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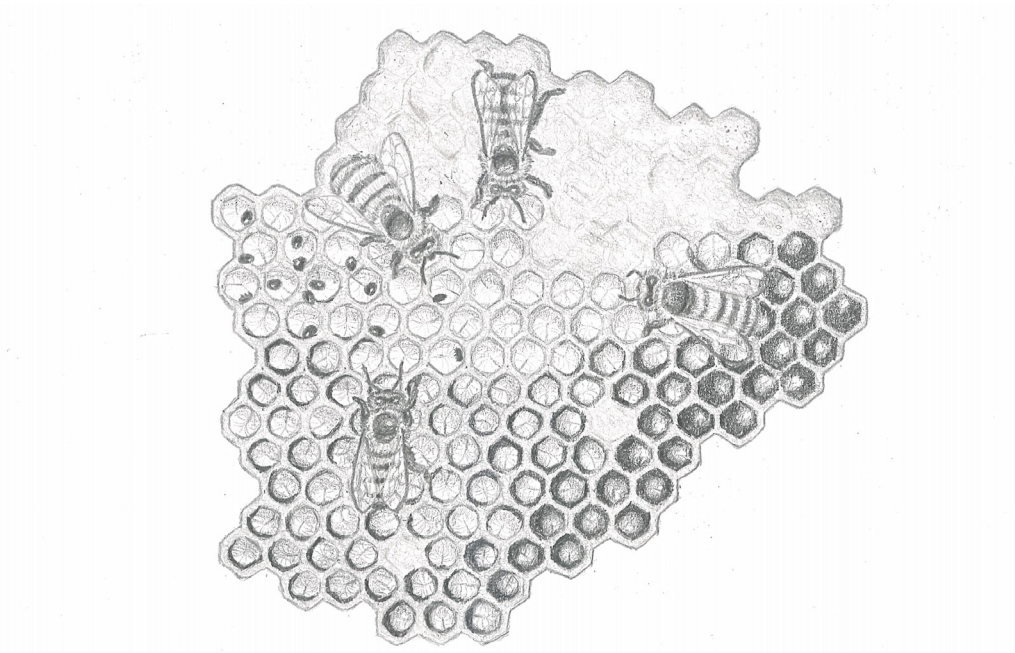
Phenotypic mechanisms of varroa resistant honey bees

From brood signalling to colony-level responses

Nicholas Scaramella



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From brood signalling to colony-level responses

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Cover: *Apis mellifera* adult worker and brood of varying stages with *Varroa destructor. Illustrator: Robyn Övergaard*

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Abstract

The parasitic mite *Varroa destructor* poses a major threat to global honey bee (*Apis mellifera*) populations, undermining colony health through parasitism and virus transmission. While most managed colonies rely on chemical treatments, several naturally surviving populations in Europe have persisted for decades without interventions, offering insights into host–parasite interactions and resistance mechanisms. This thesis investigates three such populations from Sweden, France, and Norway, focusing on suppressed mite reproduction (SMR) and chemical communication.

In Swedish and French populations, reduced mite reproductive success was primarily driven by brood traits rather than adult worker behaviour. Caging experiments confirmed that adult interaction was unnecessary to elicit SMR, underscoring intrinsic brood characteristics. In the Gotland population, resistant larvae exhibited distinct cuticular chemical profiles, notably reduced brood ester pheromones (BEP) at times critical for mite oogenesis. Additionally, the ratio of fatty acid ethyl esters (FAEE) to fatty acid methyl esters (FAME) increased when brood was infested, demonstrating phenotypic plasticity in pheromone expression. Even when treated for varroa, Gotland colonies maintained low mite reproduction, indicating SMR is partly constitutive.

These findings suggest both fixed and plastic resistance traits coexist, offering adaptive flexibility under parasite pressure. This work advances understanding of host–parasite coevolution and highlights brood pheromone modulation as a potential, heritable mechanism of resistance, informing future breeding strategies for mite-resistant bees.

Keywords: *Apis mellifera*; *Varroa destructor*; Brood Ester Pheromone; Chemical Communication; Natural Resistance; Physiology, Phenotypic Plasticity

Dedication

To my daughter Roxane,
As long as you are happy in life, I am complete.

"Rivers know this: there is no hurry.
We shall get there someday."

A.A. Milne, *Winnie-the-Pooh*

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Scaramella N., Burke A., Oddie M., Dahle B., de Miranda, J., Mondet F., Rosenkranze P., Neumann P., Locke B. (2023), Host brood traits, independent of adult behaviours, reduce *Varroa destructor* mite reproduction in resistant honeybee populations. International Journal of Parisitology. International Journal for Parisitology. 53 (10), 565-571. https://doi.org/10.1016/j.ijpara.2023.04.001

II. Scaramella, N., Glinwood, R. & Locke, B. Unique brood ester profile in a *Varroa destructor* resistant population of European honey bee (*Apis mellifera*). Sci Rep 14, 25531 (2024). https://doi.org/10.1038/s41598-024-76399-6

III. Scaramella, N., Noël, A., Glinwood, R., & Locke, B. (2026) Inducible effects of *Varroa destructor* on brood ester profiles of European honey bees (*Apis mellifera*). Manuscript

IV. Scaramella N., Noël A., Onorati P., Milbrath M., Locke B., & de Miranda JR. (2026) The effect of varroa treatment on colony development, productivity and pathogen profile in a 25-year old, naturally varroa-surviving Swedish honey bee population. Manuscript

The contribution of Nicholas Scaramella to the papers included in this thesis was as follows:

I. Data curation, formal analysis, visualization, writing: original draft, review, and editing

II. Investigation, data curation, formal analysis, visualization, writing: original draft, review, and editing

III. Investigation, data curation, formal analysis, visualization, writing: original draft, review, and editing

IV. Investigation, data curation, formal analysis, visualization, validation, writing: review, and editing

Abbreviations

RES	Varroa resistant honey bee population
SUS	Varroa susceptible honey bee population
GC	Gas chromatography
GC-MS	Gas chromatography - mass spectrometry
VSH	Varroa sensitive hygiene
SMR	Supressed mite reproduction
BEP	Brood ester pheromones
FAME	Fatty acid methyl esters
FAEE	Fatty acid ethyl esters
MP	Methyl palmitate
ML	Methyl linoleate
MLN	Methyl linolenate
MS	Methyl stearate
EP	Ethyl palmitate
EL	Ethyl linoleate
ES	Ethyl stearate
EO/BO	(*E*)-β-ocimene
DWV	Deformed wing virus
SBV	Sacbrood virus
LSV	Lake Sinai virus
BQCV	Black queen cell virus

1. Introduction

The European honey bee (*Apis mellifera*) is a eusocial insect that plays a crucial role in both maintaining ecosystem health and supporting agricultural production across the world as a highly efficient plant pollinator (Potts et al., 2010). Unfortunately, honey bee colony losses remain a persistent challenge for beekeepers (Gray et al., 2020; Insolia et al., 2022; Jacques et al., 2017), which, in combination with an increased demand for agricultural pollination, has the potential to create a serious crisis for both society and nature (Mashilingi et al., 2022).

A major cause of increased colony losses is the invasive ectoparasite *Varroa destructor* (hereafter referred to as varroa) (Boecking and Genersch, 2008; Traynor et al., 2016; Warner et al., 2024). Varroa alternates between a reproductive phase inside capped brood cells and a dispersal phase (previously called phoretic) on adult bees. In the reproductive stage, varroa invades larval cells and feeds on the haemolymph of the developing bee through its pupation phase (Han et al., 2024; Ramsey et al., 2018, 2019). This parasitic feeding also facilitates the transmission of a wide array of viruses (Bowen-Walker et al., 1999; Martin et al., 2012). Notably, varroa acts not only as a mechanical vector but also as an amplifier of pathogens, with viral replication and accumulation occurring within the mite's body prior to transmission to the bee host (Damayo et al., 2023). Most of the detrimental effects of varroa on honey bees are caused by the viruses it transmits, severely compromising bee physiology, behaviour, and survival (Eliash et al., 2022; Martin, 2001). Deformed Wing Virus (DWV) is the most prevalent viral pathogen in honey bees globally, largely due to its efficient use of varroa mites as a new vector route, including biological amplification within varroa (Damayo et al., 2023; de Miranda and Genersch, 2010; Wilfert et al., 2016). High DWV titres are strongly associated with severe developmental and physiological impairments in bees, including malformed wings, shortened abdomens, reduced body mass, impaired behaviour, and markedly shortened lifespan, with infected individuals often emerging unable to fly and prone to early death (Figure 1; Benaets et al., 2017; Brettell et al., 2017; Dubois et al., 2020; Iqbal and Mueller, 2007; Wells et al., 2016). Moreover, it was found that DWV acts an immunosuppressant in the bees, which in turn leads to increased mite reproduction, possibly due to an increased facilitation in the mites feeding due to the immunosuppressant effects (Di Prisco et al.,

2016). Beyond visible deformities, DWV also causes more subtle physiological and behavioural disruptions. Bees infected with DWV may exhibit impaired learning, memory, and foraging efficiency, all of which are critical functions for colony-level coordination (Iqbal and Mueller, 2007; Pizzorno et al., 2021; Wells et al., 2016). The resulting worker losses from virus-induced deformities and mortality can exceed replacement rates, particularly during the onset of winter, leading to compromised thermoregulation and eventual colony collapse (Boecking and Genersch, 2008; Kevill et al., 2019; Schroeder and Martin, 2012; Wilfert et al., 2016). Together, these effects make DWV, in combination with varroa infestation, one of the principal drivers of colony mortality (Barroso-Arévalo et al., 2019; Dainat et al., 2012; Kielmanowicz et al., 2015).

As mite populations increase within a colony virus infections are more likely to become widespread and the risk of collapse grows significantly. To reduce the risk of colony death, the varroa population is controlled by beekeepers typically through the use of chemical miticide treatments, which carry their own risks of the mites developing resistance to these treatments, chemical residue build-up in hive products, and a disruption to colony dynamics (Bahreini et al., 2025; Martel et al., 2007; Tihelka, 2018). However, without varroa mite control, colonies typically succumb to lethal virus infections and colony mortality occurs within 2-3 years (Rosenkranz et al., 2010).

Within Europe, some honey bee populations have been documented to survive long term with little to no varroa treatment and have adapted traits and/or behaviours to reduce the harmful effects associated with varroa infestation (Locke, 2016a). This thesis investigates the varroa resistance in mite-surviving honey bee populations from Norway (Oddie et al., 2017), France (Le Conte et al., 2007), and Sweden (Locke and Fries, 2011) (**Paper I**), with a more in depth focus on the pheromonal mechanisms of mite-resistant adaptations in the Swedish population (**Paper II** & **III**), and the influence of human intervention on their resistance (**Paper IV**).

Figure 1. Left: *Apis mellifera* with *Varroa* destructor in the dispersal stage. Right: *A. mellifera* with symptoms of deformed wing virus. Photos courtesy of Barbara Locke.

1.1 Honey bees

1.1.1 Colony development and reproduction

Honey bees (*Apis mellifera*) live in large, highly organized colonies typically ranging from 10,000 to 50,000 individuals. These colonies consist of three castes: drones, workers, and a single queen (Figure 2). Drones are haploid males whose sole role is to mate with virgin queens during mating flight away from their home colonies (Winston, 1991). They constitute a small proportion of the population, approximately 2–5%, and are present only during the reproductive season, from early to late summer. After mating, which results in their death, surviving drones are expelled from the colony in the autumn (Winston, 1991). Workers are non-reproductive diploid females, constituting around 95–98% of the colony. They perform all essential non-reproductive tasks necessary for colony maintenance and survival, including brood rearing, queen care, colony hygiene, defence, and foraging (Winston, 1991).

The queen, a single diploid female, is the sole reproductive individual within the colony and lays up to 2,000 eggs per day (Avni et al., 2014). She typically mates early in life during a single mating flight with multiple drones, often 10 to 20, storing their sperm for lifelong use (Winston, 1991). This high degree of polyandry contributes to genetic diversity within the colony, which is linked to increased resilience against diseases and environmental stressors (Seeley and Tarpy, 2007; Tarpy, 2003). Colony-level reproduction occurs through swarming, where the original queen leaves the colony with part of the worker population to establish a new colony. This process plays a key

role in honey bee population dynamics, facilitating the spread of genetic traits (Winston, 1991).

Although the social structure and reproductive ecology of honey bee colonies contribute to their complexity and resilience, they also create favourable conditions for pests, parasites, and pathogens, while complicating the study of these interactions. High population density, abundant brood, and overlapping generations provide ideal conditions for pathogen persistence and transmission through frequent social contact and shared brood environments (Laomettachit et al., 2021). Moreover, the dynamic and interconnected nature of colony life makes it difficult to isolate specific causal relationships, as effects are often indirect, synergistic, or emerge at the colony level rather than in individual bees.

1.1.2 Brood development

Honey bees undergo four major developmental stages: egg, larva, pupa, and adult (Figure 2). The duration of each stage depends on the caste of bee, with queens developing the fastest, followed by workers, and drones taking the longest (Winston, 1991). This section focuses specifically on worker development, as they are the focus for this thesis.

A single immobile egg is laid at the bottom of a brood cell, where it develops for three days. The honey bee egg hatches into a larva, a grub-like stage that is continuously fed by nurse bees. Over the next five to six days, the larva grows rapidly until the brood cell is sealed with a wax capping by nurse bees. Once capped, the larva spins a cocoon and enters a short prepupal stage of approximately two days, after which the bee begins pupal stage metamorphosis where it differentiates into its adult form, developing structures such as the head, legs, wings, and mouthparts. This pupal stage lasts around ten days, reaching the adult stage just a few hours before emerging from the cell (Winston, 1991).

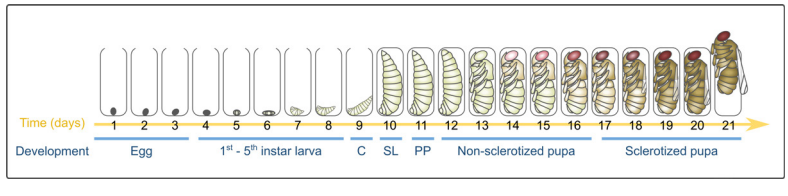

Figure 2. Timeline of *Apis mellifera* worker brood development from egg to emergence. C = Capping stage, SL = Sealed larvae, PP = Prepupae. Illustration by Fede Berckx, used with permission.

1.1.3 Pheromonal communication

One of the predominant methods of communication for insects is through the release of chemicals. When this is used within the same species in order to illicit a behavioural or physiological reaction, it is called pheromonal communication (Wyatt, 2014). This is a crucial method of communication between honey bees where each caste and life stage of bees releases their own mixture of chemical compounds (Gryboś et al., 2025; Slessor et al., 2005; Trhlin and Rajchard, 2011).

