sometimes lasted over 60 min. ETG recordings were made from 34 different mites; from these, we recorded 1711 stimulus responses. Plotting of initial data indicated variability in the electrical amplitude of responses among mites, with a majority of responses to odorants being less than they were to solvent controls. Data were further filtered to include only *V. destructor* responses to individual odorants that were greater than responses to the control stimulus (Eliash et al., 2014), leaving n = 289 responses (from the 22 remaining mites) including solvent control (Table 2.6.2). ETG responses that were less than the control stimulus indicate additional noise or other artifacts during stimulus administration. Subsequent analysis involved working with both zeroed responses compared to the control stimulus and the un-manipulated data to determine if removal of negative-relative responses changed the outcome of data interpretation.

Normalized ETG data indicated non-existent concentration-response trends within an odorant concentration series (Table 2.6.2, Fig. 2.6.3). A linear mixed effects model of normalized ETG responses with mite as a random effect indicated a significant effect of odorant ($F = 2.9$, $df = 10$, $p = 0.01$), but no effect of concentration ($F = 1.6$, $df = 3$, $p =$

0.19) or their interaction (F = 0.8, df = 30, p = 0.72). Concentration-response trends were also investigated without data filtering, results indicated a non-significance with the interaction of concentration and odor when using mite as a random effect but did detect a similar effect of odor.

Given the lack of effect of concentration, subsequent models examined average *V. destructor* normalized response to odors averaged across concentrations, with a significant effect of odor (F = 2.2, df = 24, p = 0.02). Results indicated a lack of significance among odors within concentrations in explaining *V. destructor* normalized responses.

Differences in odorant responses at a single concentration (10^4 ng) were examined. Analysis of absolute amplitude data indicated a significant difference among responses to odorants at 10^4 ng concentration (Fig. 2.6.4). When comparing normalized data, alcohols with isoprene units (e.g. α-terpineol, linalool) were more likely to elicit weaker amplitude responses relative to the solvent stimulus (Z = 5.3, df = 1 $p < 0.0001$). Attractants originating from adult honey bees or honey bee brood (e.g. ethyl palmitate, heptadecane) appeared to elicit *V. destructor* normalized responses comparable to solvent controls (Z = 2.2, df = 1 p = 0.17). Repellents (e.g. 2-heptanol, octanoic acid) generally elicited weaker normalized responses from *V. destructor* than the solvent control (Z = 3.18, df = 1 p = 0.01), and did not differ from alcohols with isoprene units (Z = 1.2, df = 1 $p > 0.99$).

Raw *V. destructor* electotarsogram responses indicated a significant effect of year in absolute responses elicited among mite preparations (F = 18.1, df = 1, p = < 0.0001). This significance was accounted for through data normalization. Models shared a significant effect of concentration (F = 13.5, df = 3, p = 0.01) and of the interaction of odor and year

(F = 25.8, df = 4, p = < 0.0001) and significance did not change when using absolute amplitude responses or normalized responses as the response variable. Closer examination of the effect of concentration indicated that this significance was due to concentration responses less than those of solvent controls. The significance of the interaction of odor and year was attributed to ethyl palmitate, although other odorants demonstrated similar non-significant effect of year on *V. destructor* electrotarsogram responses to remaining odorants with the exception for 2-heptanol, with data collected in 2017 providing both higher normalized and raw amplitude responses compared to those from 2018 (Fig. 2.6.5). Despite differences in electrotarsogams conducted between years of study, concentration responses were not identified through modelling filtered data by year.

During 2017, data for hourly outdoor temperature and relative was used to examine their effect on *V. destructor* electrotarsogram responses. Models examining raw amplitude data indicated a significant effect of temperature (F = 18.4, df = 1, p = < 0.0001). Performing the same model using normalized data indicated that normalization accounted for variability in temperature, as often temperature did not vary through time very much but more so among mite preparations. Both normalized and absolute *V. destructor* electrotarsogram responses showed a weak interaction effect of temperature × humidity × odour. This suggests that outdoor temperature and humidity may have weakly influenced *V. destructor* responses to odorants or perhaps the volatility of some odorants. Odorants showing a higher range in *V. destructor* electrotarsogram responses are not readily soluble in water (methyl palmitate, octanoic acid, benzoic acid, 2-heptanone), suggesting that both temperature and humidity only partly explained for differences in

electrophysiological responses suggesting additional influence of other unknown random variables. Although indoor temperature and relative humidity was not monitored, fluctuations of these factors within the laboratory was detected in relation to outdoor weather throughout data collection.

2.5 Discussion

Using a novel electrotarsogram technique, I screened several putative attractants and repellents, enabling us to determine whether *V. destructor* expresses ETG concentration-dependent responses. Results indicated no concentration-response trends, although significant differences were found among odorants relative to solvent control responses. Previous research focusing on *V. destructor* chemosensory-disruptive compounds used electrophysiology on excised foretarsi (Eliash et al., 2014; Singh et al., 2015; Pinnelli et al., 2016). Excised preparations may yield clearer responses given an absence of muscle responses in fresh tissue, but the quality of response from these preparations can decline relatively quickly over time, with preparations providing stable responses for at most 20 min (Eliash et al., 2014). Previous research indicated that *V. destructor* electrophysiological preparations require a 30-s recovery time in between stimulus recordings (Eliash et al., 2014).

Varroa destructor ETG responses differed significantly among odorants and may be related to the number of odorant receptors that respond to compounds of interest (Dillier et al. 2006; Fig. 2.6.4). This could indicate the relative importance of individual odorants in host detection (Nazzi et al., 2009; Ziegelmann et al., 2013; Carr and Roe, 2016).

Putative attractants (e.g. methyl oleate, methyl palmitate) elicited responses greater than putative repellents (e.g. 2-heptanol, 2-nonanol, geraniol) when examining normalized data. This corroborates prior research which has indicated that odors directly related to host detection are of primary importance in developing potential management strategies (Rosenkranz et al., 2010). Odorant responses that are less then putative attractants could suggest that a fewer number of sensilla are involved in detection of particular odorants, and may relate to sensilla specificity to these odorants (Dillier et al., 2006). 2-heptanone, trans-nerolidol, and nonanal have not been tested in previous *V. destructor* research and may be equally important in host detection as *V. destructor* had similar responses to these as to putative attractants and repellents, although absolute responses to these odorants were not significantly different from those towards hexane solvent control. Responses to trans-nerolidol at 10^4 ng were greater than responses to α-terpineol and linalool even though they share similar molecular structure. Interestingly, 2-heptanone is an attractant of small hive beetles (*Aethina tumida*) and trans-nerolidol is an attractant of two-spotted spider mites (*Tetranychus urticae*) and repellent of brown ear ticks (*Rhipicephalus appendiculatus*) (Torto et al., 2005; Carr and Roe, 2016). Moreover, 2-heptanone is found in mandibular glands of guard bees, and 2-heptanone may be associated with honey bees or nest invaders that have been bitten by defensive honey bees (Breed et al., 2004). 2-heptanone may be relevant to *V. destructor* in avoiding detection and/or damage. Previous research has reported cross-activity of putative acarine repellents towards *V. destructor* (Peng et al., 2015). Which could be an avenue for future research to investigate behavioral importance of acarine attractants to *V. destructor* (Bissinger and Roe, 2010; Carr and Roe, 2016). Both nonanal and heptadecane are components detected

from adult honey bee volatile headspace and have either never been tested or did not evoke behavioral responses from *V. destructor*, respectively (Pernal et al., 2005; Torto et al., 2005; Schmitt et al., 2007). Whereas responses by *V. destructor* to these stimuli may be determined through electrophysiology, additional research is needed to confirm if these components are behaviorally relevant.

Among odorants tested at 10^4 ng, two plant-derived compounds (α-terpineol and linalool) evoked the weakest normalized responses compared to solvent controls from *V. destructor*. This leads to questions about the activity of similar compounds on *V. destructor* receptors (Miller et al., 2007; Peng et al., 2015), and whether they have the ability to disrupt or inhibit sensory reception through neurophysiological mechanisms. Several essential oil components were previously examined for behavioral responses from *V. destructor*, including α-terpineol, which offer potential for use in pest management (Kraus et al., 1994; Imdorf et al., 1999; Peng et al., 2015). These findings suggest using electrophysiology to screen plant-derived components for their potential in eliciting chemosensory disruption by inhibiting peripheral detection of attractive stimuli (Miller et al., 2007). Future *V. destructor* research could explore whether concentration-dependent responses are present using mixtures of putative attractants and semiochemical disruptants.

Varroa *destructor* may have sensitivity to other host odorants, some of which are low-volatility (e.g. methyl palmitate, ethyl palmitate), but further research is needed (Fig. 2.6.2). Boot (1994) and Donzé et al. (1998) speculated that both low volatility and trace components of honey bee larvae are most likely responsible for evoking behavioral responses in *V. destructor*. Trouiller et al. (1992) and Pankiw and Page (2001) reported

components of honey bee larvae cuticle extractions range from $< 10^{-1}$ to $> 10^{3}$ ng per individual larva. Higher sensitivity to trace components would be advantageous in *V. destructor* life cycles (Donzé et al., 1998). Concentrations examined in my research are comparable to those previously explored with honey bees and *V. destructor* behavioral studies (Nazzi et al., 2001; Martin et al., 2002; DelPiccolo et al., 2010; Ziegelmann et al., 2013). This indicates a possible overlap in the ranges of detection for *V. destructor* and honey bees (Martin et al., 2002). Single components are often more important than complex blends in honey bee detection, and similar sensitivities may be exhibited by *V. destructor* within the colony environment (Le Conte et al., 1989; Donzé et al., 1998; Keeling et al., 2004; Reinhard et al., 2010). This finding further indicates the importance of developing comparative studies that identify differences in responses between hosts and parasites with respect to individual components and mixtures for the development of future in-colony treatment methods.

Peak amplitude responses varied in shape and strength among *V. destructor* preparations. In several instances, peak shapes were bimodal rather than unimodal (Fig. 2.6.2), consistent with prior research, and suggests double depolarizations may be both olfactory and mechanical responses occurring in quick succession (Endris and Baker, 1993). Amplitude information collected in this research focused on amplitude responses that were previously described as responses to odorants (Endris and Baker, 1993). Prior research has indicated that *V. destructor* is covered in many hair-like structures, and these may have mechanosensory capabilities as in other arthropods (Dillier et al., 2006; Ganske and Uhl, 2018). Single sensillum recording on live *V. destructor* indicated that electrode placement can greatly influence the quality of responses to odorants (Dillier et al., 2003;

Hanes, 2015). This was also observed in these data, with between-mite variation in the relative strength of odorant responses to mechanical responses. Electrophysiology using single sensilla recording may provide better signal differentiation between mechanical and odorant responses.

Varroa destructor electrotarsogram responses collected across years demonstrated an influence of year in accounting for some variability in these data. It is possible that a relocation of electrophysiological equipment to climate-controlled room altered variability in electrophysiological signal. Furthermore, during the first year of study, an influence of both outdoor temperature and relative humidity may alter amplitude responses from *V. destructor* to select odorants. It is possible that in our research, outdoor climatic conditions affected indoor temperature and humidity as well as influenced climate-control systems, resulting in the interaction identified. It is clear that outdoor temperature and relative humidity are only partly responsible for explaining some of the variability in these data collected, with individual mite variability offering the best random variable in explaining the variation among *V. destructor* electrotarsograms.

Although concentration-dependent responses were not identified in this research, concentration-dependent responses at concentrations relevant to *V. destructor* should be a future focus in developing solutions for infestation management (Plettner et al., 2017). Identifying functions of individual odorant receptors in *V. destructor* may lead to a better understanding of those in other acarine pests and the potential for cross-application of known semiochemical disruptants (Eliash et al., 2014). Improved longevity of preparations using the methods described here could be applied to future *V. destructor* chemical ecology research through their implementation in broad-scale screening of

honey bee colony volatiles through gas chromatography linked electrotarsography. Future research should explore *V. destructor* behavioral responses towards potentially relevant compounds identified here and examine possible crossover in activity with other Acari (Bissinger and Roe, 2010; Carr et al., 2013).

2.6 Figures and Tables

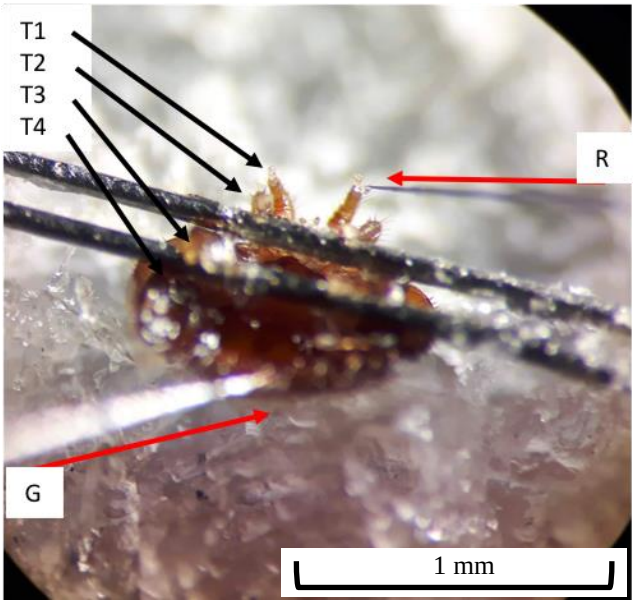

Fig. 2.6.1 Electrotarsogram preparation of a live female *Varroa destructor* with T1 – T4 indicating tarsi, R –recording electrode, and G – ground electrode.