One of the first discovered pheromonal systems in honey bee colonies is produced by the queen. She secretes pheromones that regulate both immediate behavioural responses and long-term physiological processes in drones and workers (Oreshkova et al., 2024; Princen et al., 2019). Secretions such as (2E)-9-oxo-dec-2-enoic acid and (2E)-9-hydroxydec-2-enoic acid contribute to the suppression of worker reproduction and elicit retinue behaviour, in which workers surround, groom, and feed the queen (Princen et al., 2019; Wossler and Crewe, 1999). Key among these pheromonal signals is the Queen Mandibular Pheromone (QMP), a blend of five major components (Slessor et al., 1988) with a variety of functions, such as inhibiting worker ovarian development (Mumoki et al., 2018), reducing the construction of queen cells (Winston et al., 1989), and acting as a sex attractant for drones during mating flights (Wanner et al., 2007). The five components of QMP, along with four additional compounds, including methyl oleate, form the Queen Retinue Pheromone (QRP), which specifically promotes retinue behaviours by workers toward the queen (Keeling et al., 2003).

One of the best-characterised examples of honey bee chemical communication is the alarm pheromone, a complex blend of over 40 volatile compounds that is released primarily from the sting apparatus when bees are disturbed, threatened, or killed (Blum et al., 1978; Boch et al., 1962; Ghent and Gary, 1962; Llandres et al., 2013). This pheromonal cocktail rapidly activates defensive behaviours in nestmates, including increased alertness, recruitment to the threat location, and escalated stinging responses if the perceived threat persists or intensifies (Nouvian et al., 2018).

Recent findings have revealed that the alarm response is not simply linear and that worker aggressiveness depends on the amount of alarm pheromone released. Low to moderate levels of alarm pheromone increase stinging propensity, while extremely high concentrations result in a decline in

aggression (López-Incera et al., 2021). This change in response behaviour indicates that alarm pheromone acts not only as a recruitment signal but also plays a key role in shaping collective threat assessment and adaptive behavioural responses within the colony.

Pheromonal communication is also implicated in swarming dynamics (Pankiw, 2007; Princen et al., 2019; Richards et al., 2015; Winston et al., 1982). During swarming, worker bees establish a scenting-mediated communication network using five pheromonal compounds (congregation pheromones) and QMP that helps maintain cohesion and orientation within the cluster. Bees arrange themselves in a specific spatial distribution to create a directional scent flow away from the queen, facilitating the collective movement and reformation of the swarm (Nguyen et al., 2021).

Beyond immediate behavioural effects, pheromones may also shape individual motivational states and learning capacity. Exposure to specific pheromonal cues, such as the attractant geraniol, and aversive isopentyl acetate and 2-heptane, can modulate sucrose responsiveness and improve learning to avoid negative stimuli in individual bees (Baracchi et al., 2020, 2017; Rossi et al., 2018). These findings indicate that pheromones influence not only group-level coordination but also cognitive processes that contribute to colony-level plasticity and adaptive behavioural responses.

Developing larvae also communicate with adult workers through chemical signalling. Brood-emitted pheromones play an active role in regulating colony dynamics by altering nurse bee physiology and behaviour. Two brood pheromonal signals are Brood Ester Pheromone (BEP) and (*E*)-β-ocimene (BO), which together form a brood-to-worker communication system (Le Conte et al., 1990; Maisonnasse et al., 2010, 2009). BO is a highly volatile terpene. In contrast, BEP is a blend of ten fatty acid esters, including methyl and ethyl derivatives of palmitate, oleate, linoleate, linolenate, and stearate. These compounds are classified as either fatty acid methyl esters (FAME) or fatty acid ethyl esters (FAEE). The timing, concentration, and mixture of these pheromones vary with brood age, total amount of brood, and health condition, transmitting nuanced information that modulates worker behaviour (Le Conte et al., 1990; Maisonnasse et al., 2010; Mondet et al., 2024; Noël et al., 2023). [1, 2]

BEP was first identified by Le Conte et al. (1990), who found that methyl palmitate, methyl oleate, and methyl linolenate produced by fifth-instar larvae stimulate capping behaviour by nurse bees. Maisonnasse et al. (2009,

2010) demonstrated that BO accelerates the transition of nurses to foragers and modulate reproductive physiology in workers under certain conditions. Compounds such as methyl palmitate, ethyl oleate, and BO have been shown to enhance hypopharyngeal gland development in nurse bees, thereby increasing larval feeding rates (Mohammedi et al., 1996; Pankiw, 2004; Traynor et al., 2014). The upregulation of BO specifically has been linked to larval starvation cues, indicating a mechanism by which brood can signal urgent nutritional needs (Carroll et al., 2025; He et al., 2016). Moreover, in colonies with abundant brood, where BEP and BO levels are elevated, these pheromones act to suppress worker ovarian development, reinforcing reproductive hierarchy and maintaining social cohesion (Maisonnasse et al., 2010, 2009; Mohammedi et al., 1998; Traynor et al., 2014).

BEP and BO also influence age-related division of labour among workers. High levels of these brood pheromones, typical in brood-rich colonies, increased larval feeding, and delayed transition from nursing to foraging (Le Conte et al., 2001; Ma et al., 2018; Maisonnasse et al., 2010; Pankiw, 2007, 2004). This regulatory effect ensures that adequate nurse populations are maintained to support developing brood. Conversely, when brood numbers are low and thus BEP and BO concentrations decline, the rate of worker transition to foraging accelerates, thereby increasing pollen and nectar collection (Le Conte et al., 2001; Ma et al., 2019, 2018). This dynamic adjustment maintains colony homeostasis, preventing an overabundance of nurses and ensuring sufficient resources during brood-scarce periods.

The sophisticated pheromonal communication system of honey bees enables highly nuanced and efficient coordination within the colony. However, because pheromones are generally released into the surrounding environment, they are susceptible to interception by other organisms. This can be exploited by antagonistic species, including invasive parasites, to the detriment of the individual and colony.

Figure 3. Stages of *Varroa destructor* development. Photo courtesy of Barbara Locke.

1.1.4 V. destructor history, host switch, and global invasion

Varroa destructor was originally classified as a variant of *Varroa jacobsoni*, first described by Oudemans in 1904 on *Apis cerana* in Java, Indonesia (Oudemans, 1904). This species co-evolved with the Asian honey bee over thousands of years, forming a stable host-parasite relationship. It is suspected that the first host-jump to *Apis mellifera* occurred in the 1940's when beekeepers moved their apiaries into Eastern Russia, resulting in contact between *Apis mellifera* and *Apis cerana*, and providing an opportunity for host-switching by a subset of varroa mites. Unlike *A. cerana*, *A. mellifera* had not coevolved with varroa, resulting in many populations exhibiting limited defences against varroa infestation (Grindrod and Martin, 2023, 2021; Oldroyd, 1999). *V. destructor* spread throughout Eurasia in the 1950's and 60's, reaching South America in 1971, Africa in 1975, North America in 1987 (De Jong et al., 1982; Rinderer et al., 2010; Ruttner et al., 1984), and Australia in 2022 (Chapman et al., 2023). Today it is found worldwide, with only a few isolated islands remaining mite-free.

For nearly 60 years, *V. destructor* was not viewed as an individual species, but was considered an expansion of the host range of *V. jacobsoni*. It was not

until 2000 that *V. destructor* was identified as a distinct species that was reproductively isolated and significantly larger than *V. jacobsoni* (Anderson and Trueman, 2000). Thus, studies conducted before 2000 that referred to invasive mite populations as *V. jacobsoni* are now recognized to have actually been studying *V. destructor*.

1.1.5 V. destructor reproduction

Varroa depends on honey bee for its complete life cycle, consisting of a reproduction phase and a dispersal phase. During their dispersal stage, adult female varroa mites live on the back of honey bee workers where they feed on workers' fat bodies (Han et al., 2024; Ramsey et al., 2018, 2019). When the mite is ready to enter the reproduction phase, it must locate a larval cell that is ready to be capped (approximately 8 days post-egg laying), as reproduction can only occur within capped brood cells (Martin, 1994). Once the infested worker bee nears an appropriately aged larval cell, the mite detaches from the bee and enters the cell where it crawls to the bottom, at which point it is referred to as a foundress (Figure 4). In the larval cell, the foundress buries itself in the brood food to hide, using her peritreme like a snorkel to breathe through the food, staying immobile until the brood cell is capped by nurse bees (Donzé and Guerin, 1994; Richard et al., 1990).

Once the cell is capped, the foundress begins a three-day process of embryogenesis, resulting in oviposition of the first haploid (unfertilized) male egg after 60 – 70 hours, followed by up to five diploid (fertilized) female eggs (Figure 4; Ifantidis, 1983; Martin, 1994). Male eggs are glued to the upper cell wall for protection (Donzé and Guerin, 1994; Häußermann et al., 2020). Thereafter, female eggs are laid every 30 hours, deposited sequentially further down the cell wall (Figure 4; Häußermann et al., 2020; Ifantidis, 1983; Martin, 1994). When hatched, the offspring are cared for by the foundress by guiding them to the feeding spot as well as a designated defecation spot (Donzé and Guerin, 1994). Males have much shorter lifespans than females and are never found outside the brood cells, typically dying within the cell. Males mate with females present in the cell (likely their sisters) on the communal faecal site as soon as the female has reached her first moult (around 8-9 days post-capping; Donzé and Guerin, 1994)). If a male is not hatched or is killed, the female will not mate, as this is most likely the only opportunity for insemination, though it is possible for a foundress to mate with her own son later (Häußermann et al., 2020). Around 12 days

after cell invasion, the foundress and daughters escape when the adult bee emerges, attaching themselves to the back of adult worker bees, where they repeat the cycle in the dispersal phase (Figure 4; (Donzé and Guerin, 1994; Martin, 1994).

Successful reproduction for the mite therefore is measured as the ability of the mother mite to produce at least one viable mated female offspring at the time of bee eclosion (Locke and Fries, 2011). Failed mite reproductive success would therefore occur if i) no eggs were laid (infertile mother), ii) the male mite was not present, iii) the offspring were laid too late to mature before bee eclosion (delayed egg laying), or iv) the offspring die.

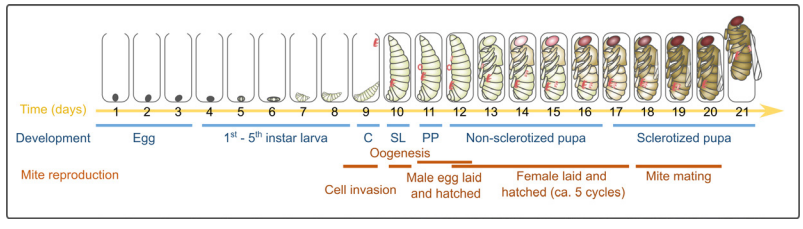

Figure 4. Timeline of *Varroa destructor* invasion and reproduction on *Apis mellifera* worker brood (Donzé and Guerin, 1994; Steiner et al., 1994). C =Capping stage, SL = Sealed larvae, Illustration by Fede Berckx, used with permission.

1.1.6 Kairomonal communication and use by V. destructor

As varroa are functionally blind and deaf, they rely heavily on their highly developed chemical sensory system to navigate their environment. The mite exploits chemical signals naturally produced by honey bees. When these pheromones are detected by an unintended receiver like varroa, they are termed kairomones (Brown, et al., 1970). Honey bee chemical signals are used to select adult bees for attachment and to locate brood cells for initiating reproduction. (Calderone et al., 2002; Frey et al., 2013; Liu et al., 2022; Piccolo et al., 2010). In the dispersal phase, varroa can differentiate between nurse and forager bees based on their distinct chemical profiles, generally preferring to infest nurse bees, which are more likely to come into contact with brood and thus offer better opportunities for reproductive success (Piccolo et al., 2010).

This chemically guided decision-making process extends into the reproductive phase, where specific compounds emitted by the brood play a key role in attracting or deterring mites from entering cells. Beyond locating

suitable environments, mites use chemical cues to assess whether brood development is at the optimal stage to initiate reproduction. Methyl and ethyl palmitate, and methyl linolenate produced by the brood have been found to be an attractant to the mite (Le Conte et al., 1989), while methyl and ethyl oleate repel varroa (Liu et al., 2022; Trouiller et al., 1994). Despite Le Conte 1989, identifying ethyl palmitate as a mite attractant, a recent study suggests a potential repellent function (Liu et al., 2022), therefore, ethyl palmitate's role remains ambiguous, and might depend on concentration and context. Brood food has also been identified as a source of varroa attractants, with 2-hydroxyhexanoic acid eliciting a strong attractive response (Nazzi et al., 2001). Differences in pheromonal composition among castes further influence mite preferences, suggesting that the chemical landscape of the brood can shape varroa invasion patterns.

Queen brood is less attractive to foundress mites than worker and drone brood, partly because it produces higher levels of repellent compounds such as ethyl oleate. In contrast, drone brood is preferentially invaded by varroa because it produces attractant compounds, such as methyl and ethyl palmitate, and methyl linolenate, at higher levels and for longer periods than worker or queen larvae (Trouiller et al., 1994, 1992). The foundress mite relies on the concentration and ratio of chemicals emitted by developing larvae to assess host age and determine whether conditions are suitable for initiating reproduction (Frey et al., 2013; Nganso et al., 2020; Trouiller and Milani, 1999).

Oogenesis begins after the mite detects the appropriate blend of kairomonal signals, which occur within a narrow developmental window following brood cell capping. If these chemical cues are absent, disrupted, or delayed, such as through brood mortality or disturbance by nurse bees within the first 12–18 hours post-capping, the mite aborts its reproductive attempt, only resuming if it subsequently enters a new brood cell that provides the correct chemical signature (Frey et al., 2013; Nganso et al., 2020). This behaviour is thought to be an adaptive strategy that conserves reproductive resources and increases the likelihood of successful offspring production in viable brood (Frey et al., 2013). Frey et al. (2013) further hypothesized that FAEE identified in larval cuticle emissions are the primary kairomonal cues involved in initiating mite reproduction, as their production declines sharply after the 12–18 hour post-capping window, mirroring the observed reduction in mite reproductive success. Additionally, the volatile alkene Z-8-

heptadecene has been detected in cells of infested brood and appears to reduce mite reproductive success (Nazzi et al., 2002). Whether this compound is produced by the brood as a defensive response to infestation or by the mite itself remains unclear and requires further investigation.

1.2 Honey bee defences against varroa

While varroa mites can severely impact honey bee colonies, some populations have strategies to reduce or cope with mite infestations. Tolerance strategies refer to the bees' ability to endure high mite loads, survive, and function despite mite infestation by limiting damage caused by the infestation without necessarily lowering the mite population. Resistance strategies refer to the bee's ability to actively remove or reduce mite infestation, through mechanisms or behaviours that reduce the mite's fitness (Reviewed in Kurze et al., 2016). Many populations exhibit a combination of these tolerance and resistance mechanisms across both colony and individual scales (Guzman-Novoa et al., 2024; Le Conte et al., 2020; Locke, 2016a), while some populations have shown tolerance and resistance mechanisms to both varroa and virus infections independently (Locke et al., 2021, 2014).

Unfortunately, the term SMR has led to some confusion based on its history of not fully understanding the trait. Subsequent findings in the SMR selective breeding program in the U.S. indicated that adult bees in this program had enhanced hygienic behaviour targeting varroa-infested pupae, particularly targeting cells with reproducing mites (Harbo and Harris, 2005; Ibrahim and Spivak, 2006). This selective removal inadvertently increased the proportion of non-reproductive mites in the colonies. To reflect this behavioural basis, the observed trait of low reproductive success rates (previously called SMR) was renamed to Varroa Sensitive Hygiene (VSH; Harris, 2007)). Selection of VSH in breeding programs focuses mainly on the frequency of non-reproducing mites, yet brood-derived cues, such as semiochemicals or physiological changes, may also disrupt mite reproduction (Mondet et al., 2016; Nazzi et al., 2004; Villa et al., 2016; Wagoner et al., 2021, 2019, 2019, 2020).

It has been proposed that honey bee populations may adapt different resistance mechanisms that result in a reduction of the mite's reproductive success rates (Locke et al., 2012a). While the ultimate cause of the trait is the

adaptive advantage of reducing the mite's fitness, the proximate cause could be based on either brood physiology, adult behaviours, or both (Locke et al., 2012a). Due to the inconsistent and overlapping use of the SMR and VSH terms, several new abbreviation have been proposed aiming to provide better clarity to describe the trait of low mite reproductive success rates, including Decreased Mite Reproduction (DMR), Reduced Mite Reproduction (RMR; Von Virag et al., 2022), and Mite Non Reproduction (MNR; Mondet et al., 2020). This thesis refers to observations of low mite reproductive success rates as SMR, as it is a resistant trait of the bees 'supressing' the ability of the mites to reproduce successfully. Whether it is caused by brood or adult bees can vary by population and until we better understand how the brood can impact mite reproductive success, we can wait to coin an appropriate term. We therefore propose to keep SMR as a loose term to define the ultimate phenotype caused by the evolutionary advantage of limiting mite reproductive success rates and that proximate causes should be considered on an individual population basis. An important distinction should be that SMR is a heritable resistance trait caused by the host rather than a non-specific inadvertent side-effect of another host trait.

1.2.1 Adult worker defences against V. destructor

Honey bees exhibit a range of behaviours that help defend against varroa infestation. Grooming behaviours, such as auto-grooming (self-grooming) and allo-grooming (grooming of nest mates), are particularly important during the dispersal phase of the mite on adult bees. These behaviours increase the likelihood that mites are detected and removed before they can infest new brood cells and initiate a reproductive cycle (Arechavaleta-Velasco and Guzmán-Novoa, 2001; Boecking and Ritter, 1993; Boecking and Spivak, 1999; Mugabi et al., 2024). Bees also exhibit hygienic behaviour, a targeted social immune response involving the detection, uncapping, and removal of diseased or dead brood from the nest (Boecking and Spivak, 1999; Gramacho and Spivak, 2003; Spivak and Gilliam, 1998). All colonies exhibit these behaviours to some degree, but those with particularly high levels of grooming or hygienic behaviour are able to more successfully reduce mite population growth (De La Mora et al., 2025). A specific type of hygienic behaviour that some colonies possess is Varroa Sensitive Hygiene (VSH), in which adult worker bees detect, uncap, and remove pupae from brood cells that are specifically infested with varroa

(Harbo and Harris, 2005; Harris, 2007; Ivanova and Bienefeld, 2023; Sprau et al., 2023). This behaviour disrupts the reproductive cycle of varroa mites, limiting the mite's population growth, and in turn the establishment of lethal virus infection levels, thereby significantly contributing to overall colony health and resilience (Boecking and Spivak, 1999; Erez et al., 2022; Masaquiza et al., 2021; Mondet et al., 2021; Mugabi et al., 2024; Spivak and Reuter, 2001). VSH appears to preferentially target brood cells with higher mite loads and mite offspring (Harris, 2007). Once a target cell is identified, workers uncap the wax capping, inspect the cell contents, and remove the pupa. This process eliminates both the parasitized brood and any immature mites present (Harris, 2007; Panziera et al., 2017).

A related behaviour, recapping, has also been associated with varroa resistance. Recapping behaviour involves adult workers that uncap a brood cell, inspect its contents, and then reseal it without removing the developing pupa. While the precise mechanism by which recapping disrupts mite reproduction is not fully understood, it is thought to interfere with the synchrony of mite development or alter the internal conditions of the cell in ways that reduce reproductive success (Hawkins and Martin, 2021; Martin et al., 2020). While there is ongoing debate about whether recapping directly causes a reduction in mite reproduction, studies have shown that many colonies exhibiting high SMR also show high rates of recapping (Hawkins and Martin, 2021; Oddie et al., 2021), suggesting a close functional association.

Early research by Nazzi et al. (2004) demonstrated that worker bees detect and remove mite infested brood in response to a semiochemical emitted by the brood. They identified (*Z*)-6-pentadecene, a volatile alkene, as significantly elevating brood-removal rates in experimental assays. Wagoner et al. (2018) found that resistant larvae could increase the rate of hygienic behaviour when introduced into non-hygienic colonies, suggesting a brood-mediated effect of these adult bee behaviours. Subsequent studies identified additional compounds linked to Varroa infestation and DWV infection that also enhanced hygienic behaviour: five by Wagoner et al. (2019, 2020, and 2021), six more by Mondet et al. (2021), and two further compounds by Liendo et al. (2021). More recently, Noël et al. (2025) profiled volatile organic compounds (VOCs) of varroa-infested and non-infested broods reared in gelatine capsule and identified five compounds released in higher quantity by varroa-infested group. Of these, five VOCs reliably triggered

both removal and recapping responses in worker bees. Low volatility compounds appeared particularly potent in inducing brood sacrifice via uncapping, while more volatile cues prompted cell inspection and recapping rather than removal (Noël et al., 2025).

1.2.2 Brood defences against V. destructor

In addition to adult-mediated defences, some honey bee populations express brood-level traits that may contribute to reduced mite reproductive success (Calderón et al., 2010; Camazine, 1986; Guzman-Novoa et al., 2024). While it remains difficult to fully disentangle these effects from adult behaviours, several brood-level characteristics have been associated with reduced mite reproductive success.

One such trait is a shortened post-capping developmental period of the pupae, which can lead to the host bee emerging before all of the mite's offspring have completed development, thereby reducing the number of viable, mated female mites, and lowering the mite's reproductive success (Moritz and Hänel, 1984; Oddie et al., 2018). Mite offspring mortality within brood cells, also impacts mite reproductive fitness. This is particularly critical for male mites since successful mating of female offspring depends on the presence of viable males (Mondragón et al., 2006; Nganso et al., 2018). The underlying causes of mite offspring mortality is not fully understood, but it has been proposed that larval movement during pupation may physically damage mite eggs, the soft-bodied immature stages of mite development, soft-bodied male mites, or disrupt mite feeding, especially during the critical transition from prepupa to pupa development(Calderón et al., 2012, 2010).

In addition to internal cell conditions, the attractiveness of brood cells to varroa mites may also play a role in limiting infestation. Mites are guided to suitable brood by chemical cues such as larval pheromones and cuticular hydrocarbons emitted shortly before cell capping (Le Conte et al., 1989; Trouiller et al., 1992). Variations in these chemical signals can affect how often mites choose to enter specific brood cells. Experimental studies have shown that larvae with lower attractiveness to mites experience significantly reduced infestation rates, even when reared under identical colony conditions. While this may not alter the reproductive success of mites once they infest a cell, it can reduce the overall reproductive potential of the mite population by lowering host cell availability (Junqueira et al., 2004).

While the honey bee-varroa relationship has been intensely studied over the last 40 years, there is still much that we do not know. To better understand the process that honey bees use to suppress mite reproduction, it is essential to study long-term naturally surviving populations to learn which life stages are primarily responsible for the resistant phenotype (**Paper I**), what adaptations these populations undergone (**Paper II**), and under what conditions these phenotypes are present (**Paper III** & **IV**).

1.2.3 Surviving populations

Most European honey bee populations rely heavily on human management and chemical treatments to control varroa infestations and ensure colony survival. However, some populations have demonstrated the ability to survive with little to no human intervention and more specifically without varroa control (Guzman-Novoa et al., 2024; Le Conte et al., 2020; Locke, 2016a). These populations employ various behavioural, physiological, and life-history traits to either tolerate or resist the negative effects of varroa mites.

One of the most notable groups exhibiting natural resistance to varroa is the African honey bee (*Apis mellifera scutellata*), which includes the Africanized honey bees in South America. Initially considered to be susceptible to varroa infestation like other populations, African and Africanized bees have since maintained survival without varroa treatments (Strauss et al., 2016; Tibatá et al., 2021). Several factors contribute to this resistance: relatively shorter brood development times reduce the window available for mite reproduction, frequent swarming and absconding reduce mite build up within colonies smaller colony sizes limit resource availability for mites (Calderón et al., 2010; Moritz and Hänel, 1984). Additionally, elevated levels of general hygienic behaviour facilitate the removal of diseased or infested brood and adults (Guerra Jr. et al., 2000). However, this trait appears to vary geographically and temporally, as other studies have failed to replicate these findings (Aumeier et al., 2000; Mondragón et al., 2005). These populations also express VSH, where worker bees detect and remove pupae infested with varroa mites, disrupting mite reproduction (Cheruiyot et al., 2018; Guerra Jr. et al., 2000; Guzman-Novoa et al., 2024). However, *Apis mellifera scutellata*, while known for their survival of varroa infestation, are not ideal candidates for widespread use in resistance breeding programs. These bees are adapted to warmer climates and are largely absent

from higher latitudes, limiting their suitability in temperate regions. In addition, they exhibit behavioural traits that are undesirable in managed beekeeping systems, including increased colony defensiveness and a higher propensity to abscond. These limitations highlight the value of investigating smaller, locally adapted populations of *A. mellifera* in the Northern hemisphere that have also demonstrated the ability to survive varroa infestation without chemical treatment. Such populations may offer more context-specific and practical insights into naturally adapted resistance mechanisms and could serve as promising genetic resources for sustainable breeding efforts (Le Conte et al., 2020; Locke, 2016a).

One such population has been documented in Østlandet, Norway, where managed *Apis mellifera* colonies have persisted without chemical varroa treatments for over two decades (Oddie et al., 2017). These bees originated from standard European stock and were not selectively bred for resistance, yet they have maintained stable colony survival and low mite loads. Studies on this population revealed reduced varroa reproductive success, a key feature of resistance, though the specific mechanisms remain under investigation. While traits like increased grooming and VSH were not markedly elevated, other behavioural and physiological traits such as frequent recapping of infested brood and a slightly shortened post-capping period appear to contribute to the observed reduction in mite population growth (Oddie et al., 2018, 2021).

After varroa invaded France in the 1980s and caused widespread colony collapse, naturally surviving untreated colonies were discovered near Le Mans in 1994 and later in Avignon (Le Conte et al., 2007). These surviving colonies maintained significantly lower mite populations, up to three times lower than in control colonies, suggesting evolved resistance mechanisms (Le Conte et al., 2007). Key traits contributing to their survival include enhanced grooming behaviour, removal of mite-infested brood (Reviewed in Le Conte et al., 2020) and SMR, particularly due to increased mite infertility (Locke et al., 2012a). Early behavioural and electrophysiological studies revealed that bees from this population exhibited heightened sensitivity to mite-derived chemical cues, suggesting improved mite detection (Martin, 2001). This was later corroborated by gene expression analyses showing the upregulation of genes involved in olfactory processing (Navajas et al., 2008). Overall, the survival of these colonies appears to result from host resistance traits that limit mite reproduction, shaped through natural selection.

One of the most studied resistant honey bee populations is from the island of Gotland in Sweden. In 1999, a large group of colonies was established and deliberately left untreated for varroa, allowing natural selection to act under high mite pressure (Fries et al., 2006). Over time, a subset of colonies survived and established a persistent population that has endured without chemical treatments for more than two decades (Beaurepaire et al., 2019). Specifically, the Gotland population exhibits a significantly reduced mite reproductive success, with a lower proportion of foundress mites producing viable offspring. This trait was also found to be heritable in both the drone and queen germ line, implying a dominant genetic component to their resistance that can potentially be useful through selective breeding programs (Locke, 2016b). In parallel, bees from this population often carry high viral loads without exhibiting the severe clinical symptoms typically associated with infection, suggesting a tolerance-based strategy that mitigates the physiological damage from viruses such as DWV, rather than preventing infection outright (Locke et al., 2014; Thaduri et al., 2021, 2019). The combination of SMR and viral tolerance allows these colonies to maintain population stability in the absence of chemical treatment.

Together, these resistant populations serve as key examples of potentially different evolutionary pathways toward varroa resilience (Locke et al., 2012a) and offer insight to natural host-parasite interactions and adaptations.

Figure 5. *Apis mellifera* populations reported to have some form of *Varroa destructor* resistant traits. Created using data from Locke 2016, Apidologie; Guzman-Novoa 2024, Frontiers in Ecology and Evolution; Le Conte 2020, Insects; Gebremedhn 2019, PloS One; Luis 2022, Scientific Reports

2. Aim

The overall aim of this thesis was to gain a deeper understanding of the honey bee host mechanisms underlying the observed reduced *Varroa destructor* mite reproduction in naturally mite-surviving honey bee colonies. My overall aim can be broken down into four specific aims, each corresponding to an individual paper.

- Investigate whether the expression of the SMR phenotype is primarily governed by nurse bees, brood, or an interaction between the two in three European mite-surviving honey bee populations (**Paper I**).
- Characterize the BEP profile of the larvae from the Gotland honey bee population during developmental stages that are highly synchronized to varroa reproduction and compare them to a non-resistant population under identical environmental conditions (**Paper II**).
- Determine whether the unique BEP profile observed in the Gotland larvae is a plastic trait influenced by the presence or absence of varroa infestation within the brood cell (**Paper III**).
- Gain a deeper understanding of the SMR trait in the Gotland honey bee population within a full-colony context. Specifically, determine whether the expression of SMR diminishes under reduced mite pressure and evaluate whether any commercially relevant traits are affected in a reduced mite environment (**Paper IV**).

3. Methods

For a more detailed description of the methodologies see the methods section within each paper. R was used for all statistical analysis.

3.1 Paper I

3.1.1 Location of experiment

All research was conducted at Swedish University of Agricultural Sciences (SLU), Uppsala, at the Lövsta research station [GPS Coordinates: N59° 50' 2.544" E17° 48' 47.447"].

3.1.2 Bee colony origin

The experimental mite-resistant colonies had their genetic origin from Norway (n = 3), Sweden (n = 5), France (n = 4) meaning the queens of these colonies were produced, mated and transported from their country of origin. A control group of colonies was included in the study with their origin from a Swedish mite-susceptible population (n = 5).

3.1.3 Experimental Design

The study was performed during August of 2017 with additional data collected in August 2019. At ~8-9 days after queen egg laying, when the majority of the larval brood cells had just been sealed for pupation, a section covering an estimated 500 sealed brood cells was designated for the exclusion treatment and isolated from contact with adult workers. Roughly 500 worker brood cells on the same frame were used as the adult honey bee exposure treatment group. Initially a metal cage was pressed into the wax around the designated brood to exclude adult bee access (Figure 6). While this metal cage generally served its purpose at excluding adult bees, it was inconsistent and adult bees managed to dig through the wax to get inside the caged area in a few colonies, which were then excluded from the analysis. Therefore, the brood exclusion method was adapted to use a nylon covering stapled to the wooden frame (Figure 6).