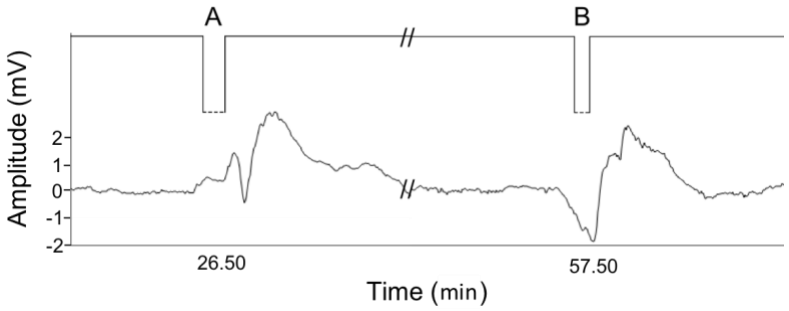

Fig. 2.6.2 *Varroa destructor* electrotarsogram responses in mV to solvent control (A) and 2-heptanone at 10^3 ng μL^{-1} stimulus concentration (B) with 30 s intervals between stimulus administration.

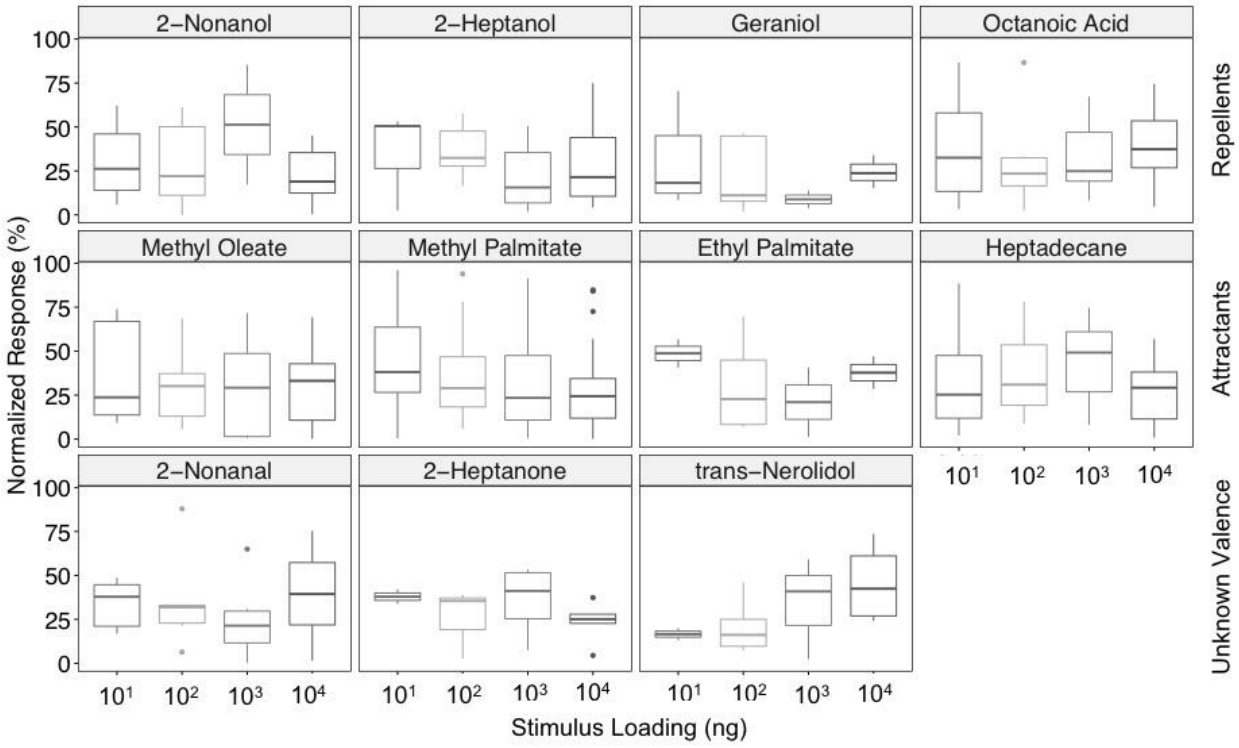

Fig. 2.6.3 *Varroa destructor* concentration-dependent responses (10^1, 10^2, 10^3, 10^4 ng in 10 µL stimulus loadings) based on electrotarsography of putative repellents (top), attractants (middle), and odorants of undetermined valence (bottom); responses were normalized to solvent control; concentration-dependent responses to odorants below those to solvent control were removed from analysis; error bars represent variance, points represent outliers, and boxes define the first and third quartiles with median indicated by bolded lines.

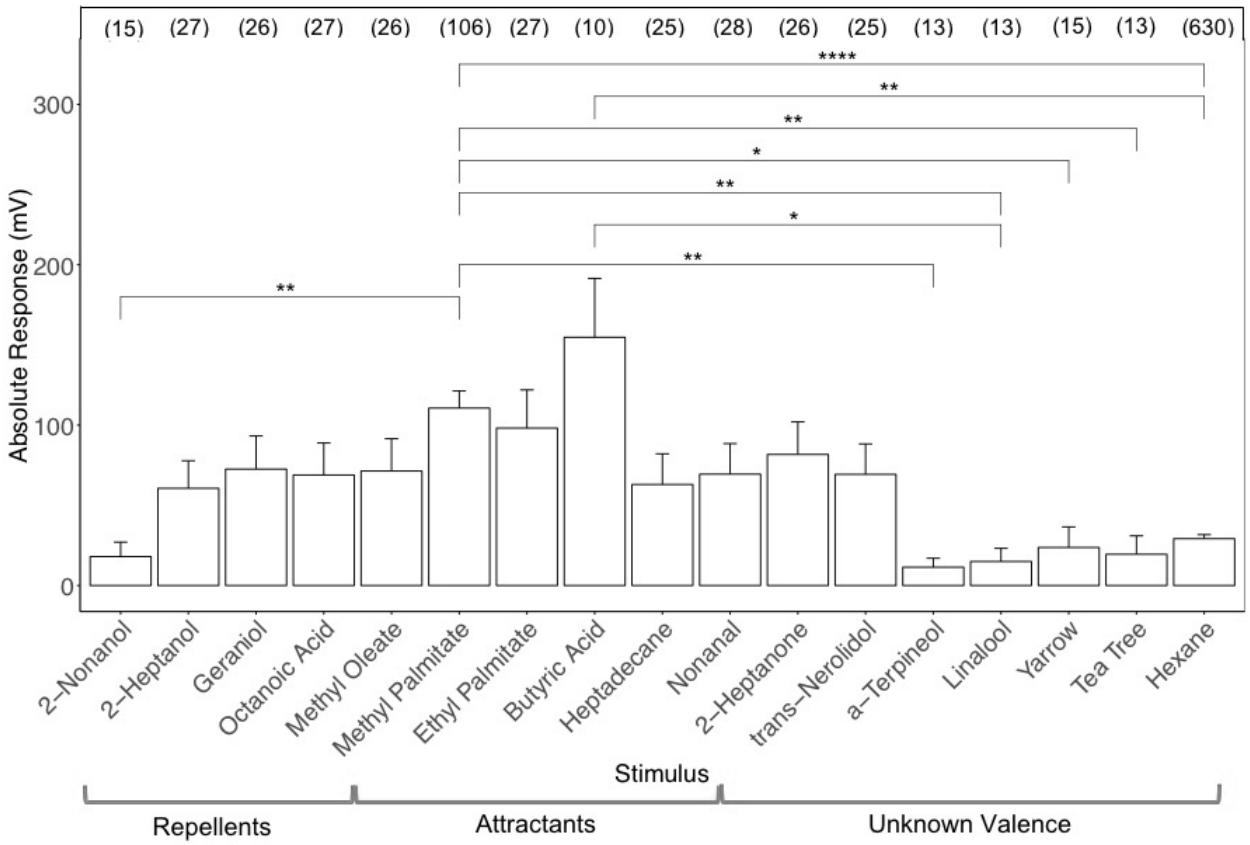

Fig. 2.6.4 Results of Mann-Whitney multiple comparisons of *Varroa destructor* electrotarsogram absolute responses (mV) at 10^4 ng in 10 µL hexane solvent using Holm's correction. Numbers within parentheses denote number of stimulus recordings. Stimuli "Yarrow" and "Tea-Tree" were essential oils (*Achillea millefolium* L. and *Melaleuca alternifolia* Cheel, respectively) tested at 10% v/v; Responses significantly different from solvent control (hexane) are represented by * $p < 0.01$, ** $p < 0.001$, and **** $p < 0.0001$. Odors are categorized as repellents, attractants, and unknown valence according to Table 2.6.1; error bars represent standard error, bars represent average absolute response.

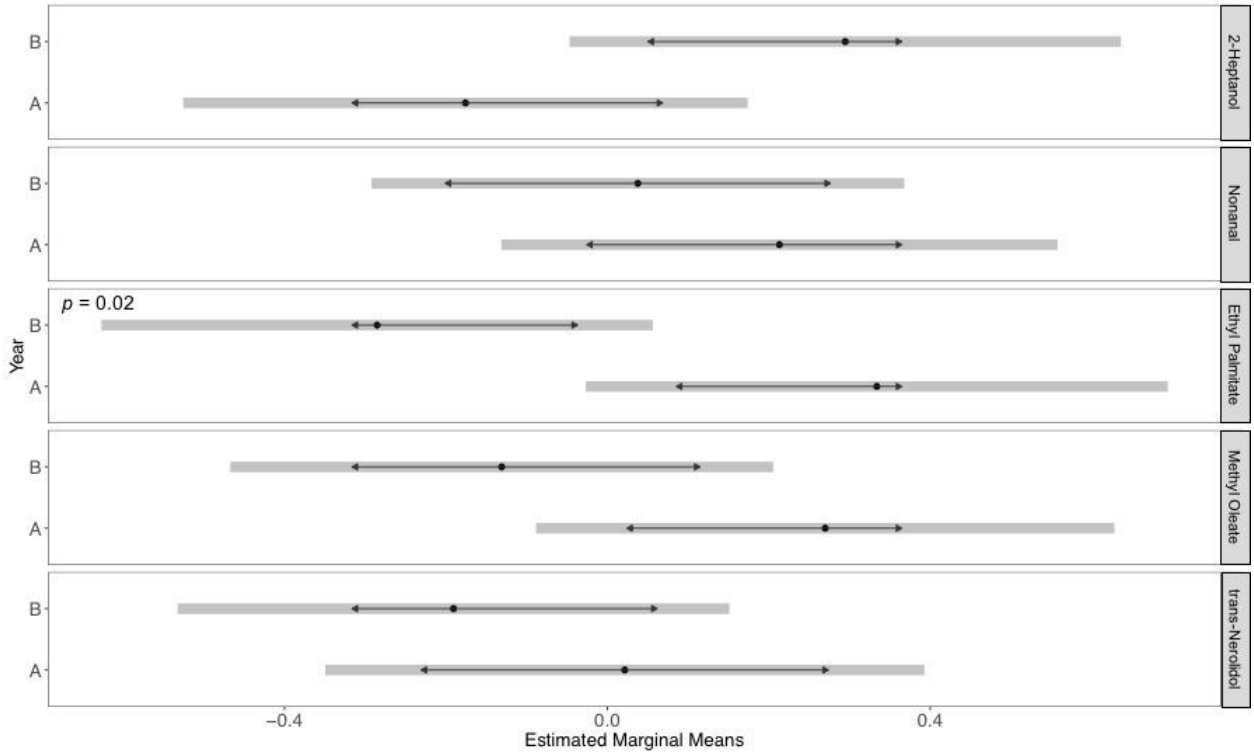

Fig. 2.6.5 Estemated marginal means with Tukey adjustment examining the influence of year on *Varroa destructor* electrotarsogram normalized responses with odor as an interaction; year is represented as 2017 (A) and 2018 (B); odorants selected are those that occur between years with relatively similar sample sizes to avoid sampling bias. Pairwise comparisons (dark arrows) differentiate whether an odorant from a particular year has significantly different estimated marginal means, with grey bars representing confidence limits.

Table 2.6.1 Previous literature examining *Varroa destructor* behavioral responses to putative attractants and repellents tested in this study.

compound	colony origin	bioassay response	citation
methyl oleate	brood cuticle	attractant	Trouiller et al. 1992
methyl palmitate	brood cuticle	attractant	Trouiller et al. 1992; Pernal et al. 2005
ethyl palmitate	brood cuticle	attractant	Le Conte et al. 1989; Trouiller et al. 1992
2-heptanol	bee alarm	repellent	Kraus 1990
2-nonanol	bee alarm	repellent	Kraus 1990
geraniol	bee alarm	repellent	Hoppe and Ritter 1988; Foster and Harris 1997
2-heptanone	bee alarm	NA	Blum 1996
octanoic acid	royal jelly	repellent	Nazzi et al. 2009
nonanal	bee cuticle	NA	Torto et al. 2005; Schmitt et al. 2007
heptadecane	bee cuticle	NS	Pernal et al. 2005; Schmitt et al. 2007
butyric acid	brood cuticle	attractant	Teal et al. 2014
a-terpineol	NA	repellent	Peng et al. 2015
trans-nerolidol	NA	NA	Carr and Roe 2016
linalool	NA	NA	Tutun et al. 2018

NA = information not available in cited literature; NS = non-significant findings.

Table 2.6.2 Results of Kruskal-Wallis rank sum test for concentration-dependent electrotarsogram responses from *Varroa destructor* across stimulus loadings (representing quantity applied to filter paper) within an odor.

stimulus	p	concentration (ng/10 µL)	n	mean	sem (±)
methyl oleate	0.95	10^1	5	27.6	10.2
		10^2	6	22.7	6.1
		10^3	5	22.8	9.4
		10^4	9	26.0	6.2
			1		
methyl palmitate	0.30	10^1	2	41.6	6.3
		10^2		30.6	5.3
		10^3	2	28.8	5.3
		10^4		31.2	5.7
			2		
ethyl palmitate	0.65	10^1	2	48.7	8.1
		10^2	4	30.5	14.7
		10^3	2	21.0	19.7
		10^4	3	30.3	9.2
2-heptanol	0.06	10^1	4	45.2	6.3
		10^2	7	32.9	4.4
		10^3	8	20.9	6.4
		10^4	1	27.2	7.1
2-nonanol	0.72	10^1	6	30.5	9.1
		10^2	7	29.4	9.2
		10^3	2	51.3	34.1
		10^4	6	22.4	7.0
geraniol	0.50	10^1	5	30.9	11.8
		10^2	5	22.4	9.6
		10^3	2	8.8	5.0
		10^4	3	24.4	5.4
octanoic acid	0.82	10^1	8	38.2	11.1
		10^2	8	28.4	9.2
		10^3	7	35.2	8.1
		10^4	5	39.4	11.8

2-heptanone	0.44	10^1	2	37.9	4.2
		10^2	3	25.7	11.5
		10^3	4	35.7	10.6
		10^4	5	23.5	5.4

Table 2.6.2 Continued

stimulus	p	loading (ng)	n	mean	sem (±)
nonanal	0.58	10^1	6	31.5	5.6
		10^2	7	34.0	9.7
		10^3	6	24.8	9.2
		10^4	6	39.5	11.1
heptadecane	0.63	10^1	8	32.3	10.0
		10^2	5	33.1	12.1
		10^3	5	40.6	11.9
		10^4	6	21.5	6.8
trans-nerolidol	0.29	10^1	2	16.6	3.4
		10^2	5	22.3	6.9
		10^3	3	34.1	16.8
		10^4	4	45.7	11.9

n = number of stimulus recordings for particular compound at a given stimulus loading; mean = average normalized response to stimulus across loadings; sem = standard error of mean normalized responses; p from Kruskal-Wallis rank sum test; concentration = ng of odorant per 10 µL of hexane solvent.

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CHAPTER 3.0 *VARROA DESTRUCTOR* MITE RESPONSES TO HONEY BEE (*APIS MELLIFERA*) COLONY VOLATILES

3.1 Abstract

Honey bees (*Apis mellifera*) produce and interpret a range of pheromones that regulate colony dynamics and allow identification nest intruders. Parasites such as *Varroa destructor* mites intercept and conceal themselves with honey bee odors. *Varroa destructor* also uses chemical detection of different host stages to complete its lifecycle and reproduce within honey bee colonies. I collected volatiles from honey bee brood at different developmental stages and screened for *V. destructor* electrophysiological responses with gas chromatography-linked electrotarsal detection. Volatile collections contained a diverse array of methyl-alkanes which evoked electrophysiological responses in *V. destructor*. Moreover, several putative plant secondary metabolites and terpenes were detected by *V. destructor*, among them (*E*)-ß-ocimene, also a colony-wide brood volatile associated with queen status and honey bee brood. Collections from mid- to late-stage larvae had greater diversity in trace volatiles. Among these, several mid- to heavy-molecular weight compounds elicited high proportional electrophysiological responses in *V. destructor* relative to their abundance but could not be identified using chemical standards of previously documented honey bee brood odors. We suggest further investigation of these unknown volatiles and future behavioral assays to determine attractiveness/repellency (valence) of volatiles that were identified through chemical standards.

3.2 Introduction

Hymenoptera produce and detect a great diversity of chemical cues (semiochemicals) which can modify behavior and physiological development (Robinson 1992; Breed et al. 2004; Johnson and Frost 2012; Keeling et al. 2004). Within honey bee (*Apis mellifera*) colonies, effects of semiochemicals are context-dependent, with the same mixture or individual odorant causing different behavioral and physiological changes in individuals both within and outside of a colony (Blum, 1996; Thom et al., 2007; Ma et al., 2018; Villar et al., 2018). Maintaining complex social structure within honey bee colonies is important in maintaining resilience to infections and colony intruders (Breed et al., 2004; Gomez-Moracho et al., 2017).

Honey bees can host numerous parasites (Shimanuki et al., 1980; De Jong et al., 1982; Schmid-Hempel, 1995; Gomez-Moracho et al., 2017). Among them, the mite *Varroa destructor* (Anderson and Trueman, 2000; Acari: Varroidae) is considered the most important and challenging apicultural pest, blamed for high annual winter colony mortalities (Beetsma, 1994; Milani, 1999; Currie et al., 2010; Rosenkranz et al., 2010). When left untreated, infested honey bee colonies usually succumb to *V. destructor* within three years (Rosenkranz et al., 2010). *Varroa destructor* rapidly developed resistance to synthetic miticides in the late 1990s in several countries, increasing motivation to develop effective alternatives and integrated approaches (Calderone, 1999; Milani, 1999; Melathopoulos et al., 2000).

Varroa destructor has a two-stage lifecycle and females repeat this cycle up to three times in their lives (Martin and Kemp, 1997). Detection and interpretation of chemical and physical cues from different honey bee hosts is important in completion of *V.*

destructor lifecycles and survival (Plettner et al. 2017) . *Varroa destructor* mites move among adult hosts in the phoretic stage, preferring 7- to 12- d old honey bees of the nurse caste (Le Conte and Arnold, 1987; Kraus, 1994; Pernal et al., 2005). The reproductive cycle occurs when *V. destructor* leave phoretic host and infiltrate a cell containing a suitable honey bee larva host (Le Conte et al., 1989, 1990; Boot et al., 1994). Approximately 70 h after cell-capping, adult *V. destructor* lay several eggs in sequence, with the first developing into a haploid male and subsequent eggs developing into diploid females (Ifantidis, 1983; Garrido and Rosenkranz, 2003). Immature *V. destructor* have two molt cycles and reproduction occurs shortly after the last moult, when molting releases a short-lived mating pheromone attractive to male *V. destructor* (Ziegelmann et al., 2013).

Host choice and reproductive development are focal areas of *V. destructor* research, given apparent importance of timing and host cues in their reproduction (Rosenkranz et al., 2010; Nazzi and Le Conte, 2016; Plettner et al., 2017). Development of effective semiochemical lures, repellents, or compounds that change detection of hosts by parasites (disruptants) to disrupt the *V. destructor* lifecycle provides a promising option in control of this widespread apicultural pest (Yoder and Sammataro, 2003; Plettner et al., 2017).

Qualitative and quantitative analysis of honey bee semiochemicals through non-destructive sampling techniques has been performed only a limited number of times (Gilley et al., 2006; Thom et al., 2007; Carroll and Duehl, 2012). To our knowledge, generic screening of *V. destructor* electrophysiological response to honey bee volatile collections has never been explored. Volatile collections from honey bee colonies may contain odors important in *V. destructor* host detection, and which are specific to

different developmental stages of honey bee brood (Carroll and Duehl, 2012). I refined methods of Carroll and Duehl (2012) for volatile collection from honey bee brood frames. Furthermore, I adapted a method from Endris and Baker (1993) and Dillier et al. (2003) for screening volatile compounds to live *V. destructor* through gas chromatography linked to electrotarsal detection (GC-ETD). Following isolation of electrophysiologically-active honey bee volatiles, I identified some of these honey bee colony volatiles through cross reference using databases and chemical standards.

3.2 Methods

3.2.1 *Varroa destructor* collection

From July through August 2018, three privately owned Langstroth bee hives located in Berwick, Nova Scotia (NS) (45.0452° N, 64.7347° W), Canada were used to rear worker and drone brood via queen trapping. Collection and maintenance of *V. destructor* followed protocols developed by Dietemann et al. (2013). Drone frames containing brood were transferred from donor colonies to an untreated *V. destructor*-infested colony in Coldbrook, NS (45.0585° N, 64.5925° W). Drone frames were reared by the untreated colony until cells were capped and then were transferred to environmentally controlled chambers (1.3 × 1.3 × 1.8 m; 32°C and 65 % relative humidity; Conviron - Controlled Environments Ltd., Model E-16; Winnipeg, Manitoba, Canada) at Acadia University. All frames were placed on wooden racks that had similar dimensions to a honey bee nucleus box (53 × 21 × 24 cm), all bees and frames on wooden racks were kept inside nylon insect-rearing tents (90 × 60 × 60 cm) within environmental chambers. Worker honey

bees ranging in age from 1 to 10 d old were added to drone frames at a ratio of 2:1 for every drone to maintain colony structure and in prolonging *V. destructor* lifespan within environmental chambers. Queen mandibular pheromone (Intko Supply Ltd - Vancouver, British Columbia, Canada) was applied on a glass coverslip every 48 h at a concentration of 0.1 queen equivalents (42.2 ng in 10 µL of 2-propenol) to promote honey bee health and longevity (Grozinger et al., 2007). Adult worker and drone honey bees were transferred in groups of 10 to 20 into wooden hoarding cages (17 × 12 × 13 cm) using a hand-held vacuum modified as a bee-aspirator (Dewalt DCV517B; Baltimore, MD, USA; Rogers and Williams, pers. comm.). Honey bees in hoarding cages were then individually examined for phoretic *V. destructor*. Mites were removed from honey bees using both a moistened paintbrush and aspirator and transferred in groups of three to 50-mL falcon tubes (Thermo Fisher Scientific; NY, USA) containing 2- × 4-mm moistened filter paper. All *V. destructor* gathered in falcon tubes were used for electrophysiological experiments on the same day as collection.

3.2.2 Collecting honey bee brood frames

All honey bee brood rearing and volatile collections were performed using food-grade plastic Langstroth frames (Pierco Inc., Riverside, CA, USA) with wax comb produced within the same year of volatile collections. Prior to brood rearing, high density polyethylene (HDPE-2) 300-mL cups were used to create indentations of cup diameter on drawn-out Langstroth frames. This prevented queens from laying on cup peripheries where indentations in wax comb occurred, later avoiding volatiles produced through incidental damage to honey bee brood (injury volatiles) during volatile collection (Fig.

3.6.1). All honey bee brood rearing and volatile collections were done using a single honey bee colony located in Coldbrook, NS (45° 3’ N, 64° 36’ W). The honey bee queen was located and kept on frames for a period of 24 h to ensure uniform age of brood to be reared and later used for volatile collection (Human et al., 2013). Brood age was then determined by development time in days post egg laying; volatile collections were categorized accordingly.

Volatile collections were performed in environmentally-controlled chambers (1.3 × 1.3 × 1.8 m; 32°C and 65% relative humidity; Conviron - Controlled Environments Ltd.; Model E-16, Winnipeg, Manitoba, Canada) without adult honey bees on the frames of interest. Frames containing early-instar larvae or capped brood were transported using a colony nucleus box from the donor honey bee colony to environmentally controlled chambers for volatile collections (K.C. Irving Environmental Centre, Acadia University, NS, Canada). Frames with uncapped cells containing larvae were returned to the same colony immediately after volatile collection. Capped brood frames were maintained in the incubator along with 100 to 200 worker nurse bees following volatile collection for honey bee rearing and subsequent estimation of percent *V. destructor* infestation.