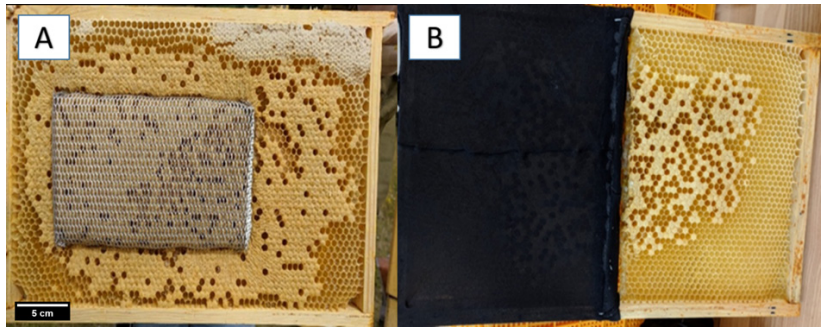

Figure 6. Cages placed in brood frames to exclude *Apis mellifera* adult worker access to brood. A. Metal cage. B. Nylon cage

3.1.4 Frame dissection

When the brood cells were ~9 days post capping, at which time the mite reproductive success is possible to assess, the frames were removed from the colonies for dissection. Individual cell content was analysed using a stereoscopic microscope (Leica MZ75, 6.5X magnification).

3.1.5 Varroa reproduction evaluation

The pupae developmental stage, the number of mite offspring and their developmental stage were recorded and compared to each other to evaluate mite reproductive success. A mite was considered to have successfully reproduced if it had produced a male offspring and a viable female offspring that will mature and mate with each other before the bee emerges from the brood cell as an adult (Dietemann et al., 2013). If a mite failed to reproduce, the reason for failure (absence of a male, delayed egg laying, dead progeny or infertility of the mother mite) was recorded along with mite fecundity (total number of offspring produced; Dietemann et al., 2013). Brood cells were opened until a minimum of thirty infested cells were uncovered, or until all available cells were opened.

3.1.6 Data analyses

A linear mixed effect model was performed using rate of mite reproductive success as the response variable, population origin and excluder treatment as fixed variables, with colony and year as random effect variables. Least square means of the model were used to compare treatments between each population (R Core Team, 2024).

3.2 Paper II & III

3.2.1 Location of experiment

All experiments for **Paper II** and **III** were conducted at in a single apiary at the SLU main campus apiary [GPS Coordinates: N 59° 48' 55.60596", E 17° 39' 54.39866"].

3.2.2 Bee colony origin

Colonies for both experiments were established during the summer months but followed different procedures. For **Paper II** (2021), six non-resistant honey bee colonies were equally split. Half retained their original queens to serve as the control group, while the other half were requeened with mated queens from the mite-surviving Gotland population. The colonies were allowed to develop for four weeks to ensure complete brood turnover, establishing the desired genetic background in the developing brood.
For **Paper III** (2024), colony establishment followed a different approach. Four resistant colonies were transported directly from Gotland, Sweden, while four non-resistant colonies were acquired from a local beekeeper in Uppsala, Sweden, maintaining distinct genetic lineages from the outset.

3.3 Experimental Design

Eight-day old larvae were checked hourly to identify the time of cell capping. A transparent acetate sheet was used to overlay the brood frame, allowing the marking of newly capped cells as the 0-hour time point. Individual pupae were then sampled from their brood cells at specific time intervals post-capping (Figure 7). For **Paper II**, pupae were collected at 0, 6, 12, 18, 24, and 36 hours after capping. For **Paper III**, the same experimental procedure was followed, with the exception that the 36-hour time point was omitted, as it was determined to not be relevant to the study.

Figure 7. Use of transparent plastic sheets placed over *Apis mellifera* brood frames to mark and track the age of capped brood. Individual cells were labelled on the sheet at the time of capping to allow later determination of brood age.

3.3.1 Collection of pupae

In both **Paper II** and **III**, frames were removed from their respective colonies and brought to a designated indoor workspace for larval collection. Using sterilized forceps, cell cappings were carefully opened, and developing larvae were gently extracted. Each larva was placed on filter paper to inspect for cuticle damage and to assess for the presence of varroa. To prevent cross-contamination of volatile compounds, forceps were flamed between each colony and time point.

In **Paper II**, only larvae that were free of varroa and had intact cuticles were retained for analysis; all others were discarded. In contrast, **Paper III** used the presence or absence of varroa infestation to define the experimental groups: infested and uninfested. Additionally, due to logistical considerations, larvae in **Paper III** were snap-frozen in liquid nitrogen immediately after collection and stored at –80°C until chemical extraction (Figure 8).

3.3.2 Extraction of volatiles

For both **Paper II** and **III**, each chemical sample contained four pupae pooled together for each time point per colony. The pupae were submerged in 2 ml (1.25g) of n-pentane for 10 minutes, following the procedure detailed in Frey et al., 2013 (Figure 8). Chemical extracts were stored in glass vials and immediately put in a -20 °C freezer before being transferred to a -80 °C freezer. Vials were removed from the freezer and left at room temperature for 5 minutes to thaw completely. They were then agitated to homogenize the mixture before being concentrated under a gentle nitrogen flow.

Figure 8. (A) *Apis mellifera* larvae immersed in pentane for extraction of cuticular semiochemicals for later analysis. (B) Larvae placed in a screw-cap glass vial prior to

snap-freezing in liquid nitrogen for subsequent chemical extraction. (C). Chemical extraction concentrated under a gentle nitrogen flow.

In **Paper II**, tentative identification of compounds was performed using gas chromatography-mass spectrometry (GC-MS), which identified three FAME, methyl palmitate, methyl linoleate, and methyl stearate; three FAEE, ethyl palmitate, ethyl linoleate, and ethyl stearate; and one terpene, (*E*)-β-ocimene. Quantification and compound identification were then conducted using gas chromatography (GC).
In **Paper III**, a similar method was applied, but the entire analysis, both identification and quantification, was performed using GC-MS. This study identified all BEP components except methyl and ethyl linoleate.

3.3.3 Data analyses

Both **Paper II** and **Paper III** employed generalized linear mixed-effects regression models (glmer) to analyse compound quantities as the response variable, with colony included as a random effect (R Core Team, 2024).
In **Paper II**, background and time points were included as fixed effects. Estimated marginal means post-hoc comparisons assessed background effects on compound quantities within and between time points (R Core Team, 2024).
In **Paper III**, the model included background, infestation status, and time points as fixed effects. Post-hoc estimated marginal means comparisons evaluated background effects on compound quantities within and between time points, accounting for infestation status.

3.4 Paper IV

3.4.1 Location of experiment

The experiment was conducted on the isolated southern peninsula of Gotland [GPS Coordinates: N 57° 04’ 30” E 18° 12’ 43.199”].

3.4.2 Bee colony origin

All colonies used originated from the Gotland mite-surviving honey bee population.

3.4.3 Experimental Design

Nine varroa resistant colonies were dequeened and split in the summer of 2020 and places into two treatment groups, those receiving varroa treatment (treated group), and those not receiving varroa treatment (control group). The two groups were placed five km apart and allowed to requeen before being returned to separate apiaries 1 month later. The apiaries were roughly 200 meters apart in order to limit drifting. Both groups were treated with identical beekeeping practices with the exception of standard anti-varroa treatment for the treated group.

3.4.4 Sample collection

Over the summer of 2021 and 2022 data on colony production, performance, and varroa infestation levels were collected in both early and late summer to determine overall colony health. This included: honey yield, estimate number of adult bees, estimate number of brood, pollen, and nectar cells, varroa infestation and reproduction, and the incidence and load of six bee viruses (Deformed Wing Virus, Sac Brood Virus, Black Queen Cell Virus, Lake Sinai Virus, Acute Bee Paralysis Virus, and Chronic Bee paralysis Virus).

3.4.5 Varroa infestation estimation

Infestation levels were determined by calculating varroa infestation and fertility rate. Infestation rate was determined by washing around 200 adult bees in soapy water and counting the mites that were collected (Bava et al., 2022).

3.4.6 Varroa reproduction evaluation

Mite fertility rate was determined by examining the contents of frozen brood cell containing pink to red-eyed and white/yellow bodied pupae. Cells found to contain at least one foundress mother with visible offspring were considered to be fertile and mother mites lacking visible offspring were considered infertile. To ensure adequate infestation of brood cells in the treated colonies, a frame of brood about to be capped were transferred to a separate mite donor colony.

3.4.7 Colony strength assessments

Counts of adult bees and cell types were performed with digital pictures taken of all frames from each colony using the Liebenfeld method to determine estimates (Dainat et al., 2020).

3.4.8 Virus Extraction

RNA was extracted from a pool of 30 adult bees per extraction. RNA was converted to cDNA using standard methodologies (de Miranda et al., 2013).

3.4.9 Virus detection and quantification

The cDNA templates were assayed for the presence and loads of common RNA viruses (DWV, SBV, LSV, BQCV, CBPV, and ABPV) using a set of standardized broad-range qPCR assays (de Miranda et al., 2021).

3.4.10 Data analyses

A general mixed effect model was used, with individual quantification (i.e. Rate of successful mite reproduction) used as the response variable, varroa treatment, month, and year used as fixed variables, and colony used as a random effect variable. Estimated marginal means was used on all models to compare individual factors (R Core Team, 2024). All viral data were natural log transformed. CBPV and ABPV were not found in any samples and were removed from the analysis.

4. Results and discussion

4.1 Host brood traits reduce *V. destructor* mite reproduction independent of adult behaviour

To determine whether varroa mite reproductive success is influenced by adult bee behaviours or by traits of the worker brood, mite reproduction was compared between brood cells either exposed to adult bees or caged to exclude adult interactions in three mite-resistant honey bee populations from Sweden, France, and Norway and a local non mite-resistant honey bee population as a control group.

Mite reproductive success rates did not significantly differ between treatment groups, either brood exposed to adult bees and their potential removal behaviours, or caged brood that were excluded from adult interaction, irrespective of the population's genetic background (Figure 9). The only variable that influenced varroa mite reproductive success was the population's genetic background, irrespective of treatment.

The average mite reproductive success rates and average mite fecundity were both significantly lower in the French and Swedish mite-resistant populations compared to the mite-susceptible control group (roughly 50% versus 80% successful mite reproduction; Figure 9), while the mite's reproductive success and fecundity in the Norwegian population was slightly lower than in the mite-susceptible controls, though not significantly (Figure 9).

Delayed egg laying was the most common reason for failed reproduction across all populations, while the absence of male mites occurred more frequently in the French and Swedish colonies than in the Norwegian and control colonies (Figure 10).

In the surviving populations studied, SMR was primarily associated with traits of the brood and adult worker behaviour did not differentially affect or add to the SMR trait. This indicates that brood effects play a major role in reducing mite reproductive success in these resistant populations, providing a crucial foundation for future research aimed at identifying specific brood traits underlying SMR and improving mite-resistant breeding programs. Moreover, the underlying causes of mite reproductive failure differed among populations, supporting the theory that resistance mechanisms may evolve independently through distinct evolutionary pathways shaped by local

environmental conditions and selective pressures (Locke et al., 2012a)**Paper I**). In contrast, breeding programs have traditionally focused on adult-expressed behaviours such as VSH, but achieving sustainable resistance through these traits has proven difficult due to their complex, polygenic nature and the labour-intensive selection process. For example, Tsuruda et al. (2012) identified only two quantitative trait loci explaining 10% of the variance in VSH, and subsequent studies found different associated genes, suggesting it is a multi-locus trait involving many genes of small effect (Scannapieco et al., 2017; Spötter et al., 2016). Additionally, the evolution of novel behaviours like VSH is inherently constrained, even under strong selection pressures like high parasite load (Sokolowski, 2001). In comparison, brood-based resistance may present a simpler and more evolutionarily accessible pathway. Subtle shifts in brood volatile or cuticular compound profiles, such as those involved in capping signals, may interfere with mite reproduction by disrupting their ability to identify or exploit suitable hosts (Frey et al., 2013; Nazzi and Le Conte, 2016) offering a potentially more stable and effective strategy for resistance.

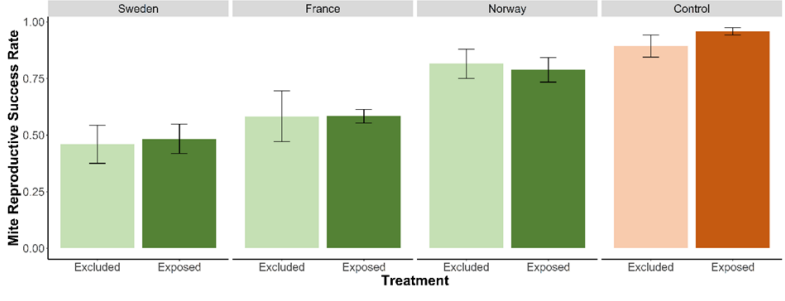

Figure 9. The average rates of *Varroa destructor* mite reproductive success (means +/- se) examined in four *Apis mellifera* populations (n indicates number of colonies) with error bars indicating standard error. Green bars represent the three mite-resistant populations examined from: Sweden (n = 6), France (n = 5), and Norway (n = 3), and the orange bars represent the mite-susceptible control group (n = 4). Within each population, treatment groups are differentiated between caged brood excluded from adult bees (light colour) and brood exposed to adult bees (dark colour).

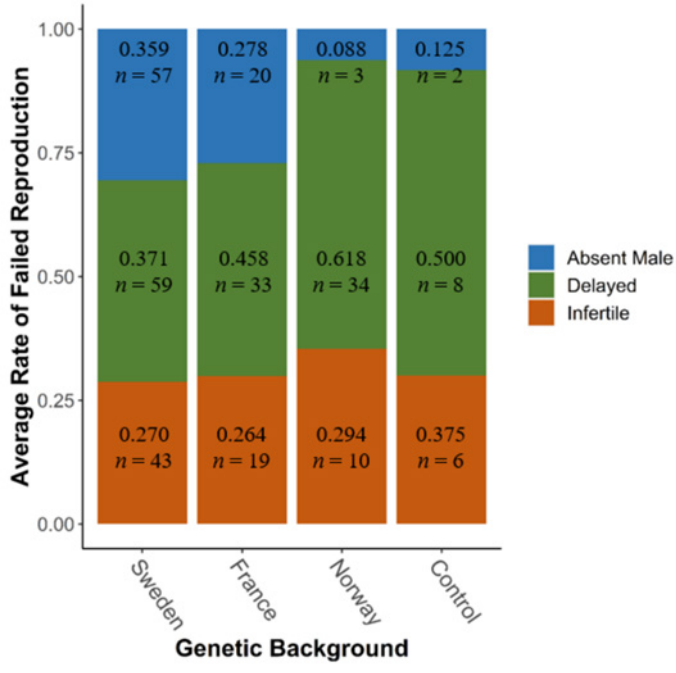

Figure 10. Average rate of reasons for the failed *Varroa destructor* reproductive success in the three naturally adapted *Apis mellifera* populations and control group, exposed and excluded groups pooled. The recorded reasons are: A) absence of a male (blue); B) delayed egg laying as mite offspring were too young to successfully reproduce (green); and C) infertility of the foundress (orange).

4.2 Unique brood ester pheromone profile in resistant brood

Varroa reproduction is closely synchronized with honey bee brood development, mediated by brood BEP. Therefore, alterations in the pheromone profile could potentially disrupt the mite's reproductive timing. To explore this possibility, cuticular chemical profiles of a mite-resistant Gotland honey bee population were analysed and compared to a non-resistant control group. BEP and BO were extracted at specific developmental time points that are critical for initiating mite reproduction

and analysed using gas chromatography to identify differences potentially linked to resistance.

The Gotland population consistently produced lower amounts of BEP at critical time points for varroa reproduction (Figure 11, Figure 12, **Paper II**). This pattern is most pronounced at the 00H time point, but across all time points, there is a clear trend of lower BEP production by the Gotland population in 38 out of 46 comparisons (83%) (**Paper II**). There is no significant difference in (*E*)-β-ocimene levels, with only time affecting the amount produced.

The Gotland population was found to exhibit a distinct BEP profile during developmental stages critical for varroa reproduction, marked by a significant reduction in BEP production. This reduction may serve as a form of chemical camouflage, referred to here as "chemical whispering", potentially disrupting the mite's ability to synchronize its reproduction with host development by making the brood signal more difficult to detect, while still allowing recognition by adult bees with heightened sensitivity to chemical cues. Frey et al. (2013) demonstrated that mite reproduction was significantly reduced if not exposed to the necessary pheromonal signals produced by the brood in the first twelve hours post capping. While the specific chemical triggers of varroa oogenesis remain unknown, BEPs are strong candidates for kairomonal cues used by the mite (Frey et al., 2013; Nazzi and Le Conte, 2016).

Theoretical models of signal–receiver co-evolution support the possibility that shifts in chemical communication can emerge rapidly under strong selective pressure. These models predict that receivers, in this case, adult bees, can adapt more quickly than signallers, and that novel or modified signals may be retained when the benefits of reduced parasitism outweigh any colony-level costs to communication (Phelan, 1997; Roelofs et al., 2002). Given varroa's low genetic diversity and dependence on inbred reproduction (Beaurepaire et al., 2017; Solignac et al., 2005), host shifts in pheromonal cues may pose a significant challenge to the parasite's adaptive capacity. Analogous systems show that even small genetic changes in signal-emitting organisms can lead to modified pheromone blends that evade detection by parasites or predators, sometimes within just a few generations (Löfstedt, 1990; Rewitz et al., 2010; Schulte et al., 2010). For instance, bark beetles (*Ips pini*) altered their pheromonal blend over only three years to fall between the preferences of two predators, while also adding a synergistic

compound that enhanced conspecific attraction without increasing predator detection (Rewitz et al., 2010).

Similar dynamics may be occurring in the Gotland honey bee population, which developed resistance phenotypes after only a few generations of natural varroa infestation. In host–parasite and parasitoid systems more broadly, many parasites rely on cuticular kairomones to locate hosts or determine reproductive timing. Disruption of these chemical cues—through altered timing, composition, or signal strength—can reduce parasite success by impairing host location or synchrony with optimal conditions (Conti and Colazza, 2012; Himeidan et al., 2013; Mwingira et al., 2020; Renwick, 1989; Wang et al., 2017; Xiaoyi and Zhongqi, 2008). These parallels suggest that the Gotland bees may have evolved subtle changes in their brood semiochemicals that interfere with varroa reproduction, representing an underexplored but plausible mechanism of resistance. Bees may reflect a rare case in host-parasite dynamics where the host gains an edge via subtle, rapidly evolving changes in semiochemical communication. These findings deepen our understanding of brood chemical communication as an adaptive strategy against varroa mites and provide a foundation to further elucidate how pheromonal shifts affect mite reproduction.

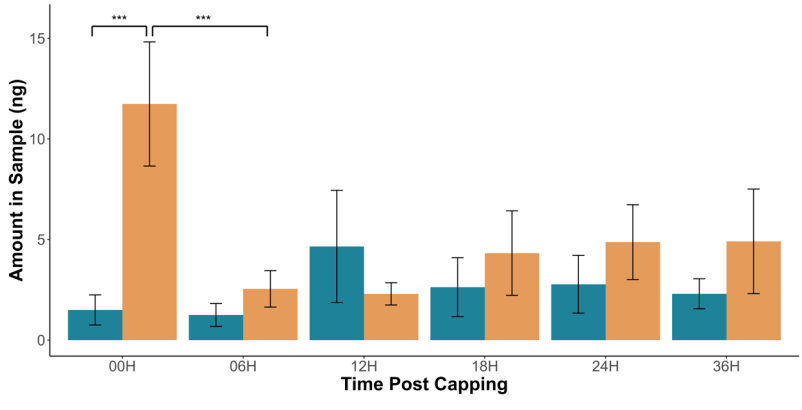

Figure 11. Amount of total fatty acid methyl esters (FAME) extracted the cuticle of *Apis mellifera* worker brood 00, 06, 12, 18, 24, and 36 hours after cell capping. Blue = Resistant, Orange = Non-Resistant. n = 18 (Resistant 00H, 06H, 12H, 18H, 36H; Non-Resistant 00H); n = 17 (Resistant 24H; Non-Resistant 06H, 12H, 18H, 24H, 36H) p value of significant differences added. Errors bars represent standard error. Statistically significant p values indicated by stars: * < 0.05, ** < 0.01, and *** < 0.001.

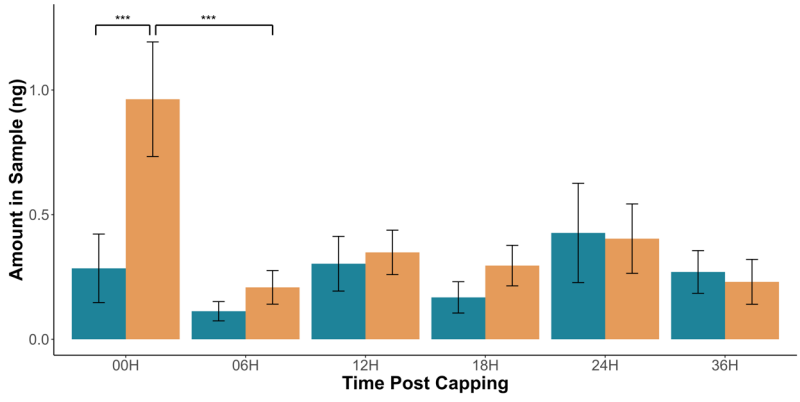

Figure 12. Amount of total fatty acid ethyl esters (FAEE) extracted from the cuticle of *Apis mellifera* worker brood 00, 06, 12, 18, 24, and 36 hours after cell capping. Blue = Resistant, Orange = Non-Resistant. n = 18 (Resistant 00H, 06H, 12H, 18H, 36H; Non-Resistant 00H); n = 17 (Resistant 24H; Non-Resistant 06H, 12H, 18H, 24H, 36H) p value of significant differences added. Errors bars representing standard error used. Statistically significant p values indicated by stars: * < 0.05, ** < 0.01, and *** < 0.001.

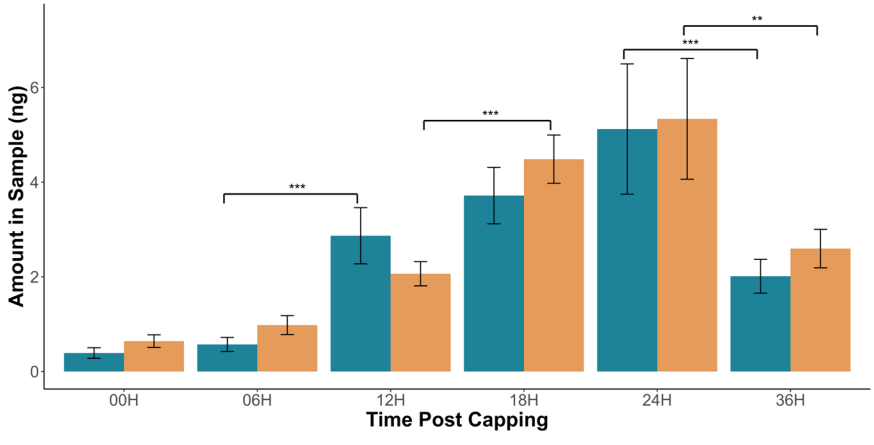

Figure 13. Amount of (*E*)-β-ocimene extracted from the cuticle of *Apis mellifera* worker brood 00, 06, 12, 18, 24, and 36 hours after cell capping. Blue = Resistant, Orange = Non-Resistant. n = 18 (Resistant 00H, 06H, 12H, 18H, 36H; Non-Resistant 00H); n = 17 (Resistant 24H; Non-Resistant 06H, 12H, 18H, 24H, 36H) p value of significant differences added. Errors bars representing standard error used. Statistically significant p values indicated by stars: * < 0.05, ** < 0.01, and *** < 0.001.

4.3 Plasticity in resistant BEP production

The reduction in pheromonal communication observed in **Paper I** may entail an undiscovered trade-off for developing larvae under mite-free conditions. To explore whether this effect is constitutive or condition-dependent, this study investigated the potential for phenotypic plasticity in the chemical signalling of the resistant Gotland honey bee population. Specifically, BEP and BO production were compared between varroa-infested and uninfested brood in both resistant and non-resistant colonies, to assess whether chemical expression shifts in response to mite presence.

Varroa destructor reproduction is tightly synchronized with host brood development, relying on chemical cues to initiate oogenesis during the first 12 hours post-capping (Frey et al., 2013; Nganso et al., 2020). In our study, infestation altered pheromone profiles differently, depending on the population. At 6 hours post-capping, infestation reduced FAME levels only in resistant larvae (Figure 15), whereas BO production at 18 hours increased more strongly in resistant than susceptible populations (Figure 16). The FAEE/FAME ratio showed the most pronounced differences (Figure 17), increasing under infestation in resistant brood but decreasing in susceptible brood across several time points (0, 6, 12, and 24 hours post-capping).

Chemical communication between brood and adult workers regulates colony behavior, including larval feeding, division of labor, and brood differentiation (Le Conte et al., 1990; Maisonnasse et al., 2009, 2010b). Variations in BEP and BO ratios can trigger different worker responses, and infestation-induced changes are consistent with this signalling plasticity. Parasites like varroa, and associated viruses such as DWV, can modify brood chemical profiles, either by producing novel compounds or altering key pheromonal quantities, potentially interfering with colony-level defences such as VSH (Nazzi et al., 2004; Mondet et al., 2021; Wagoner et al., 2019).

Varroa mites rely heavily on chemical cues to locate suitable hosts (Calderone et al., 2002; Liu et al., 2022). Brood-emitted esters such as methyl palmitate attract mites, while others like ethyl oleate can repel them, with some compounds showing context-dependent roles. Consistent with previous work, absolute FAEE levels declined after 12 hours post-capping. However, examining the FAEE/FAME ratio revealed significant plasticity under infestation: resistant brood increased this ratio early post-capping, potentially disrupting the chemical cues mites require to initiate oogenesis.

This suggests a previously unrecognized mechanism by which resistant larvae may reduce varroa reproductive success.

Adaptive modulation of chemical cues is observed across host–parasite systems, where changes in signalling can reduce parasite success (Conti and Colazza, 2012; Raffa et al., 2007). In honey bees, resistant populations may alter brood pheromone ratios to make larvae appear suboptimal for mite reproduction, while still maintaining fidelity for worker recognition and brood care. Phenotypic plasticity, activating defensive chemical profiles only in the presence of mites, may balance the trade-off between parasite defence and colony communication, minimizing potential fitness costs.

Temporal and environmental variation further shapes these strategies. Varroa infestation levels fluctuate seasonally with brood availability and colony dynamics (Traynor et al., 2020; Medina-Flores et al., 2024). VSH activity and brood volatile emission may be upregulated during periods of high mite pressure, such as late summer (Tison et al., 2022). The observed increase in the FAEE/FAME ratio in resistant populations under infestation may reflect an adaptive, plastic response to dynamic parasitic pressures, enhancing colony-level defence while balancing costs to communication. Effective defence also depends on signal perception, and resistant nurse bees may exhibit heightened sensitivity or alternative cue processing, emphasizing co-evolutionary dynamics of chemical signalling in honey bees.

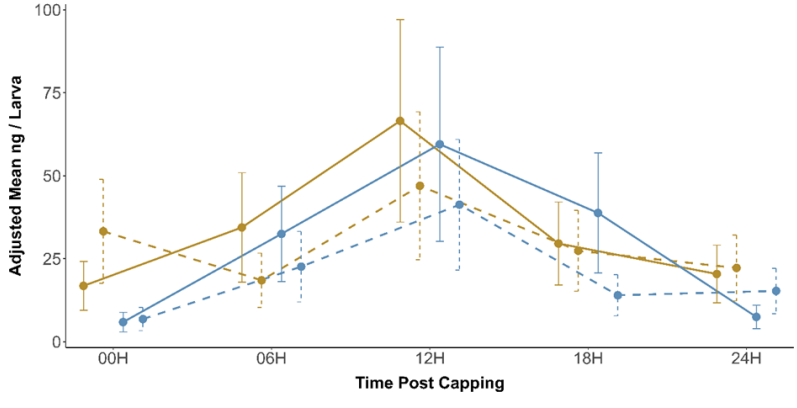

Figure 14. EM means ng/pupae of combined fatty acid ethyl esters (FAEE) from the cuticle of *Apis mellifera* worker brood 00, 06, 12, 18, and 24 hours after cell capping. Blue = Susceptible population, Yellow = Resistant population. Dotted line = Not infested by varroa, Solid line= Infested by varroa. Errors bars represent standard error.

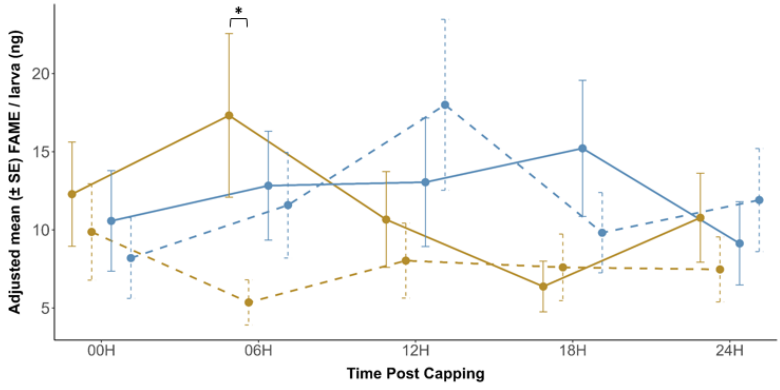

Figure 15. EM means ng/pupae of combined fatty acid methyl esters (FAME) from the cuticle of *Apis mellifera* worker brood 00, 06, 12, 18, and 24 hours after cell capping. Blue = Susceptible population, Yellow = Resistant population. Dotted line = Not infested by varroa, Solid line= Infested by varroa. Errors bars represent standard error. Statistically significant p values indicated by stars: * < 0.05, ** < 0.01, and *** < 0.001.