3.2.3 Volatile collection

Pre-packed volatile collection traps (HayeSep-Q 80/100 mesh, 27+ mg; Volatile Assay Systems; Rensselaer, NY, USA) were cleaned prior to use following adapted methods (Kunert et al., 2009; Molnár et al., 2015). Traps were cleaned by flushing sequentially with 1 mL each of methanol (100%; Bebbington Ind., Dartmouth, NS, Canada), acetone (>98.5% pure; Sigma-Aldrich, St. Louis, MO, USA), and HPLC grade hexane (Fisher

Scientific, Fair Lawn, NJ, USA) and dried under compressed ultra-high purity nitrogen following each solvent flush. Volatile collection traps were then kept in an oven at 75 °C for 2 h before use. Prior to volatile collection, traps were flushed with 1 mL HPLC Hexane and dried under compressed ultra-high purity nitrogen.

Drone frames were held vertically inside insect-rearing tents using wooden racks. Volatile collections were performed in tandem, allowing for two volatile collections to commence simultaneously on different areas of a brood frame. This method allowed for two replicate volatile collections from brood of a particular age to be collected within the same time span (Table 3.6.1; Raguso and Pellmyr 1998; Kunert et al. 2009; Carroll and Duehl 2012). Sample replication was performed to compare for possible contamination of volatile collection or contamination of adsorbent. Volatile collections used a Portable Volatile Assay System (PVAS22; Rensselaer, NY, USA) carbon-filtered continuous airflow pump (0.3 L/min push and 0.1 L/min pull with volatile collection traps in-line) using Fisherbrand™ Tygon™ (0.64 cm I.D. x 15.24 cm length) tubing fitted with Teflon™ connectors (Fisher Scientific, Pittsburgh, PA, USA). Conditioned volatile collection traps were then connected to the airflow system. HDPE-2 300-ml cups were used to isolate areas of interest in volatile collections. Cups were pushed into initial indentations made in wax comb, forming a seal with the frame. Elastic bands were used to hold cups to drone frames during volatile collections. Volatile collections were performed for three hours within environmental chambers maintained at above mentioned climatic conditions comparable to in-colony environment (Carroll and Duehl, 2012).

Following collection, volatile traps were sealed with Teflon tape and wrapped in aluminum foil. Solvent elution was performed the day of volatile collection to reduce

sample degradation and contamination. HPLC-grade hexane (Fisher Scientific, Fair Lawn, NJ, USA) was used to elute volatile collection traps at 1 mL volume (Raguso and Pellmyr, 1998). Extractions were kept in Teflon™-sealed 2-mL vials at -20°C prior to analysis.

All volatile collection equipment was washed with warm unscented soap and water, and then subsequently flushed with 100% ethanol and air dried overnight between volatile collections. Blank volatile collections were performed in the same conditions as above, with volatile collection cups wrapped in aluminum foil forming a seal using elastic bands. Prior to blank collections, aluminum foil was cleaned using ethanol and air-dried for 1 h.

3.2.4 Preparation of extracts

Volatile collection replicates were initially tested for contamination of sorbents using gas chromatography mass spectrometry (GC-MS; see 3.2.7 for parameters). Sample replicates that indicated no contamination were then pooled together to provide a larger sample volume for screening against multiple *V. destructor* preparations through electrotarsography. Volatile collection samples were divided into 200-µL aliquots and individually concentrated under compressed ultra-high purity nitrogen to 20 µL using 250-µL glass vial inserts. Nonyl acetate was used as an internal standard forming final concentration of 3 ng/µL for quantification of proportional abundance (Sigma Aldrich, St. Louis, MO, USA) (Carroll and Duehl, 2012). Concentrated samples were then immediately used for electrophysiological recording using gas chromatography

electrotarsal detection (GC-ETD) and subsequent compound identification using GC-MS, with remaining solvent extract stored at -20 ℃.

3.2.5 Gas chromatography electrotarsal detection specifications

GC-ETG recordings were performed using Varian 450-GC fitted with a flame ionization detector (FID) in which CIP SIL8-CB (30 m; 0.25 mm ø; 25 μm) non-polar column was used (Varian Inc., Lake Forest, CA, USA). Oven temperature was held at 50 ℃ for 5 min, then increasing at 5 ℃/min to 200 ℃ with a final temperature ramp of 25 ℃/min to 280 ℃, holding this temperature for 5 min. Concentrated volatile extracts were manually injected at a volume of 1μL, with the inlet held at 250 ℃. Helium was used as a carrier gas at a rate of 1.2 L/min. The GC column effluent was split with a sample ratio of 50:50, where half of the sample was delivered to a heated transfer line held at 280 ℃ (Syntech Temperature Controller TC-02; Syntech, Kirchzarten, Germany) and introduced into carbon-filtered humidified airstream at 0.5 L/min directed over *V. destructor* preparations (see below).

3.2.6 Electrophysiology

Varroa destructor were chilled on ice for 2-3 s in plastic petri dishes to reduce mobility (55 mm; Fisher Scientific; Ottawa, Ontario, Canada) and then mounted on a microscope slide coated in dental wax (Electron Microscopy Sciences - Hatfield, PA, USA). A single *V. destructor* was placed on its dorsum, without pressing into the dental wax, and held in place with two parallel, horizontally-positioned Minuten pins (ENTO SPHINX - Černá za Bory, Czech Republic) to reduce motor activity.

GC-ETDs were performed using methods adapted from previous research (Endris and Baker 1993; see methods in Chapter 2). Changes in electrical potential were measured from either the left or right foretarsus using tungsten recording electrodes. Recordings were repeated on up to nine different *V. destructor* preparations for a given concentrated volatile extraction to ensure consistency in electrophysiological responses. Varroa destructor electrotarsograms were performed among six volatile collections of honey bee larvae and capped pupae of varying stages of development (Table 3.6.1). GC-ETD signals were collected and amplified by Intelligent Data Acquisition Controller-2 (IDAC-2) (Syntech, Kirchzarten, Germany). Syntech GC-EAD software was used to analyze results (Filter Low Cut-off: 0.05 Hz, Offset: 0, Ext amp: 10; Version: V 1.2.5, © 2010).

All electrophysiological recordings were manually integrated and peak amplitude (mV) responses to volatile collections were determined from averaged responses from *V. destructor* preparations (Fig. 3.6.2).

3.2.7 Chemical identification

Concentrated volatile extractions were analyzed using a Scion 456 Gas Chromatogram - Single Quad Mass Spectrometer (SCION Instruments, Livingston, UK) for identification of compounds eliciting electrophysiological responses with electron impact ionization mode at 70 eV, scanning m/z 40-350 (Restek Rxi-5MS; 30 m; 0.25 mm Ø; 0.25 µm; Restek Corporation, State College, PA, USA). The same oven temperature specifications were used to compare peak retention times with GC-ETD output. Helium was used as a carrier gas at a flow rate of 1.2 mL min^{-1}. Concentrated volatile samples were manually injected at 250 °C in splitless mode, with split closed for 1 min.

Quantitation was performed using the following chromatogram integration parameters: peak width = 4.0 s; slope sensitivity (SN) = 10; tangent = 10%; peak size reject = 2000; using RMS noise calculation, mean three-point smoothing, and a spike threshold factor of 10. All peak areas were then compared to internal standards and then quantified as ng μL^{-1} of sample before concentration. GC-ETD and GC-MS methods for volatile screening and identification were designed to reduce the potential of co-elution of similar molecular weight compounds while at the same time minimizing duration of a single run (39 min) to ensure reliable sustained electrophysiological recordings from *V. destructor*.

Differences in retention times between GC-ETD and GC-MS were calculated and accounted for through a hydrocarbon standard series (Sigma Aldrich, St. Louis, MO, USA). Compound identification was performed using a combination of National Institute of Standards and Technology (NIST) Database, Kovats retention index calculated using equation for temperature programmed chromatography, and chemical standards when available. Compounds were then classified into three categories of confidence (Stein et al., 2011). Compounds identified with a "high" level of confidence were confirmed through chemical standards. Compounds labeled as "medium" confidence had a NIST reverse match ranging from 700 to 900. Compounds with "low" confidence had a NIST reverse match of < 700. Kovats retention indices were compared to possible matches for temperature programmed runs using same column active phase through online chemical databases. Compounds deemed through NIST as low confidence and lacking a matching Kovats retention index were categorized as "unknown" (n = 13) and compiled in Appendix Table B1. Peak identities that could not be narrowed to a single possible

compound match through both NIST and Kovats (e.g. dimethyl-, trimethyl-, and tetramethyl-alkanes) were deemed low confidence.

Nonyl acetate was used as an internal standard in all volatile collections to identify relative abundance of individual volatiles (Torto et al., 2013). Moreover, we discovered *V. destructor* had electrophysiological responses towards nonyl acetate, allowing for identification of proportional peak response (in mV). Peak abundance was then compared to peak response relative to the internal standard.

3.2.8 Statistical analysis

Non-parametric pairwise comparisons were performed for incomplete unreplicated data with non-normal residuals to examine possible differences among volatile collections and compound functional groups in relation to proportional response/abundance (R Foundation for Statistical Computing 2014; using software packages: PMCMRplus, ggplot2, dplyr, coin). A general linear model was used to identify possible interactions among occurrence of volatiles of a particular functional group among volatile collections (software packages: lme4, emmeans, car).

3.4 Results

Volatile collections yielded a range of new (n = 99) and previously detected (n = 9) compounds from honey bee colony frames. In particular, identification of ETD-active volatile compounds through GC-MS indicated a number of methyl-alkanes, possibly originating from wax substrates, with Kovats retention indices between 860-1060 (Table 3.6.3).

Several suspected plant secondary metabolites (methyl salicylate, α-pinene, citronellal, ß-ocimene, menthol; Table 3.6.4) were detected which have been previously tested for *V. destructor* electrophysiological response or detected in volatiles from honey bee colonies (Endris and Baker, 1993; Dillier et al., 2003; Carroll and Duehl, 2012; Peng et al., 2015). Moreover, several aromatic compounds were identified (ethylbenzene, naphthalene, and acetophenone; Table 3.6.5) which may be emitted from nectar or pollen resources (Dillier et al., 2003; Torto et al., 2007; Carroll and Duehl, 2012; Molnár et al., 2015).

Volatile collections yielded some suspected contaminants (xylenes, butylated hydroxytoluene, methyl 2-methylhexyl phthalate); several of these compounds were easily identifiable through their mass spectra. I also identified putative colony volatiles in blank (control) volatile collections that elicited *V. destructor* electrophysiological responses in addition to known contaminants originating from plastics (Table 3.6.6).

Several GC-ETD-active compounds were detected in volatile collections but varied in their abundance among brood developmental stages (e.g. limonene and 3,7-dimethylnonane). 3,7-dimethylnonane was detected in all volatile collections, with higher abundance detected in early instar brood and brood that was killed through chilling. Limonene was detected in highest abundance from volatile collection of early instar brood and had low abundance in capped pupae and brood killed through chilling. Volatile collection from early-stage larvae produced fewer alkanes and a greater number of putative plant secondary metabolites (e.g. menthol, citronellal, methyl salicylate; Table 3.6.7). In general, plant volatiles were detected in trace quantities in volatile collections of late-stage larvae and chilled brood; however, they still evoked relatively high electrophysiological responses from *V. destructor* suggesting importance in its lifecycle

(Fig. 3.6.3, Fig. 3.6.4). The greatest number and relative quantity of GC-ETD-active alkanes were detected in late-stage larvae volatile collections, suggesting their importance in host detection. Volatile collections from early-stage larvae compared to late-stage larvae indicate that wax may contribute to some background odors. The overall greater number of alkanes detected in late-stage larvae suggest that these are likely originating from larvae rather than the wax comb (Table 3.6.7).

Comparison of proportional abundance to proportional electrophysiological responses relative to a nonyl acetate internal standard revealed potential volatiles important to *V. destructor* in host detection (Fig. 3.6.3, Fig. 3.6.4). Among the top ten greatest relative responses from each volatile collection, two components (identified as 6,9-dimethyltridecane; Kovats 1490, and "unknown13"; Kovats 1095) elicited proportional responses approximately 14- and 3-fold greater than responses to other odorants, respectively.

We identified several dimethyl- and trimethyl-alkanes that may be important in host detection (Fig. 3.6.3, Fig. 3.6.4). Among these, odorants from volatile collections containing mid- to late-stage larvae (WkrL4/5 50 and DrnL4/5 70) elicited the strongest relative electrophysiological responses compared to other volatile collections ($Z = 6.74$, $df = 1$, $p < 0.0001$). Higher relative proportional responses were also detected in late-stage larvae volatile collections (Fig. 3.6.3). No difference in *V. destructor* relative proportional response was detected when comparing functional groups of compounds identified among the different volatile collections ($F = 1.2$, $df = 5$, $p = 0.35$). Several putative plant secondary metabolites (e.g. α-cumyl alcohol, citronellal, limonene, m-cymene, menthol, methyl benzoate, and methyl salicylate) also elicited higher relative

electrophysiological responses, primarily originating from early-stage larvae and dead pupae volatile collections.

3.5 Discussion

For the first time, we were able to screen a range of honey bee colony volatile components to live *V. destructor* through gas chromatography linked electro-tarsal detection (GC-ETD). Methods for electrophysiological recordings were modified from previous research to maximize longevity of preparations (Endris and Baker, 1993). Our technique allowed *V. destructor* preparations to respond to volatile compounds reliably for the duration of the GC-ETD temperature programmed run, with prepared *V. destructor* demonstrating mobility following discarding from recording. Concurrent work exploring concentration-dependent responses in *V. destructor* to selected odorants suggested interaction of mechanoreceptors to individual puffs of stimuli using the same method (Chapter 2). Here we show that performing gas chromatography electro-tarsal detection under a constant humidified air stream can be a reliable method of screening honey bee colony volatiles. GC-ETD recordings contained suspected mechanical responses, typically following responses to odorants; performing repeated GC-ETD recordings allowed confirmation of electrophysiologically-active odorants.

In contrast to previous research on honey bee volatile collections, we identified fewer suspected plant secondary metabolite compounds (Carroll and Duehl, 2012). By isolating volatile collections to the wax comb, this study eliminated background volatiles which may be associated with propolis and wax (Carroll and Duehl, 2012; Popova et al., 2014). In addition, we used food-grade plastic frames in our volatile collections and suggest that

these may eliminate background odors associated with older wood frames and wax foundations (Carroll and Duehl, 2012; Torto et al., 2013). Torto et al. (2013) suggest using honey bee equipment < 2 years old to avoid saturation of volatile collections with frame background odors associated with consecutive brood rearing and propolis buildup.

Honey bee larval volatiles of high molecular weight previously identified as important in *V. destructor* host detection were infrequently detected in our volatile collections, although some evoked *V. destructor* electrophysiological responses (Le Conte et al., 1990). Although potential compounds related to infested and dead pupae (e.g. oleic acid, (*E*)-ß-ocimene) have infrequently been explored, it is possible that brood esters are important in eliciting honey bee hygienic behavior (cell uncapping and removal of infected or dead pupae; Martin et al. 2002; Frey et al. 2013; McAfee et al. 2017).

Volatiles specific to late-stage larvae (DrnL4/5 70 and WkrL4/5 50) evoked greater electrophysiological responses relative to nonyl acetate internal standard from *V. destructor*. This suggests that in addition to brood methyl- and ethyl-esters, other compounds (like methyl-alkanes identified in this research) may be important for host or conspecific detection (Martin et al., 2002; Fig. 3.6.3). Late-stage drone larvae contained a number of electrophysiologically active, heavy molecular weight compounds in trace quantities. Several of these trace compounds were not identifiable or were identified with low confidence.

Volatile collections from early-stage drone larvae contained the most ETD-active compounds. Compounds unique to this stage were frequently plant secondary metabolites, potentially originating from larval food and/or royal jelly (e.g. menthol, limonene; Drijfhout et al. 2005; Nazzi et al. 2006). (*E*)-ß-ocimene was detected in mid- to

late-stage larvae volatile collections only and was found in low proportional abundance, consistent with previous research on brood volatiles (Carroll and Duehl, 2012). For the first time, we identified electrophysiological responses from *V. destructor* to (*E*)-ß-ocimene. Further examination of *V. destructor* arrestment responses towards (*E*)-ß-ocimene indicated increasing time spent arrested to increasing logarithmic concentrations (10^1 to 10^3 ng per 10 µL of ethanol) using an arrestment behavioral assay design (Light et al. in prep.). Together, these findings suggest importance of (*E*)-ß-ocimene to *V. destructor* in host detection.

Among suspected plant secondary metabolites I detected (Table 3.6.4), geranyl acetone and menthol (which activate *V. destructor* noxious stimulus receptor TRPA1, suggesting potential repellent or aversive compounds) were present in volatile collections from early- and mid-stage development drone larvae (Peng et al., 2015). Butylated hydroxytoluene, a suspected contaminant, also elicited ETD responses from *V. destructor*. Previous research in vertebrates identified toluene as a noxious stimulus (Nilius et al., 2012). It is possible that butylated hydroxytoluene has properties similar to toluene. It is unknown if either of these compounds are repellent to *V. destructor*. Furthermore, 2,6-di-tert-butylbenzoquinone, found exclusively in volatile collections from honey bee brood killed with cold treatment, may possess insecticidal properties or similarly involve the TRPA1 channel (Miller et al., 2007; Nilius et al., 2012). Methyl salicylate was identified in several volatile collections as ETD-active, but it does not activate the TRPA1 receptor (Peng et al., 2015). A dosage of 5 mg of methyl salicylate per a cage of honey bees infested with *V. destructor* resulted in an average mortality of 22% for honey bees and 100% mortality for *V. destructor* (Lindberg et al., 2000;

Rosenkranz et al., 2010). Single sensillum recordings within *V. destructor* tarsal pit organ also found methyl salicylate activated olfactory sensilla (Dillier et al., 2003). Methyl salicylate may be behaviorally relevant to *V. destructor* because it is an component of aggregation pheromone in other species of ticks and predatory mites (de Bruyne et al., 1991; Carr and Roe, 2016). In the context of the honey bee colony, methyl salicylate was not identified previously from volatile collections, but is a common floral component (Dillier et al., 2003; Clavijo Mccormick et al., 2014). Behavioral responses of *V. destructor* to methyl salicylate at concentrations relevant to the colony environment are still unclear.

Of those we evaluated, I identified the top ten compounds eliciting strong relative electrophysiological responses compared to relative abundance (Fig. 3.6.3, Fig. 3.6.4). Alkanes with di- and tri-methyl groups, in the range of C-10 to C-15 carbon atoms, elicited strong relative responses. These alkanes were more commonly detected from volatile collections of capped brood stages. Several of these branched alkanes may be important in *V. destructor* host or conspecific detection (Martin et al., 2002).

Compounds eliciting the strongest relative responses from early stage honey bee drone larvae and eggs were putatively identified as (*E* or *Z*)-2-nonenal and menthol. Early stage larvae between 1 and 3 d old are typically provisioned royal jelly. Volatile collection from this brood stage contained plant secondary metabolites in higher abundance relative some alkanes. These compounds may be important in eliciting *V. destructor* repellence towards royal jelly, although they were not detected in previous research (Drijfhout et al., 2005; Nazzi et al., 2009). Moreover, volatile collections from pupae at the pink eye stage and chilled brood potentially offer additional insight into possible volatiles involved in

interrupting *V. destructor* reproductive cycles (Frey et al., 2013). Volatile collections from larvae of mid- to late-stage development (DrnL4/5 70 and WkrL4/5 50) stimulated a greater number of high proportional electrophysiological responses relative to volatiles collected from capped pupae stages. Among these, several unknown compounds (Fig. 3.6.3, Fig. 3.6.4, Appendix Table B1) of moderate to heavy molecular weight appear to be important to *V. destructor* based on electrophysiological responses. Kovats retention indices of these compounds ranged from 1390 to 2029 and did not match retention times of previously identified brood or *V. destructor* pheromone components (Le Conte et al., 1989; Pankiw and Page, 2001; Ziegelmann et al., 2013). Further investigation in these unknown volatiles may lead to new discoveries in integrated management of *V. destructor*.

Among previously identified fatty acid and respective methyl and ethyl esters from cuticle extractions of late-stage worker and drone larvae, we detected palmitic and stearic acid consistently in volatile collections from uncapped brood of various ages. Identification of these volatiles was possible only by using chemical standards, due to the relatively low abundance of these brood pheromone components. Identification of stearic acid and attendant ETD responses suggests the possible influence of this fatty acid outside the context of *V. destructor* reproduction (Ziegelmann et al. 2013; Chapter 4).

The absence of several putative attractive brood pheromone components (e.g. methyl linoleate, methyl linolenate, ethyl palmitate) from volatile collections of late-stage brood brings into question the importance of these components compared to methyl-alkanes identified to have high relative responses (Le Conte et al., 1989; Trouiller et al., 1992). Development of a generic screening method and exploration of relative

electrophysiological responses to volatile components has provided a baseline for further research to investigate behavioral relevance of these honey bee brood volatiles. Compounds eliciting a strong relative electrophysiological response from *V. destructor* can further narrow research focus towards new, potentially behaviorally relevant, semiochemicals. Broad scale screening techniques used here could be similarly applied to other acarine pests. This may also lead to better understanding whether particular sensitivities to compounds (e.g. methyl salicylate) are conserved across species.

3.6 Figures and Tables

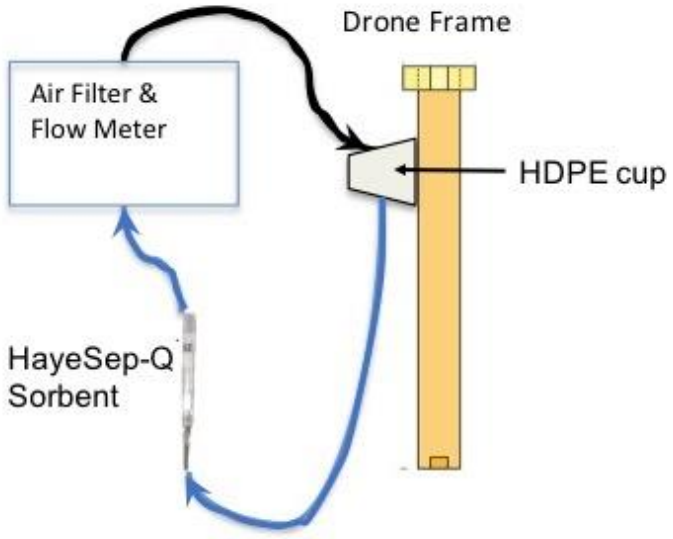

Fig. 3.6.1 Example of a closed system volatile collection on a drone frame with a HayeSep-Q volatile collection trap and high-density polyethylene cup (HDPE cup); arrows indicate direction of flow; air flow was regulated by Portable Volatile Assay System (PVAS22; Rensselaer, NY, USA).

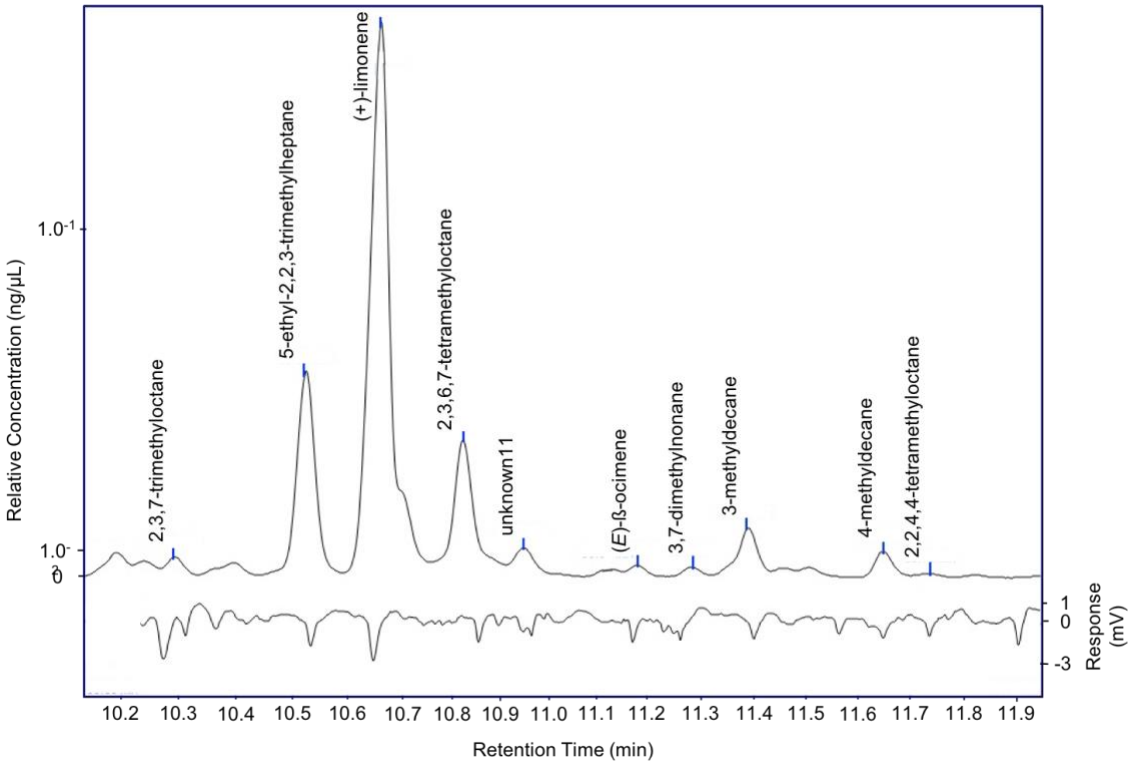

Fig. 3.6.2 Example of responses from *Varroa destructor* adult female to volatile compounds from worker larvae of the fourth to fifth instar stage of development. Compounds (+)-limonene (3.1 mV response) and (*E*)-ß-ocimene (1.1 mV response) were identified through chemical standards. Bottom, tarsal recording signal (electro-tarsal detection); top, gas chromatography flame ionization detection trace on CIP SIL8-CB column. Relative concentration is represented by proportional peak area relative to known amount (3 ng μL^{-1}) of internal standard, nonyl acetate.