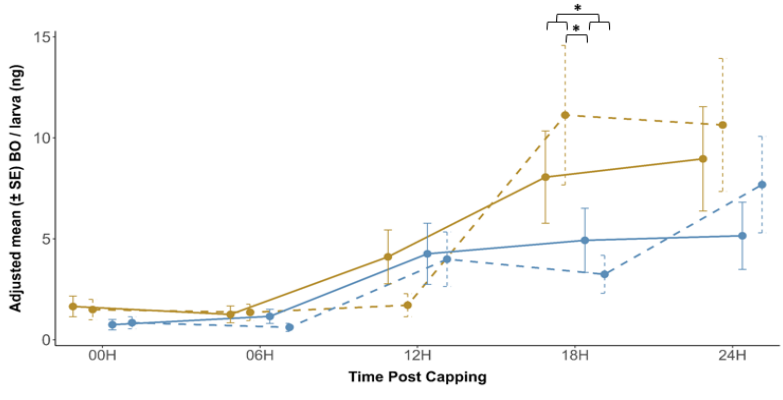

Figure 16. EM means ng/pupae of (*E*)-β-ocimene from the cuticle of *Apis mellifera* worker brood 00, 06, 12, 18, and 24 hours after cell capping. Blue = Susceptible population, Yellow = Resistant population. Dotted line = Not infested by varroa, Solid line= Infested by varroa. Errors bars represent standard error. Statistically significant p values indicated by stars: * < 0.05, ** < 0.01, and *** < 0.001.

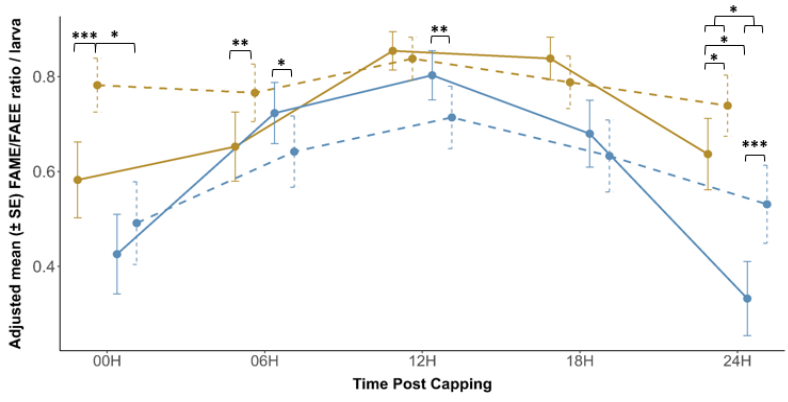

Figure 17. EM means relative amount of FAEE in relation to FAME from the cuticle of *Apis mellifera* worker brood 00, 06, 12, 18, and 24 hours after cell capping. Blue = Susceptible population, Yellow = Resistant population. Dotted line = Not infested by varroa, Solid line= Infested by varroa. Errors bars represent standard error. Statistically significant p values indicated by stars: * < 0.05, ** < 0.01, and *** < 0.001.

4.4 Varroa treatment increases colony strength and productivity without affecting mite fertility

To evaluate whether resistance traits in the Gotland population are fixed or phenotypically plastic, trait expression was compared between treated and untreated colonies across multiple seasons and years. Colony health and productivity were assessed using proxy measures including total numbers of adult bees and brood, as well as pollen, honey stores and honey yield. Varroa resistance was quantified by measuring mite fertility. The incidence and loads of various bee pathogens were determined by extracting RNA from a pool of 30 adult bees. Passive external RNA reference nucleic acids were included during extraction. The RNA was then converted to cDNA and assayed for a range of viruses using a standardized set of broad-range, optimized qPCR assays (Locke et al., 2012b).

When comparing the health and performance of resistant colonies treated for varroa to those that did not receive treatment, improvements were observed. Over the two-year study period we see an increase in total number of bees (Figure 18), amount of open (Figure 19) and closed (Figure 20) brood, pollen (Figure 21) and nectar cell count (Figure 22), although only early in the season, with values equalling the control group later in the

season. Honey yield also improved, but only early in season and in the second year (Figure 23). Viral dynamics were also significantly altered by treatment. Levels of DWV were markedly reduced in treated colonies, particularly in the first year, and SBV and LSV increased early in the season, with BQCV increasing during the first and final years (Figure 24). The only metric that showed no change with varroa treatment was the rate of mite fertility (Figure 25).

The observed increase in adult bee numbers in treated colonies likely reflects improved bee health and survival following reduced varroa pressure and lower DWV levels (Locke et al., 2012b). High titres of DWV are known to severely disrupt bee development and physiology, leading to deformities such as malformed wings, shortened abdomens, reduced mass, impaired behaviour, and shortened lifespan (Figure 1; Benaets et al., 2017; Brettell et al., 2017; Dubois et al., 2020; Iqbal and Mueller, 2007; Wells et al., 2016). Additionally, both varroa and DWV act as immunosuppressants, weakening the bees' ability to combat pathogens and environmental stressors, further compromising individual and colony resilience (Becchimanzi et al., 2025; Di Prisco et al., 2016; Yang and Cox-Foster, 2005). With lower mite burdens during the dispersal stage, the corresponding reduction in DWV likely contributed to enhanced colony health, including greater adult bee populations and improved brood production. An increased number of nurse bees and better resource availability may explain the observed rise in both open and capped brood in treated colonies. Varroa has also been shown to impair foraging efficiency through effects on flight performance, homing ability, and cognitive function (Blanken et al., 2015; Iqbal and Mueller, 2007; Monchanin et al., 2019; Pizzorno et al., 2021), all of which impact resource collection. Consequently, the increase in pollen and nectar stores observed in treated colonies may reflect improved foraging performance, as a result of a reduced DWV burden.

Viral dynamics in the first year of the study revealed a sharp decline in DWV in treated colonies, alongside early-season increases in SBV and LSV. The DWV decrease was expected given its strong, well-documented association with varroa infestation (Beaurepaire et al., 2020; Doublet et al., 2024; Traynor et al., 2020; Yañez et al., 2020). The SBV and LSV increases, however, are more complex. Interestingly, SBV levels in this study did not correlate closely with varroa levels but instead peaked in mid-summer, mirroring seasonal trends in foraging activity and resource collection. SBV

has previously been linked to foraging differences at both individual and colony levels (Anderson and Giacon, 1992; Bailey and Fernando, 1972; Bailey and Milne, 1969), suggesting that behavioural or environmental plasticity, rather than mite infestation, may influence its dynamics. A similar pattern may apply to LSV, although the mechanisms remain unclear and fall beyond the scope of this study. Viral interactions at multiple biological scales (molecular to colony level) may also contribute to these patterns.

Interestingly, there was no observed difference in mite fertility between treated and untreated colonies. If the resistant trait were plastic and dependent on mite pressure for their expression, treated colonies would be expected to show increased mite fertility closer to non-resistant populations as a result of allocating resources away from resistant traits and into traits such as colony growth (Strauss et al., 2002). Resistance to parasites is often assumed to carry fitness costs due to resource allocation trade-offs (Sheldon and Verhulst, 1996). However, the genetic basis of resistance may allow for beneficial mutations that minimize or mitigate such costs (Björkman et al., 1998; ffrench-Constant and Bass, 2017; Lenormand et al., 2018; Rigby et al., 2002). While traits associated with Gotland bees, such as smaller colony size and reduced honey yield, are commonly viewed as trade-offs (Guichard et al., 2023), these fitness costs remain unquantified. Understanding these costs is critical to elucidating the selective pressures shaping the population's resistance and to improving bee health management strategies.

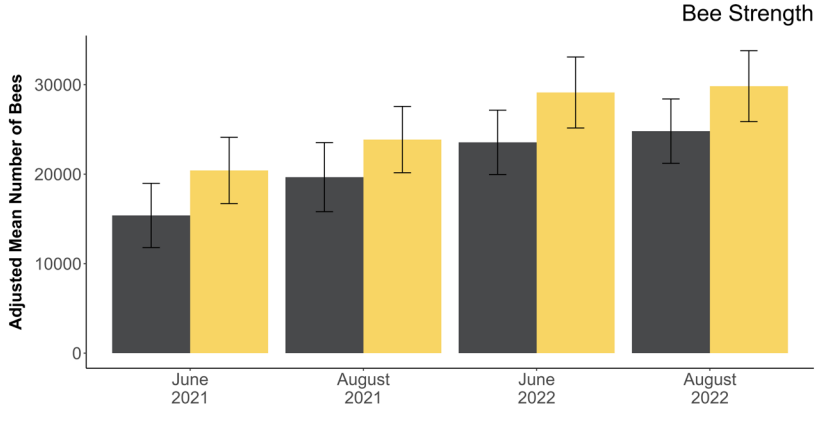

Figure 18. Estimated marginal means of the number of *Apis mellifera* adult bees per colony over four visits between June 2021 and August 2022 in treated (yellow) and untreated (black) bee hives. n = 6 (June 2021 Treated & Untreated, August 2021 Treated,

June & August 2022 Untreated); n = 5 (August 2021 Untreated, June & August 2022 Untreated).

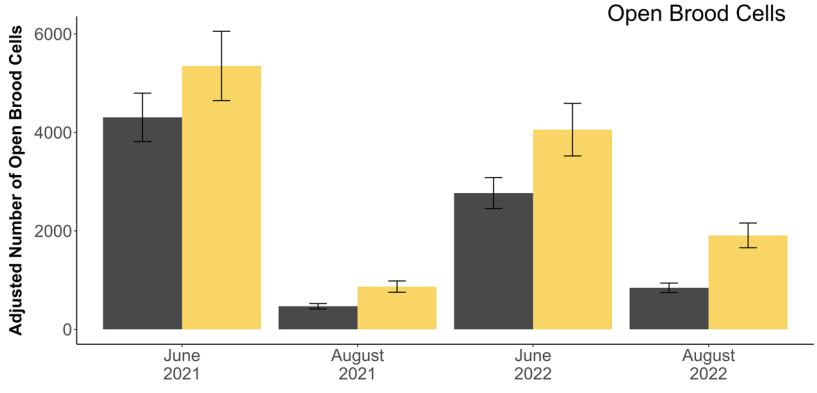

Figure 19. Estimated marginal means of the number of *Apis mellifera* open brood cells per colony over four visits between June 2021 and August 2022 in Treated (yellow) and untreated (black) bee hives. n = 6 (June 2021 Treated & Untreated, August 2021 Treated, June & August 2022 Untreated); n = 5 (August 2021 Untreated, June & August 2022 Treated).

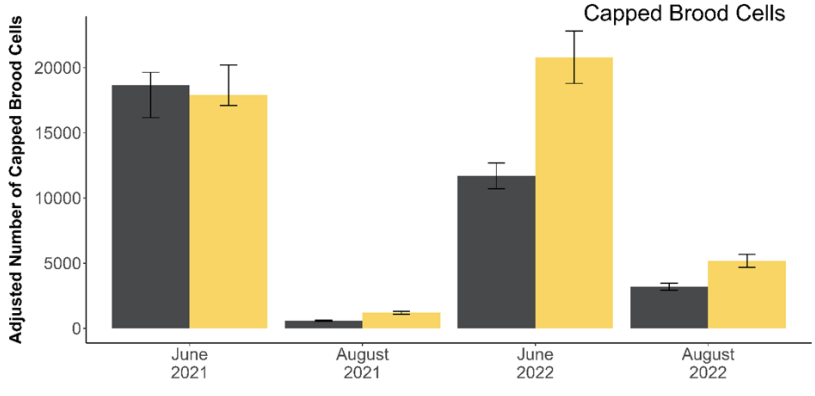

Figure 20. Estimated marginal means of the number of *Apis mellifera* closed brood cells per colony over four visits between June 2021 and August 2022 in Treated (yellow) and Untreated (black) bee hives. n = 6 (June 2021 Treated & Untreated, August 2021 Treated, June & August 2022 Untreated); n = 5 (August 2021 Untreated, June & August 2022 Treated).

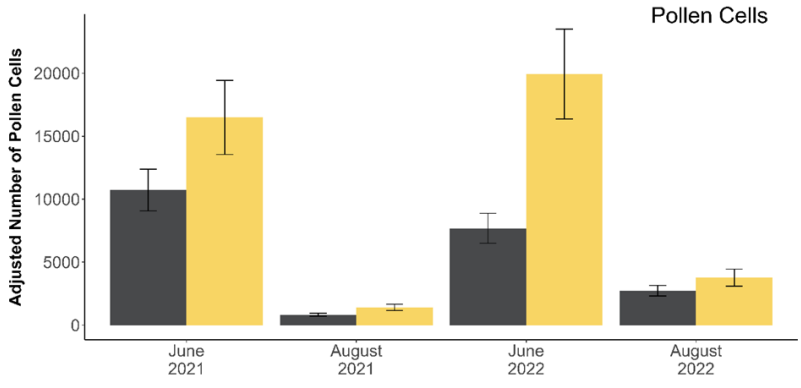

Figure 21. Estimated marginal means of the number of pollen cells per *Apis mellifera* colony over four visits between June 2021 and August 2022 in Treated (yellow) and Untreated (black) bee hives. n = 6 (June 2021 Treated & Untreated, August 2021 Treated, June & August 2022 Untreated); n = 5 (August 2021 Untreated, June & August 2022 Treated).

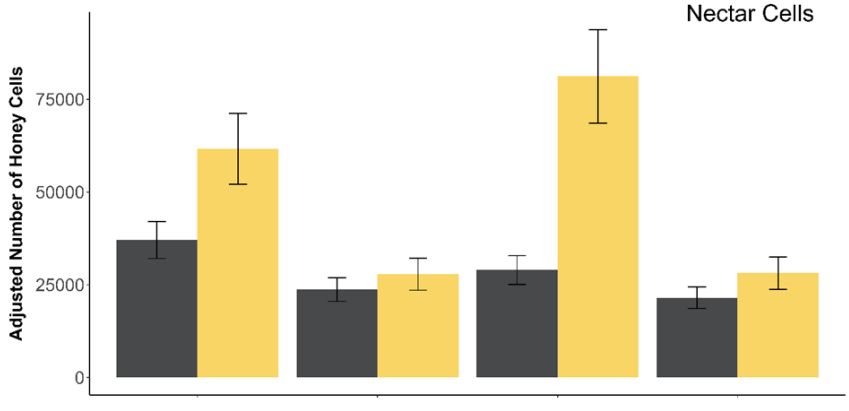

Figure 22. Estimated marginal means of the number of nectar cells per *Apis mellifera* colony over four visits between June 2021 and August 2022 in Treated (yellow) and Untreated (black) bee hives. n = 6 (June 2021 Treated & Untreated, August 2021 Treated, June & August 2022 Untreated); n = 5 (August 2021 Untreated, June & August 2022 Treated).

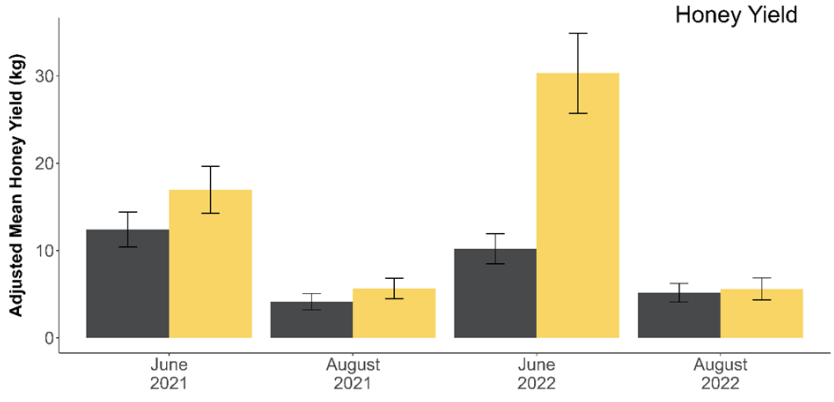

Figure 23. Estimated marginal means of the kg of honey extracted per *Apis mellifera* colony over four visits between June 2021 and August 2022 in Treated (yellow) and Untreated (black) bee hives. n = 6 (June 2021 Treated & Untreated, August 2021 Treated, June & August 2022 Untreated); n = 5 (August 2021 Untreated, June & August 2022 Treated).