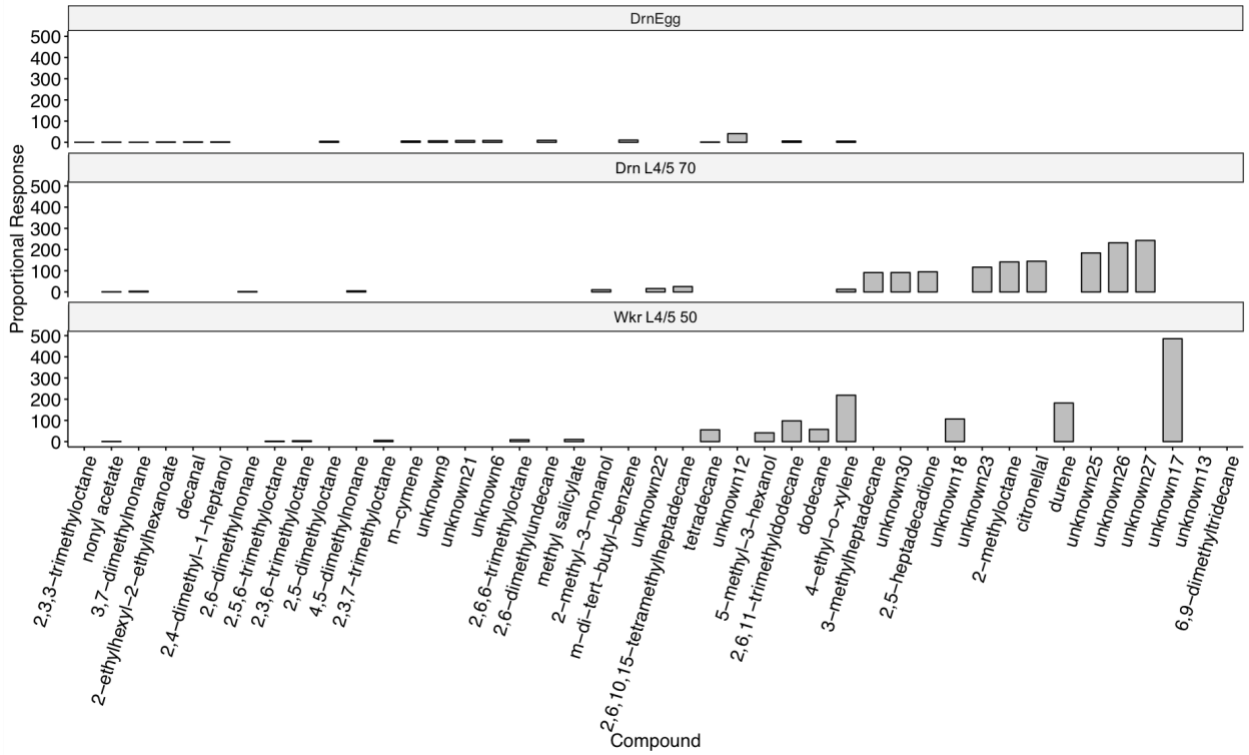

Fig. 3.6.3 *Varroa destructor* relative electrophysiological response (mV) compared to relative compound abundance (peak area) identified as proportions based on nonyl acetate internal standard across three honey bee larvae volatile collections; see Table 3.6.1 for abbreviations. Compounds are arranged according to ascending Kovats retention index, with missing data indicating compounds not detected by *V. destructor* through electrotarsography. Responses to volatiles 6,9-dimethyltridecane and unknown13 were removed to improve visualization because relative responses to these compounds were respectively 14- and 3-fold greater than most other responses.

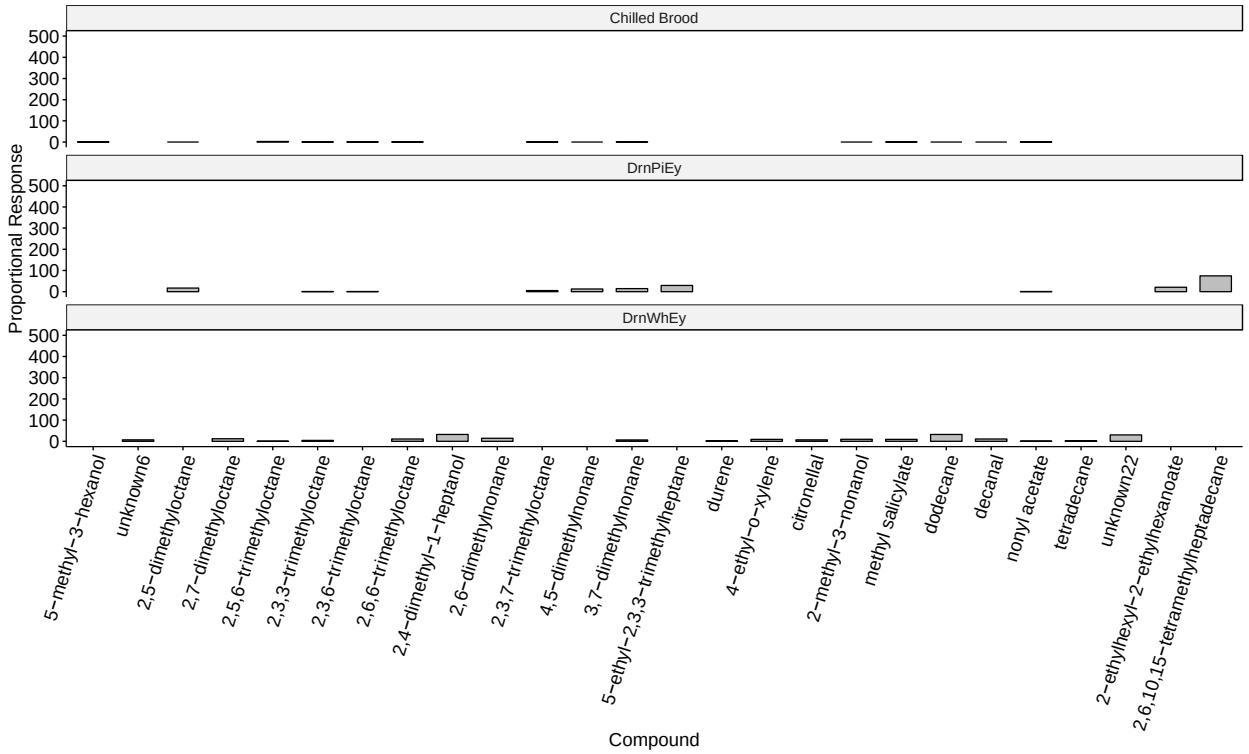

Fig. 3.6.4 *Varroa destructor* relative electrophysiological response (mV) compared to relative compound abundance (peak area) identified as proportions based on nonyl acetate internal standard across three honey bee pupae volatile collections; see Table 3.6.1 for abbreviations. Compounds are arranged according to ascending Kovats retention index, with missing data indicating compounds not detected by *V. destructor* through electrotarsography.

Table 3.6.1 Stages of honey bee (*Apis mellifera*) brood development in which volatiles were collected and analyzed for electrophysiological response from *Varroa destructor*.

volatile collection abbreviation	**brood stage volatiles collected**
DrnEgg	drone eggs and 1st instar larvae
WkrL4/5 50	worker larvae between 4th and 5th instars, 50% cell occupancy
DrnL4/5 70	drone larvae between 4th and 5th instars, 70% cell occupancy
DrnWhEy	capped drone pupae white eye stage
DrnPiEy	capped drone pupae pink eye stage
Chilled Brood	capped drone pupae pink eye stage killed through cold treatment (7 h at 11°C)

Percent occupancy was estimated based on number of larvae within areas in which volatiles were collected. Stages of pupal development were identified by uncapping 10 brood cells in areas of volatile collection and referring to literature (Frey et al., 2013; McAfee et al., 2017). Each volatile collection was composed of two replicates collected simultaneously containing brood of the same age from the same frame; replicates were combined and concentrated under ultra-high purity nitrogen for GC-ETD and GC-MS analysis.

Table 3.6.2 Alcohols, aldehydes, esters, and ketones that elicited *Varroa destructor* electrophysiological responses through gas chromatography linked electrotarsal detection of honey bee brood volatiles ranging in developmental stage.

compound	retention (min)	Kovats	concentration (ng/µL)	se (±)	n	confid
4-hexen-3-one	4.25	825	0.011	0.032	2	med
5-methyl-3-hexanol	4.38	832	0.051	0.024	2	med
2-methyl-3-penten-1-ol (*E* or *Z*)	4.48	836	0.001		1	med
2,5-dimethyl-5-hexen-3-ol	6.24	920	1.551		1	low
2,4-dimethyl-1-heptanol	8.37	1003	0.017	0.006	2	low
2-nonenal (*E* or *Z*)	11.97	1130	0.001	0.012	2	med
3-decyn-2-ol	12.40	1146	NA	<0.001	2	low
2-methyl-3-nonanol	12.56	1151	0.907	0.443	3	low
4,6-dimethyloctanoate	12.87	1162	0.010		1	med
5-ethyl-4-nonanone	14.84	1232	1.187	0.572	2	low
2-ethyl-2-propyl-1-hexanol	15.44	1254	0.004		1	low
ethyl 4-methyloctanoate	15.76	1266	0.003		1	low
2-undecanone	16.63	1297	0.005	0.001	2	med
nonyl acetate	16.91	1308	IS		1	high
2-ethylhexyl-2-ethylhexanoate	24.09	1596	0.009		1	low
methyl palmitate	31.04	1930	NA		1	high
palmitic acid	31.68	1971	0.003		1	high
2,5-heptadecadione	32.50	2041	NA		1	low
2-(octadecyloxy)-ethanol	32.56	2049	NA		1	low
methyl oleate	33.02	2100	NA		1	high
stearic acid	33.43	2161	0.003		1	high

Compounds are listed according to gas chromatographic retention time GC-MS (Rxi-5MS). NA indicates peak did not meet requirement for integration. Concentration was calculated using proportional peak area relative to internal standard and calculated to sample volume before concentration under ultra-high purity nitrogen. Compounds were detected across honey bee brood volatile collections of varying developmental stages; confid = confidence in identification NIST reverse match < 700 and multiple Kovats

matches (low), NIST reverse match 700 – 900 and single Kovats match (med), confirmation using chemical standards and Kovats match (high); se = standard error for volatiles detected in more than one volatile collection; n = number of volatile collections where particular volatile was detected and *V. destructor* elicited electrophysiological response to. Response was determined by consistent depolarization across three or more *V. destructor* electrophysiological preparations.

Table 3.6.3 Alkanes, cycloalkanes, and alkenes that elicited *Varroa destructor* electrophysiological responses through gas chromatography linked electrotarsal detection of honey bee brood volatiles ranging in developmental stage.

compound	retention (min)	Kovats	concentration (ng/µL)	se (±)	n	confid
4-methyloctane	4.91	859	2.043		1	med
3-methyloctane	4.95	860	NA		1	med
2-methyloctane	5.20	873	NA		1	med
2-methyl-1-octene	5.34	881	2.625		1	med
2,5,5-trimethylheptane	5.66	897	0.001		1	low
nonane	5.70	899	0.765		1	high
2-nonene (E or Z)	5.99	910	0.002		1	med
2,3,6-trimethylheptane	6.07	914	0.137	0.025	5	low
3,5-dimethyloctane	6.20	919	NA		1	low
2,5-dimethyloctane	6.29	922	0.219	0.101	3	low
2,3,6-trimethylheptane	6.34	924	0.011	0.025	5	low
3,5-dimethyloctane	6.53	932	0.072		1	low
2,7-dimethyloctane	6.57	933	0.004		1	low
2,6-dimethyloctane	6.66	937	0.022	0.018	5	low
3-ethyl-4-methylheptane	6.83	943	0.001		1	low
2,3-dimethyloctane	7.04	952	0.011	0.001	4	med
2,4,6-trimethyloctane	7.20	958	0.085		1	low
2-methylnonane	7.37	964	NA		1	med
2,2,6-trimethyloctane	7.54	971	0.045	0.003	3	low
2,2,3-trimethyloctane	7.61	974	0.052	0.013	6	low
2,5,6-trimethyloctane	7.80	981	0.051	0.021	3	low
2,4,6-trimethyloctane	7.85	983	NA		1	low
2,3,3-trimethyloctane	8.02	990	0.087	0.013	6	low
2,3,6-trimethyloctane	8.08	992	0.040	0.004	3	low
2,6-dimethylnonane	8.15	995	NA	0.001	2	med
decane	8.24	999	0.065		1	high
2,3,6,7-tetramethyloctane	8.41	1005	NA	<0.001	2	low
2,2,4,6,6-pentamethylheptane	8.55	1009	0.013	0.001	2	med

Table 3.6.3 Continued

compound	retention (min)	Kovats	concentration (ng/µL)	n	confid
2,2,6-trimethyloctane	8.60	1011	0.006	1	low
2,3,7-trimethyloctane	8.70	1015	0.056	1	low
5-ethyl-2-methyloctane	8.79	1018	0.080	1	low
5-ethyl-2,2,3-trimethylheptane	8.89	1022	0.062	1	low
2,3,6,7-tetramethyloctane	9.20	1032	0.046	1	low
4,5-dimethylnonane	9.43	1041	0.194	1	low
3,7-dimethylnonane	9.60	1047	0.011	1	low
3-methyldecane	9.73	1051	0.047	1	med
5-ethyl-2,3,3-trimethylheptane	9.80	1054	NA	1	low
5-methyldecane	9.87	1056	0.001	1	low
4-methyldecane	10.04	1062	0.013	1	low
2,2,4,4-tetramethyloctane	10.13	1066	NA	1	low
3-methyldecane	10.37	1074	0.990	1	low
3,4-dimethyldecane	10.97	1095	NA	1	med
4-methylundecane	12.92	1164	NA	1	med
3-methyl-5-undecene (*E* or *Z*)	13.11	1170	1.404	1	med
(1,2-dimethylpropyl)-cyclohexane	14.03	1203	0.001	1	low
2,6-dimethylundecane	14.32	1213	0.005	1	low
2,3-dimethylundecane	15.57	1259	NA	1	low
2,6,11-trimethyldodecane	16.02	1275	0.009	1	low
2,6,11-trimethyldodecane	17.23	1320	NA	1	low
2,6,10-trimethyldodecane	17.27	1322	NA	1	low
farnesane	18.00	1349	0.007	1	low
tetradecane	19.33	1400	0.017	1	high
6,9-dimethyltridecane	21.58	1490	NA	1	low
heptadecane	26.41	1699	0.748	1	high
5-methylheptadecane	27.35	1743	0.001	1	low
3-methylheptadecane	27.55	1753	NA	1	low
2,6,10,15-tetramethylheptadecane	30.77	1912	0.001	1	low

Compounds are listed according to gas chromatographic retention time GC-MS (Rxi-5MS). NA indicates peak did not meet requirement for integration. Concentration was calculated using proportional peak area relative to internal standard and calculated to

sample volume before concentration under ultra-high purity nitrogen. Compounds were detected across honey bee brood volatile collections of varying developmental stages; confid = confidence in identification NIST reverse match < 700 and multiple Kovats matches (low), NIST reverse match 700 – 900 and single Kovats match (med), confirmation using chemical standards and Kovats match (high); se = standard error for volatiles detected in more than one volatile collection; n = number of volatile collections where particular volatile was detected and *V. destructor* elicited electrophysiological response to. Response was determined by consistent depolarization across three or more *V. destructor* electrophysiological preparations.

Table 3.6.4 Terpenes and suspected plant secondary metabolites that elicited *Varroa destructor* electrophysiological responses through gas chromatography linked electrotarsal detection of honey bee brood volatiles ranging in developmental stage.

compound	retention (min)	Kovats	concentration (ng/µL)	se (±)	n	confid
α-pinene	6.55	932	NA		1	med
(+)-limonene	9.08	1028	0.080	0.324	3	high
ϱ-sylvesterene	9.12	1030	2.064		1	low
β-ocimene	9.54	1045	0.003		1	high
m-cymene	10.45	1077	0.008		1	low
α-cumyl alcohol	10.61	1083	0.002		1	med
4-thujanol	10.66	1084	0.001	<0.001	2	med
citronellal	11.99	1131	0.002		1	low
menthol	13.28	1177	0.029	0.009	3	med
methyl salicylate	13.67	1190	0.080		1	high
pinanediol	15.85	1269	NA		1	low
longifolene	19.60	1411	NA		1	low
α-cedrene	19.75	1417	NA		1	med
geranyl acetone	20.46	1445	NA		1	high
lapachol	32.53	2045	NA		1	low

Compounds are listed according to gas chromatographic retention time GC-MS (Rxi-5MS). NA indicates peak did not meet requirement for integration. Concentration was calculated using proportional peak area relative to internal standard and calculated to sample volume before concentration under ultra-high purity nitrogen. Compounds were detected across honey bee brood volatile collections of varying developmental stages; confid = confidence in identification NIST reverse match < 700 and multiple Kovats matches (low), NIST reverse match 700 – 900 and single Kovats match (med), confirmation using chemical standards and Kovats match (high); se = standard error for volatiles detected in more than one volatile collection; n = number of volatile collections where particular volatile was detected and *V. destructor* elicited electrophysiological response to. Response was determined by consistent depolarization across three or more *V. destructor* electrophysiological preparations.

Table 3.6.5 Aromatic compounds that elicited *Varroa destructor* electrophysiological responses through gas chromatography linked electrotarsal detection of honey bee brood volatiles ranging in developmental stage.

compound	retention (min)	Kovats	concentration (ng/µL)	se (±)	n	confid
ethylbenzene	4.87	856	NA		1	med
tetrahydro-2-furanmethanol	5.95	909	0.016		1	med
acetophenone	10.06	1063	0.140	0.012	2	med
durene	11.50	1114	0.013	<0.001	2	med
4-ethyl-o-xylene	11.61	1118	0.001	0.001	4	low
napthalene	13.46	1183	0.004	0.084	4	med
m-di-tert-butyl-benzene	15.26	1247	0.183		1	low

Compounds are listed according to gas chromatographic retention time GC-MS (Rxi-5MS). NA indicates peak did not meet requirement for integration. Concentration was calculated using proportional peak area relative to internal standard and calculated to sample volume before concentration under ultra-high purity nitrogen. Compounds were detected across honey bee brood volatile collections of varying developmental stages; confid = confidence in identification NIST reverse match < 700 and multiple Kovats matches (low), NIST reverse match 700 – 900 and single Kovats match (med), confirmation using chemical standards and Kovats match (high); se = standard error for volatiles detected in more than one volatile collection; n = number of volatile collections where particular volatile was detected and *V. destructor* elicited electrophysiological response to. Response was determined by consistent depolarization across

Table 3.6.6 Background contaminants detected among volatile collections and blank collections at same relative concentrations to amount of internal standard (3 ng/µL) that elicited *Varroa destructor* electrophysiological responses through gas chromatography linked electrotarsal detection of honey bee brood volatiles ranging in developmental stage.

compound	**retention**	**Kovats**	**concentration (ng/µL)**	**se** (±)	**n**	**confid**
p-xylene	5.05	866	0.003		1	med
o-xylene	5.12	869	NA		1	med
styrene	5.49	888	0.705		1	med
2-ethyl-1-hexanol	9.01	1026	0.023		1	low
methyl benzoate	10.87	1092	0.188	0.091	5	high
undecane	11.09	1100	0.004		1	high
nonanal	11.18	1103	0.064	0.030	2	high
dodecane	13.95	1200	0.358	0.173	3	high
decanal	14.07	1204	0.081	0.030	3	high
benzothiazole	14.57	1222	0.001		1	med
tridecane	16.71	1300	0.035	0.020	2	high
1-cyclohexyloctane	20.54	1449	NA		1	low
2,6-di-tert-butylbenzoquinone	20.81	1459	NA		1	low
butylated hydroxytoluene	21.85	1501	0.062	0.004	3	med
2,4-bis(1,1-dimethylethyl)-phenol	21.92	1504	0.074	0.030	4	med
methyl 2-methylhexyl phthalate	33.17	2123	0.144		1	low

Compounds are listed according to gas chromatographic retention time GC-MS (Rxi-5MS). NA indicates peak did not meet requirement for integration. Concentration was calculated using proportional peak area relative to internal standard and calculated to sample volume before concentration under ultra-high purity nitrogen. confid = confidence in identification NIST reverse match < 700 and multiple Kovats matches (low), NIST reverse match 700 – 900 and single Kovats match (med), confirmation using chemical standards and Kovats match (high); se = standard error for volatiles detected in more than one volatile collection; n = number of volatile collections where particular volatile was detected to elicit a electrophysiological response from *V. destructor*. Response was determined by consistent depolarization across three or more *V. destructor* electrophysiological preparations.

Table 3.6.7 Ranked five most produced honey bee brood volatiles that elicited responses from *Varroa destructor* at different developmental stages (described in Table 3.6.1) in ascending order (A to E) for each volatile collection; each run corresponds to one volatile collection analyzed.

run and rank	compound	Kovats	prop area	prop ratio (amp/area)
DrnEgg				
A	4-methyloctane	859	68.1	0.02
B	5-ethyl-4-nonanone	1232	78.8	0.02
C	2-methyl-1-octene	881	87.5	0.02
D	2-methyl-3-nonanol	1151	90.6	0.01
E	unknown14	1127	376.0	0.00
WkrL4/5 50				
A	unknown11	1035	0.6	8.35
B	unknown10	963	0.9	3.07
C	2,3,6-trimethyloctane	992	1.2	4.04
D	2,2,6-trimethyloctane	971	1.7	2.53
E	2,5,6-trimethyloctane	981	1.7	2.36
DrnL4/5 70				
A	2,3,6-trimethylheptane	924	0.2	3.58
B	2,6-dimethylnonane	1014	0.3	2.01
C	2,3-dimethyloctane	952	0.3	1.52
D	4-methyldecane	1062	0.5	0.97
E	3,7-dimethylnonane	1051	0.8	0.63
DrnWhEy				
A	2,2,6-trimethyloctane	971	1.7	1.23
B	2,3,3-trimethyloctane	990	1.7	4.52
C	butylatedhydroxytoluene	1500	1.9	2.41
D	5-ethyl-2,2,3-trimethylheptane	1020	2.3	0.59
E	(+)-limonene	1030	5.1	0.53
DrnPiEy				
A	2,3,3-trimethyloctane	990	0.5	1.00
B	2,4-bis(1,1-dimethylethyl)-phenol	1504	0.7	1.66
C	2,3,6-trimethyloctane	992	1.0	0.86
D	2,2,6-trimethyloctane	971	1.1	1.08
E	2,3,6,7-tetramethyloctane	1032	1.5	0.85

Table 3.6.7 Continued.

Chilled Brood				
A	ethylbenzene	856	53.8	0.02
B	4-methyloctane	859	68.1	0.02
C	5-ethyl-4-nonanone	1232	78.8	0.02
D	2-methyl-1-octene	881	87.5	0.02
E	2-methyl-3-nonanol	1151	90.6	0.01

Kovats = retention index determined from hydrocarbon standards; prop area = proportional peak area relative to internal standard nonyl acetate; prop ratio = proportional amplitude over proportional peak area relative to internal standard nonyl acetate.

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CHAPTER 4.0 CONCLUSIONS

4.1 Summary

Varroa destructor continues to be a virulent parasite of honey bees, and its recent arrival in New Zealand makes this parasite a nearly global challenge for apiculture (Iwasaki et al., 2015). Current treatments are variable in effectiveness and often negatively affect honey bee health, so that an integrated approach may be required (Mondet et al., 2011; Bonnafé et al., 2017; Ferland et al., 2017; Dai et al., 2018). Despite this, alternatives to current management approaches are still in early development. Previous behavioral and electrophysiological studies indicate odor detection is important in *V. destructor*'s lifecycle. Findings presented in this thesis and previous studies underline potential to develop new techniques for *V. destructor* management that rely on olfaction (Nazzi and Le Conte, 2016; Plettner et al., 2017). Furthermore, I offer new directions for future research on honey bee colony odorants putatively important in *V. destructor* host detection where I used approaches adapted from GC-ETG.

Although concentration-dependent responses towards putative chemical disruptants can interrupt host detection (Eliash et al., 2014; Singh et al., 2015), electrophysiological results in this thesis indicated a lack of concentration-dependent responses towards putative attractants and repellents of *V. destructor*. A lack of significance among concentrations for select odorants suggests that concentration responses may be present at ranges greater than those explored in this research. Putative plant secondary metabolites elicited significantly weaker electrophysiological responses from *V. destructor* in contrast to solvent controls. Dampening of electrophysiological responses from these compounds, some of which are putative *V. destructor* repellents, suggests possible semiochemical

disruption. Additional research is needed in identifying mode of action of putative repellent plant secondary metabolites and their potential as semiochemical disruptants.