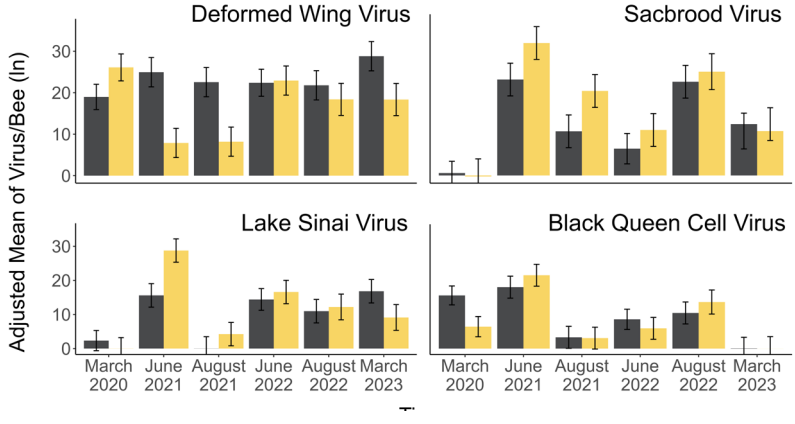

Figure 24. Estimated marginal means of natural log-transformed stated viral titers from *Apis mellifera* adults over four visits between June 2021 and August 2022 in Treated (yellow) and Untreated (black) bee hives. n = 6 (June 2021 Treated & Untreated, August 2021 Treated, June & August 2022 Untreated); n = 5 (August 2021 Untreated, June & August 2022 Treated).

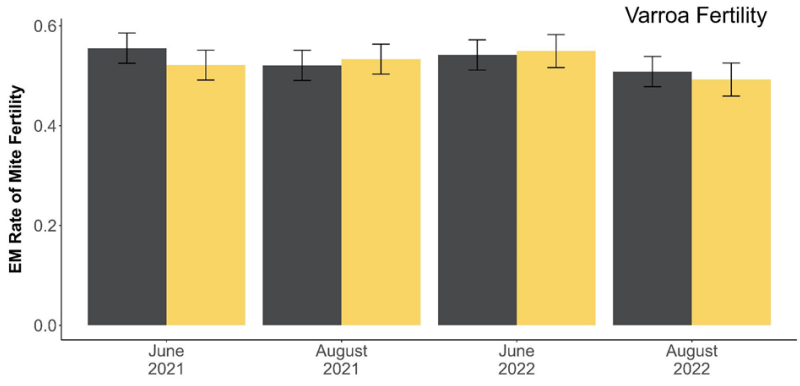

Figure 25. Estimated marginal means of the rate of *Varroa destructor* fertility per *Apis mellifera* colony over four visits between June 2021 and August 2022 in Treated (yellow) and Untreated (black) bee hives. n = 6 (June 2021 Treated & Untreated, August 2021 Treated, June & August 2022 Untreated); n = 5 (August 2021 Untreated, June & August 2022 Treated).

5. Conclusion and future perspective

This thesis provides valuable insight into the natural interactions and adaptations between *Apis mellifera* and *Varroa destructor*, using surviving populations that persist without beekeeping intervention or chemical mite control. These populations offer a unique opportunity to investigate resistance mechanisms in their natural context, contributing to our broader understanding of host–parasite dynamics. The findings presented here highlight the importance of brood adaptations in affecting mite reproductive success (**Papers I–III**), and demonstrate that these traits exhibit conditional expression depending on environmental and colony-level factors (**Papers III–IV**). This knowledge supports the development of more sustainable honey bee management strategies and lays a foundation for future research into the evolutionary and ecological dynamics of parasite resistance.

While the findings presented in this thesis provide important insights into the mechanisms underlying varroa resistance in naturally adapted honey bee populations, several limitations should be acknowledged. One limitation of **Paper I** is that, although the design enabled comparisons between resistant and control populations, incorporating an exchange of capped brood between resistant and control colonies in a cross-placement experiment (both excluded and exposed), would have allowed a clearer distinction between brood-specific effects and potential influences from adult workers. The chemical analyses in **Papers II** and **III** involved extraction methods that, while effective for obtaining FAME and FAEE, required killing the larvae during sample collection. Consequently, it was not possible to monitor the same individuals over time, limiting longitudinal observations of pheromonal dynamics within brood. Additionally, these methods did not capture highly volatile compounds that may play a role in brood–mite interactions. Headspace extraction could have addressed this issue; however, such an approach presents its own challenges, including the need for larger sample sizes and the co-extraction of unwanted volatiles from sources such as wax, which complicates analysis and interpretation. Finally, in **Paper IV**, mite fertility was measured as a proxy for reproductive success due to the limitation of using frozen brood frames. However, this metric alone does not fully capture the entirety of the suppressed mite reproduction trait, since it does not include features such as the absence of male mites, delayed egg laying, or dead progeny – all of which impact the mite's reproductive success

and ultimately mite fitness. Using frozen brood for this assessment removes the possibility to exam intricate features of mite reproductive success fully.

A key question emerging from this work is how adult nurse bees have adapted to the reduced pheromonal signalling emitted by resistant brood. Investigating whether nurse bees in the Gotland population exhibit heightened sensitivity to these pheromonal cues could shed light on potential compensatory mechanisms that maintain brood care despite altered brood communication signals to avoid mite interception.

Additionally, it would be valuable to experimentally determine the effect of reduced BEP production on mite reproductive success. Although current methods for assessing mite reproduction using artificial brood dummies remain underdeveloped, advances in technology and methodology may allow for the application of synthetic BEP levels, mirroring those observed in the Gotland population, to artificial brood. This would enable controlled testing of how specific pheromone profiles influence varroa reproductive outcomes. Further, a deeper mechanistic understanding may be achieved by employing multi-omics approaches, such as genomics, transcriptomics, and proteomics, to identify the genetic and molecular pathways responsible for the altered pheromone profiles observed in the Gotland bees.

The individual effects of some compounds of the BEP mixture, as well as their effects in different combinations, remain insufficiently characterized (Le Conte, 1995; Le Conte et al., 2001, 1994, 1990; Maisonnasse et al., 2010; Mohammedi et al., 1998). Several compounds, such as methyl and ethyl stearate, ethyl linoleate, and ethyl linolenate, have not yet been shown to elicit specific physiological or behavioural responses in either adult honey bees or varroa when tested individually (Le Conte et al., 1990). It is possible that these compounds exert their effects only synergistically, as part of pheromonal blends, rather than in isolation. Investigating the roles of these individual compounds and their combinatorial interactions, particularly in terms of both honey bee and varroa responses, represents a valuable and underexplored research direction.

A thorough understanding of the naturally adapted defence mechanisms in varroa resistance also has broad implications for apicultural management. It presents an opportunity in the future to develop breeding programs that integrate resistance mechanisms into economically valuable bee populations. Although the Gotland bees can survive without chemical treatment, they are currently not well suited for commercial apiculture due to their relatively

small colony size and low honey yield. By selectively breeding for resistance traits in more productive bee lineages, or by crossbreeding while maintaining desirable commercial characteristics, it may be possible to establish sustainable, mite-resistant populations that are also viable for pollination and honey production. Additionally, beekeepers, researchers, and policymakers can base their decisions on sound biological understanding. Continued research, alongside improved outreach and education, will be essential in ensuring that future management strategies are both scientifically robust and ecologically sustainable.

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Popular science summary

The invasive mite *Varroa destructor* is one of the greatest threats to honey bees and beekeeping today. Now found worldwide, it can wipe out an untreated colony in under three years. Wild honey bee populations have been devastated, and managed colonies require constant monitoring and treatment, at the expense of beekeepers. Chemical treatments are currently the most reliable defence against varroa, but they come with side effects and can lead to mite resistance. As a result, researchers and beekeepers are seeking more sustainable, low intervention strategies to manage mite populations.

One promising approach is to work with honey bees that can manage mite's infestation themselves. Some populations show natural resistance or tolerance to varroa. For instance, some resistant populations have developed a varroa specific cleaning behaviour, finding and removing mites at a much higher rate than susceptible populations. However, even resistant colonies often still require chemical support, and such traits can be lost over generations or bring trade-offs like lower honey yields, higher rates of absconding, or increased aggression.

However, some colonies seem to interfere with the mite's ability to reproduce through unknown mechanisms. Understanding these mite resistance traits is important as they could be selected for and possibly even bred into commercially desirable colonies, benefiting both honey bee and beekeeper.

Adult bees are known to reduce mite reproduction in some populations, but the specific contribution of the brood itself is less studied even though the varroa mite relies on brood for reproduction, so we set out to separate these effects. We looked at three varroa resistant colonies in Europe (from Gotland, Sweden; Avignon, France; and Østlandet, Norway) that are able to reduce the mite's ability to reproduce successfully. We found that in two of these resistant populations, Gotland, Sweden and Avignon, France, adult presence did not impact mite reproduction. This suggested that the brood itself was the key factor in resistance.

Focusing on the Gotland population, we examined the role of chemical communication. In honey bee colonies, chemical signals are a key to interactions between adults and brood, but varroa mites also exploit these

cues to find larvae and time their reproduction. This made larval chemical signalling a likely target for resistance.

We compared chemicals produced by the larvae that are important for varroa reproduction from resistant Gotland bees to those from a non-resistant control. Early in the mite's reproduction, the resistant larvae produce significantly lower amounts of these important chemicals compared to the control, essentially, "whispering" instead of "broadcasting". This could make it harder for mites to detect when to begin reproduction.

The next question asked was if this change in communication is always present, regardless of the presence of the mite. To answer this, we measured chemical signals from infested and uninfested larvae and compared them between the resistant Gotland population and a non-resistant control. We found that the ratio of chemicals used to communicate changed when mites were present, indicating that larvae modulate their signals depending on threat level. This suggests that there may be a cost to changing their way of communication, which larvae avoid when mites are absent.

We also tested whether reducing mite pressure would affect honey bee health and resistance. Still using the resistant Gotland honey bee, we compared bees treated with anti-varroa chemicals to bees left untreated. Treated colonies had improved productivity, particularly early in the season, with increased honey and pollen reserves. The colonies ability to repress the mite's reproduction remained unaffected. These results suggest that this population or the traits they possess might be more economically viable to beekeepers than previously thought, but longer-term, multi-generation studies would need to confirm this.

Overall, this thesis shows that honey bee brood can play a central role in resisting varroa. In Gotland bees, larvae emit altered signals used for mite reproduction, particularly when the mite itself is present. When mite pressure is managed, these colonies can become even more productive early in the season without losing resistance traits. This work highlights the potential of larval communication in developing more

Populärvetenskaplig sammanfattning

Det invasiva kvalstret *Varroa destructor* är idag ett av de största hoten mot honungsbin och biodling världen över. Utan behandling leder varroakvalsterangrepp ofta till att bisamhället kollapsar inom loppet av tre år. Vilda honungsbipopulationer har drabbats hårt, och inom biodling krävs kontinuerlig övervakning och behandling, vilket innebär stora kostnader för biodlarna.

I dagsläget är kemiska bekämpningsmedel det mest pålitliga sättet att behandla för varroakvalster. Tyvärr kan sådana medel ha negativa bieffekter och leda till resistensutveckling hos varroakvalstret. Därför vill forskare och biodlare hitta mer hållbara och skonsammare metoder för att bekämpa varroakvalstret.

En intressant väg framåt är att studera bin som uppvisar naturlig motståndskraft eller tolerans mot varroakvalstret. Det finns flera kända exempel på populationer som utvecklat särskilda egenskaper, såsom ökat hygieniskt beteende, det vill säga beteenden som gör att bina snabbt upptäcker och avlägsnar varroakvalster. Trots detta krävs i många fall kemisk behandling även i dessa populationer. Motståndskraften kan också försvinna med tiden eller medföra oönskade egenskaper, såsom minskad honungsproduktion, ökad svärmning eller mer aggressivt beteende.

Intressant nog har man observerat bisamhällen där varroakvalstrets förmåga att föröka sig minskats genom hittills okända mekanismer. En ökad förståelse för hur detta går till skulle möjliggöra avel för sådana egenskaper, möjligtvis även inom kommersiell biodling. Det är något som skulle kunna gagna både bin och biodlare.

Det är känt att vuxna bin i vissa populationer kan påverka varroakvalstrets förmåga att föröka sig men vilken roll ynglet har är mindre utforskat trots att varroakvalstret är beroende av yngel för sin förökning. För att studera detta närmare utgick vi från tre populationer av honungsbin i Europa; Gotland (Sverige), Avignon (Frankrike) och Østlandet (Norge), där man tidigare observerat att varroakvalstrets förmåga att föröka sig är påverkad. Våra jämförelser visade att i två av populationerna, Gotland och Avignon, hade de vuxna bina ingen direkt påverkan på varroakvalstrens förökning.

Detta indikerar att biynglet kan spela en avgörande roll i den observerade motståndskraften.

Vi valde att fokuserade vidare på populationen av bin från Gotland och den kemiska kommunikationen inom ett bisamhälle. Bisamhället styrs mycket genom samspelet mellan vuxna bin och yngel genom kemiska signaler. Varroakvalstret använder sig av denna kemiska kommunikation för att hitta yngel och veta när det är dags att börja föröka sig. Detta ledde till att ynglens kemiska signaler blev intressanta i arbetet mot att lära oss mer om motståndskraften hos dessa honungsbin.

Vi analyserade de kemiska föreningar som produceras av biyngel och som varroakvalstret är beroende av för sin förökning och fann att det fanns skillnader mellan den motståndskraftiga populationen av bin från Gotland och en kontrollgrupp utan motståndskraft. Yngel från de gotländska bina producerade betydligt mindre av de kemiska ämnen som utsöndras när varroakvalstret börjar föröka sig. Man kan säga att ynglena ”viskade” istället för att de ”ropade". Vilket skulle försvåra varroakvalstret förmåga att hitta rätt tidpunkt att föröka sig.

Nästa fråga vi ställde oss var om den här förändringringen i kemisk kommunikationen hos de gotländska bina alltid finns där, eller om den påverkas av om varroakvalstret är närvarande eller inte. För att ta reda på det jämförde vi de kemiska signalerna från yngel hos de gotländska bina med en kontrollgrupp, både när varroakvalstret finns där och när det inte gör det. Vi kunde se att närvaro av varroakvalster påverkade nivåerna av de kemiska ämnen som produceras av ynglen. Detta tyder på att bina inte aktiverar sitt försvar mot varroakvalster i onödan, kanske för att det kräver mycket energi eller innebär andra kostnader för dem.