Varroa destructor electrophysiological responses to stearic acid from drone and worker larvae suggest that this odorant may be important in host detection, in addition to being a component of *V. destructor* mating pheromone. Screening honey bee colony odors identified a range of compounds predominantly in late-stage larvae which elicited high proportional electrophysiological responses relative to proportional abundance. Identification of some of these honey bee odors points to additional semiochemical cues not previously explored in Varroa research that may be important in *V. destructor* reproduction and host detection. Volatile collections from pupae killed through cold treatment revealed several dimethyl- and trimethyl-alkanes that may be important in detecting host status or presence of brood infested with *V. destructor* mites (Martin et al., 2002), but not oleic acid or (*E*)-ß-ocimene, previously cited volatiles indicative of pupae death (McAfee et al., 2017). Trace amounts of palmitic and stearic acids, methyl oleate, and methyl palmitate were detected through chemical standards. Concentrations of some of these putatively important brood odors were below integration parameters for peak detection, and as a result, *V. destructor* preparations inconsistently elicited responses to these odors among volatile collections likely due to their weak relative concentrations when compared to 3 ng/µL nonyl acetate internal standard. The occurrence of honey bee brood pheromone components following pupation suggests possible importance in later developmental stages of the *V. destructor* reproductive cycle (Frey et al., 2013).

4.2 Future directions

This thesis further uncovered the relative importance of several branched alkanes, terpenes (notably (*E*)-ß-ocimene), and unidentifiable compounds within volatile collections from honey bee larvae that may be important in host detection by *V. destructor*. Given the high electrophysiological responses they generated compared to relative abundance, future research should focus on identification of these unknown odors and examine behavioral valence towards *V. destructor*.

Electrophysiology provides a method for screening plant secondary metabolites as potential repellents or semiochemical disruptants (Miller et al., 2007). Results identified in this thesis may suggest a reduction of responses from *V. destructor* when exposed to some of the tested plant secondary metabolites (e.g α-terpineol, linalool). Putative plant secondary metabolite volatiles that elicit a dampening of electrophysiological responses from *V. destructor* could be investigated for similar activity in other acarine pests, given cross-species behavioral and electrophysiological responses shown elsewhere (Bissinger and Roe, 2010; Peng et al., 2015).

Honey bees often respond to dominant odorants within complex floral bouquets (Masterman et al., 2000; Reinhard et al., 2010). This in particular has promise for developing management techniques involving semiochemicals specific to *V. destructor*. Subsequent analysis of honey bee responses to electrophysiologically active honey bee volatiles detected by *V. destructor* in this thesis could further narrow research to those with weak influence on honey bee behavior.

I evaluated effects of outdoor humidity and temperature on electrotarsography results and identified an effect of temperature in influencing *V. destructor* electrotarsogram

responses. Additional behavioral assays, not included in this thesis, focused on identifying *V. destructor* responses to single components and synthetic mixtures under conditions similar to an in-colony environment. Previous research often neglected to report factors such as temperature, humidity, and light exposure; these factors should be accounted for to facilitate comparison among studies.

Broad scale screening of honey bee volatiles towards electrophysiological responses from *V. destructor* identified several potentially important volatiles among honey bee developmental stages. Electrophysiologically active odorants identified here could be further confirmed for possible relevance to *V. destructor* through behavioral assays, and importance to honey bee behavior through *in-situ* testing. Results support new methods for screening volatiles to differentiate putative attractants from repellents. Moreover, methods used in this thesis offer approaches to further examine differences in brood stage development and the relative importance of various colony and conspecific odors to *V. destructor* through electrotarsograms.

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APPENDIX A

Table A1 List of putative attractants associated with honey bee brood pheromone (bp) and *Varroa destructor* pheromone (Vdp), CAS = Chemical Abstracts Service registry number, ns = non-significant responses.

compound	CAS	response	source	citation
methyl linoleate	112-63-0	attractant	bp	Le Conte et al. 1989; Trouiller et al. 1992; Pernal et al. 2005
methyl linolenate	301-00-8	attractant	bp	Calderone and Lin 2001
ethyl linolenate	1191-41-9	ns	bp	Le Conte et al. 1989
ethyl palmitate	628-97-7	attractant	bp Vdp	Le Conte et al. 1989; Trouiller et al. 1992; Ziegelmann et al. 2013
methyl palmitate	112-39-0	attractant	bp Vdp	Le Conte et al. 1989; Rickli et al. 1992; Trouiller et al. 1992; Boot et al. 1994
palmitic acid	57-10-3	attractant	bp Vdp	Rickli et al. 1992; Donzé et al. 1998; Ziegelmann et al. 2013
stearic acid	57-11-4	attractant	Vdp	Ziegelmann et al. 2013
oleic acid	112-80-1	attractant	Vdp	Ziegelmann et al. 2013
ethyl stearate	111-61-5	attractant	Vdp	Ziegelmann et al. 2013
ethyl oleate	111-62-6	attractant	Vdp	Ziegelmann et al. 2013
guanine	73-40-5	attractant	Vdp	Yoder and Sammataro 2003

Table A2 List of compounds from pre-pupare cocoon (ppc), foundress *V. destructor* (fVd), and honey bee foragers (hbf), which elicit arrestment in *Varroa destructor*, CAS = Chemical Abstracts Service registry number.

compound	**CAS**	**source**	**citation**
docosanol	30303-65-2	ppc	Donzé et al. 1998
eicosanol	629-96-9	ppc	Donzé et al. 1998
henicosanal	51227-32-8	ppc	Donzé et al. 1998
henicosanol	15594-90-8	ppc	Donzé et al. 1998
heptadecanol	67762-27-0	ppc	Donzé et al. 1998
hexadecanol	36653-82-4	ppc	Donzé et al. 1998
nonadecanal	17352-32-8	ppc	Donzé et al. 1998
nonadecanol	1454-84-8	ppc	Donzé et al. 1998
nondecane	629-92-5	ppc	Donzé et al. 1998
octadecanal	638-66-4	ppc	Donzé et al. 1998
octadecanol	112-92-5	ppc	Donzé et al. 1998
eicosanal	2400-66-0	ppc, fVd	Donzé et al. 1998; Martin et al. 2002
docosanal	57402-36-5	ppc, hbf	Donzé et al. 1998; Schmitt et al. 2007
eicosane	112-95-8	ppc, hbf	Donzé et al. 1998; Schmitt et al. 2007

Table A3 List of compounds from drone larval food (df), worker mandibular gland (wm), royal jelly (rj), pollen (p), and honey (h), that have been assayed for valence or electrophysiological responses from *Varroa destructor*, CAS = Chemical Abstracts Service registry number, ns = non-significant responses.

compound	CAS	response	source	citation
2-hydroxyhexanoic acid	6064-63-7	attractant	dlf	Nazzi et al. 2004
3-hydroxyhexanoic acid	10191-24-9	attractant	dlf	Nazzi et al. 2004
(Z)-3-hexenoic acid	1775-43-5	ns	dlf	Nazzi et al. 2004
2-ethylhexanoic acid	149-57-5	ns	dlf	Nazzi et al. 2004
2-methylbutanoic acid	116-53-0	ns	dlf	Nazzi et al. 2004
3-methylbutanoic acid	503-74-2	ns	dlf	Nazzi et al. 2004
3-methylpentanoic acid	105-43-1	ns	dlf	Nazzi et al. 2004
benzoic acid	65-85-0	ns	dlf	Nazzi et al. 2004
hexanoic acid	142-62-1	ns	dlf	Nazzi et al. 2004
nonanoic acid	112-05-0	ns	dlf	Nazzi et al. 2009
phenylacetic acid	103-82-2	ns	dlf	Nazzi et al. 2004
heptanoic acid	111-14-8	repellent	dlf, wm	Nazzi et al. 2009
octanoic acid	124-07-2	repellent	dlf, wm	Nazzi et al. 2004a, 2009; Carroll and Duehl 2012
methylsalicylate	119-36-8	response	rj	Endris and Baker 1993; Dillier et al. 2003
salicylaldehyde	90-02-8	response	rj	Endris and Baker 1993; Dillier et al. 2003
benzaldehyde	100-52-7	response	rh, p, h	Dillier et al. 2003; Torto et al. 2007

Table A4 List of honey bee alarm pheromones from Koschevnikov gland (k) and Nasonov gland (n) previously assayed for valence or electrophysiological responses from *Varroa destructor*, CAS = Chemical Abstracts Service registry number, ns = non-significant responses.

compound	CAS	response	source	citation
1-hexanol	111-27-3	ns	k	Kraus 1990
hexyl acetate	142-92-7	ns	k	Kraus 1990
isopentyl acetate	123-92-2	ns	k	Kraus 1990
1-octanol	111-87-5	repellent	k	Kraus 1990
2-heptanol	543-49-7	repellent	k	Kraus 1990
2-methyl butanol	75-85-4	repellent	k	Kraus 1990
2-nonanol	628-99-9	repellent	k	Kraus 1990
2-nonyl acetate	143-13-5	repellent	k	Kraus 1990
butyl acetate	123-86-4	repellent	k	Kraus 1990
octyl-acetate	112-14-1	response	k	Endris and Baker 1993
geranic acid	459-80-3	ns	n	Pernal et al. 2005
nerol	106-25-2	ns	n	Pernal et al. 2005; Torto et al. 2005
geraniol	106-24-1	repellent	n	Hoppe and Ritter 1988; Endris and Baker 1993; Pernal et al. 2005; Torto et al. 2005; Schmitt et al. 2007
nerolic acid	4613-38-1	repellent	n	Pernal et al. 2005

Table A5 Honey bee colony odorants evoking behavioral responses in *Varroa destructor* when tested within colonies, CAS = Chemical Abstracts Service registry number.

compound	**CAS**	**response**	**source**	**citation**
(Z)-8-heptadecene	16369-12-3	repellent, reduced reproduction	capped brood honey bees	Nazzi et al. 2002, 2004b; Milani et al. 2004; Pernal et al. 2005; DelPiccolo et al. 2010
capro-lacetone	502-44-3	reduced brood cell invasion	drone larvae	Boot 1994
valero-lacetone	108-29-2			

Table A6 Previously tested cuticle extractions, fractions, and synthetic mixtures eliciting responses in *Varroa destructor*.

source	**response**	**tested compounds**	**citation**
brood pheromone	attractant	fatty acids and their methyl and ethyl esters	Le Conte et al. 1989; Trouiller et al. 1992; Boot 1994; Aumeier et al. 2002
pre-pupae cocoon	arresting	saturated hydrocarbons, alcohols, aldehydes, fatty acids	Donzé et al. 1998
nurse honey bees	attractant	saturated and unsaturated hydrocarbons	Pernal et al. 2005
royal jelly	repellent	fractionations	Drijfhout et al. 2005; Nazzi et al. 2009
brood cuticle	arresting	saturated and unsaturated hydrocarbons	Rickli et al. 1994
larval food	attractant	acidic fractions	Nazzi et al. 2004b, 2006
Nasonov pheromone	repellent	gland secretions	Hoppe and Ritter 1988

APPENDIX B

Table B1 Unknown compounds eliciting high relative electrophysiological responses from *Varroa destructor* compared to proportional peak area relative to nonyl acetate internal standard, shown in Fig. 3.6.3.

unknown number	run	retention (min)	Kovats	amp (mV)	prop amp	prop area	ratio (amp/area)
6	DrnWhEy	5.95	909	6.4	6.29	0.884	7.1
6	DrnEgg	5.96	909	1.3	1.53	0.178	9.0
9	DrnEgg	6.98	948	1.6	1.94	0.275	7.1
12	DrnEgg	10.27	1070	1.9	2.31	0.057	41.0
13	WkrL4/5Cap50	10.96	1095	4.7	3.67	0.002	1561.0
17	WkrL4/5Cap50	17.15	1317	4.4	3.42	0.007	485.4
18	WkrL4/5Cap50	17.52	1331	4.6	3.60	0.034	107.0
21	DrnEgg	19.12	1391	2.0	2.39	0.286	8.4
22	DrnWdL4/5-70	20.75	1457	1.7	0.80	0.050	16.2
22	DrnWhEy	20.75	1457	1.5	1.47	0.049	30.2
23	DrnWdL4/5-70	22.11	1512	2.1	0.99	0.008	117.0
25	DrnWdL4/5-70	24.99	1636	1.9	0.91	0.005	184.1
26	DrnWdL4/5-70	25.46	1657	1.8	0.85	0.004	232.2
27	DrnWdL4/5-70	25.57	1662	1.4	0.67	0.003	243.1
30	DrnWdL4/5-70	32.39	2029	1.8	0.86	0.009	92.0

Unknown number = correlates to number of unknown listed in Fig. 3.6.3; run = volatile collection in which the unknown was detected; retention = retention time using Rxi-5MS column; amp = absolute amplitude response elicited by *V. destructor*; prop amp and prop area = proportional amplitude (mV) and proportional peak area compared to nonyl acetate internal standard respectively; ratio = difference in proportional amplitude over proportional area.