Vi ville även ta reda på om minskade nivåer av varroakvalstret skulle påverka honungsbinas hälsa och motståndskraft. Därför fortsatte vi att studera honungsbina från Gotland och jämförde två olika grupper: en som fick behandling med kemiska bekämpningsmedel mot varroakvalster, och en som inte fick behandling. När vi jämförde de behandlade och obehandlade honungsbina från Gotland såg vi att bisamhällen som fått behandling var mer produktiva, särskilt i början av säsongen, och producerade och samlade in mer honung och pollen. Däremot verkade behandlingen inte påverka deras motståndskraft mot varroakvalstret. Det här tyder på att de motståndskraftiga honungsbina, eller de egenskaper de

har, kan vara mer ekonomiskt hållbara för biodlare än man tidigare trott. För att vara säkra och för att få en tydligare bild behöver vi göra fler och längre studier.

Sammanfattningsvis visar studien att ynglen spelar en viktig roll i bisamhällets försvar mot varroakvalstret. I den motståndskraftiga populationen av bin på Gotland verkar ynglen kunna ändra sina kemiska signaler så att varroakvalstrets förökningsförmåga påverkas. De anpassar även signalerna beroende på om varroakvalstret är närvarande eller ej. Om trycket från varroakvalstret hålls nere, kan dessa samhällen dessutom bli mer produktiva utan att tappa sin motståndskraft.

De här resultaten öppnar upp för nya möjligheter att utveckla mer hållbar biodling, där binas egen kommunikation får spela huvudrollen.

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Host brood traits, independent of adult behaviours, reduce *Varroa destructor* mite reproduction in resistant honeybee populations

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ABSTRACT

The ectoparasitic mite *Varroa destructor* is an invasive species of Western honey bees (*Apis mellifera*) and the largest pathogenic threat to their health world-wide. Its successful invasion and expansion is related to its ability to exploit the worker brood for reproduction, which results in an exponential population growth rate in the new host. With invasion of the mite, wild honeybee populations have been nearly eradicated from Europe and North America, and the survival of managed honeybee populations relies on mite population control treatments. However, there are a few documented honeybee populations surviving extended periods without control treatments due to adapted host traits that directly impact *Varroa* mite fitness. The aim of this study was to investigate if *Varroa* mite reproductive success was affected by traits of adult bee behaviours or by traits of the worker brood, in three mite-resistant honey bee populations from Sweden, France and Norway. The mite's reproductive success was measured and compared in broods that were either exposed to, or excluded from, adult bee access. Mite-resistant bee populations were also compared with a local mite-susceptible population, as a control group. Our results show that mite reproductive success rates and mite fecundity in the three mite-resistant populations were significantly different from the control population, with the French and Swedish populations having significantly lower reproductive rates than the Norwegian population. When comparing mite reproduction in exposed or excluded brood treatments, no differences were observed, regardless of population. This result clearly demonstrates that *Varroa* mite reproductive success can be suppressed by traits of the brood, independent of adult worker bees.

1. Introduction

The *Varroa destructor* mite is an invasive ectoparasite of the Western honey bee (*Apis mellifera*) and undeniably the largest pathogenic threat to honey bee health, severely impacting apiculture and agricultural crop production that relies on honey bees for pollination services. The *Varroa* mite is completely dependent on the honey bee colony for survival with a reproduction cycle tightly synchronized to pupa development inside brood cells (Steiner et al., 1995; Rosenkranz et al., 2010). In the mid-20th century, the *Varroa* mite made a host jump from the Asian honey bee (*Apis cerana*) to the Western honey bee species and has successfully spread throughout the world, with only a few isolated locations remaining mite-free (de Guzman and Rinderer, 1999; Oldroyd, 1999; Rosenkranz et al., 2010).

One of the most significant factors influencing the successful invasion and expansion of the *Varroa* mite with its new host is the ability of the mite to exploit and capitalize on the worker brood for reproduction. In contrast, Asian honey bees exhibit a variety of host traits that limit the ability of mites to reproduce in worker brood cells, acting as a natural control of the mite population growth (Lin et al., 2018; Wang et al., 2020). While some similar host traits exist in Western honey bees, they are far less pronounced and highly variable between subspecies (Corrêa-

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0020-7519/

Marques et al., 2002; Danka et al., 2011; Lin et al., 2016). Unrestricted access to thousands of worker brood cells in colonies of Western honey bees provides the mite with many more opportunities to reproduce, compared with Eastern honey bees. This contributes to an exponential population growth rate of the mite in this new host.. During the mite's reproductive phase, it feeds on developing pupae and vectors detrimental honey bee viruses, in particular *Deformed wing virus* (DWV), causing crippled, flightless adult honey bees with significantly shortened life spans, ultimately resulting in the loss of colony function (de Miranda and Genersch, 2010; Wilfert et al., 2016). To avoid viral infections killing the honey bee colony, mite population control treatments are required in apiculture. The *Varroa*-virus complex has caused a near complete eradication of wild honey bee colonies in Europe and North America (Le Conte et al., 2010). However, there are small subpopulations that have survived extended periods without *Varroa* mite control treatment and have documented resistant and tolerant host phenotypes to both the *Varroa* mite and their viruses (Locke et al., 2012; Locke, 2016a; Oddie et al., 2018).

Within populations of *A. mellifera* there is large natural variation in the mite's reproductive success, which is rarely 100% (Gregorc et al., 2016; Mondet et al., 2020). Mite reproductive success is defined as the ability of a mother mite to produce a viable mated female offspring before the bee emerges from its brood cell as an adult. Suppressed mite reproduction (SMR), is a term first coined by Harbo and Harris (1999), referring to a hereditary phenotype of a honey bee colony that causes *Varroa* mites to have a reduced reproductive success rate. This phenotype will undoubtedly have a significant influence on mite population growth and thus the development of virus infections and the life-span of the colony. It is also a trait of economic importance as a selection criterion for honey bee mite-resistant breeding programs. In naturally adapted mite-resistant honey bee populations, the mite's reproductive success rate has been recorded to be as low as 50% (Locke et al., 2012; Locke, 2016a; Oddie et al., 2018). However, the underlying host mechanisms responsible for expression of the SMR phenotype in any honey bee population, those in breeding programs or those that are naturally mite-resistant, remain elusive. It has been proposed that SMR is related to adult honey bee hygienic behaviors (Harbo and Harris, 2005; Harris, 2007). An example is Varroa Sensitive Hygiene (VSH) behavior, where adult bees selectively remove brood parasitized with reproducing mites while ignoring brood with non-reproductive mites. This behavior results in the appearance of a higher rate of non-reproducing mites (Ibrahim and Spivak, 2006; Danka et al., 2011; Harris et al., 2012). Another honey bee behviour that could relate to the SMR phenotype is uncapping and recapping of the wax cap placed over the brood cell by adult workers. This behavior could potentially disrupt the timing of mite reproduction, or even physically displace or damage the mites in the brood cell (Oddie et al., 2018, 2021). Another explanation for the SMR phenotype is related to traits of the worker brood such as altered volatile expression patterns that could inhibit mite reproduction (Locke et al., 2012; Frey et al., 2013). The mite uses volatile compounds from the cuticle of the larvae and pupae, that vary during specific developmental stages through pupation, as the signal to either initiate or inhibit the onset of egg laying (Frey et al., 2013; Nazzi and Le Conte, 2016).

The aim of this study was to gain a better understanding of the honey bee host mechanisms responsible for the SMR phenotype. This was approached by separating the adult bee behaviors from brood traits and measuring the rate of *Varroa* mite reproductive success. We examined three naturally adapted mite-resistant honey bee populations from Sweden, Norway and France that express SMR (Locke and Fries 2011; Locke et al., 2012; Oddie et al., 2017) and compared them with a local mite-susceptible population as a control group. The origin and phenotypes of the three naturally surviving honey bee populations examined in this study have been abundantly described (Locke, 2016a; Oddie et al., 2017). Briefly, these populations have evolved independently without mite control since 1994 (Avignon, France; (Le Conte et al., 2007)), 1999 (Gotland, Sweden; (Fries et al., 2003)) and 2001 (Oslo, Norway; (Oddie et al., 2017)). Adult bees were restricted from sections of brood on the same hive frame as brood that was exposed to adult bees. The hypothesis was that if mite reproductive success was reduced in the worker brood that was excluded from adult bees, then brood traits would be a significant contributor to the SMR expression in these populations, independent of the adult worker behaviors. Specific reasons for failed mite reproduction were also examined to compare and identify differences between the mite-resistant populations.

2. Materials and methods

2.1. Genetic background and colony establishment

During the summer of 2016, queens from each of these three populations were produced, mated in their original geographic locations and transported to Sweden according to European Union (EU) legislation guidelines. Queens from a local Swedish mite-susceptible honey bee population were similarly produced and used as controls. All queens were established in Swedish standard hives (Lågnormal, LP Biodling, Sweden) at a single apiary located at the Swedish University of Agricultural Sciences, Uppsala, at the Lövsta research station (GPS Coordinates: 59° 50′ 2.544″N, 17° 48′ 47.447″E). In the autumn of 2016, all colonies were treated against *Varroa* mites using tai-fluvalinate (ApistanRegisted, Vita Europe, UK) to equalize the mite infestation pressure.

2.2. Experimental design

The study was performed during August of 2017 with additional data collected in August 2019. The experiemental mite-resistant colonies had their genetic origin in Norway (n = 3), Sweden (n = 5) and France (n = 4), meaning the queens of these colonies were produced, mated and transported from their country of origin. A control group of colonies was included in the study with their origin being a Swedish mite-susceptible population (n = 5). The queens from each colony were confined to a single frame of drawn-out wax using a queen-excluder frame-cage in order to obtain frames with brood of uniform age. After 48 – 72 h, when the frames were full of eggs, the queen excluder was removed. Then, frames were checked daily to monitor the brood development and observe when the brood started to be capped. At ∼ 8–9 days after queen egg laying, when the majority of the larval brood cells had just been sealed for pupation, a section covering an estimated 500 sealed brood cells was designated for the exclusion treatment and isolated from contact with adult workers. Initially a metal cage was pressed into the wax around the designated brood to exclude adult bee access (Fig. 1A). While this metal cage generally served its purpose in excluding adult bees, it was inconsistant and adult bees managed to dig through the wax to get inside the caged area in a few colonies, which were then excluded from the analysis. Therefore, the brood exclusion method was adapted to use a nylon covering stapled to the wooden frame (Fig. 1B). This method was more consistent and effective at excluding adult bees from the brood. Approximately 500 worker brood cells on the same frame were used as the adult honey bee exposure treatment group.

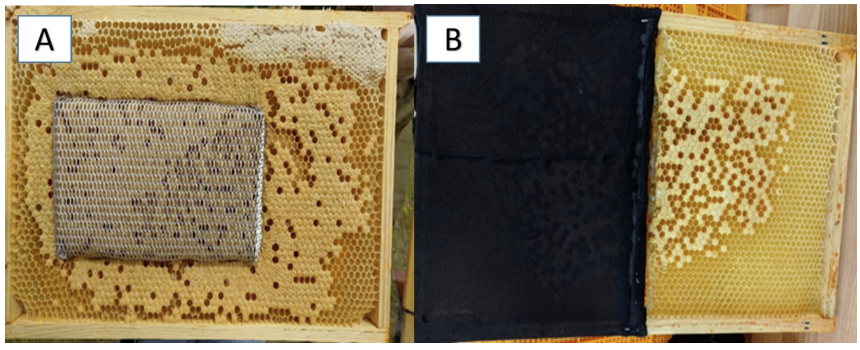

Fig. 1. Photographs of the two types of experimental frames used to exclude approximately 500 sealed worker brood cells from adult bees (*Apis mellifera)*. (A) Wire mesh cage; (B) nylon mesh cage. The frame size used is called Swedish Lågnormal, with dimensions 222 mm height × 366 mm width.

2.3. Frame dissection and mite reproduction evaluation

When the brood cells were ~ 9 days post capping, at which time mite reproductive success is possible to assess, the frames were removed from the colonies for dissection. In order to evaluate the mite reproductive success in individual brood cells, cell caps were removed using a scalpel, and the pupa and mite families were carefully removed from the cell using forceps and a fine paint brush according to standard methods (Dietemann et al., 2013; Table 1). Individual cell content was analyzed using a stereoscopic microscope (Leica MZ75, 6.5X magnification, Leica Microsystems, Germany). The pupal developmental stage, the number of mite offspring and their developmental stage, were recorded and compared with each other to evaluate mite reproductive success (Supplementary Table S1). A mite was considered to have successfully reproduced if it had produced a male offspring and a viable female offspring that would mature and mate with each other before the bee emerges from the brood cell as an adult (Dietemann et al., 2013). If a mite failed to reproduce, the reason for failure (absence of a male, delayed egg laying, dead progeny or infertility of the mother mite) was recorded (Supplementary Table S1), together with mite fecundity (total number of offspring produced; Dietemann et al., 2013). Brood cells were opened until a minimum of 30 infested cells were uncovered, or until all available cells were opened.

Table 1
Number of examined honey bee (*Apis mellifera)* worker brood cells, how many were opened, examined, naturally infested by mites (Varroa destructor), and how many had mites that reproduced successfully.

Genetic background	Measurement	Exposed brood	Caged brood
Norway	opened cells	772	937
	infested cells	89	73
	reproductive mites	70	58
France	opened cells	1965	1135
	infested cells	81	76
	reproductive mites	46	39
Sweden	opened cells	1204	796
	infested cells	161	133
	reproductive mites	76	59
Control	opened cells	536	797
	infested cells	120	94
	reproductive mites	115	83

2.4. Statistical analyses

Statistical analyses were performed using R version 4.0.1 R Development Core Team, 2010. A language and environment for statistical computing: reference index. R Foundation for Statistical Computing, Vienna) and R Studio Version 1.3.959 (R Studio Team, 2020. RStudio: Integrated Development for R). Data was shown to be normally distributed using a Shapiro normality test. A linear mixed-effect model was performed with rate of mite reproductive success as the response variable, population origin and excluder treatment as the independent variables and colony and year as random effect variables. This was done to compare treatments across populations, to compare treatments within each population, and to compare fecundity using the packages "multcomp", "lme4", "nlme", "car", "lmertest", "lsmeans", and "dplyr". Least-square means of the model were used to compare treatments between individual populations using the package "emmeans". Interactions were included in the model and sequentially removed when significance was not detected. *P* value threshold of 0.05 was used to determine significance. All graphs were made using the package "ggplot2".

2.5. Data accessibility

The datasets generated and/or analysed during the current study are available at the Swedish National Data Service, https://doi.org/10.5878/znc2-9b12.

3. Results

Mite reproductive success rates did not significantly differ between treatment groups of either caged brood or brood exposed to adult bees and their possible removal behaviors, irrespective of the population's genetic background (χ^2 = 2.45, degrees of freedom (df) = 1, $P > 0.11$). The only variable that did influence *Varroa* mite reproductive success was the population's genetic background, irrespective of treatment (χ^2 = 44.51, df = 3, $P < 0.005$).

The average mite reproductive success rates were significantly lower in the French (estimate = 0.326, df = 14, t.ratio = 3.89, $P = 0.008$) and Swedish (estimate = 0.125, df = 14, t.ratio = 0.0784, $P < 0.005$) mite-resistant populations compared with the mite-susceptible control group (Fig. 2). The mite reproductive success in the Norwegian population was slightly lower than in the mite-susceptible controls, but was not significantly different (estimate = 0.125, df = 14, t.ratio = 1.35, $P = 0.55$; Fig. 2), while the aver-

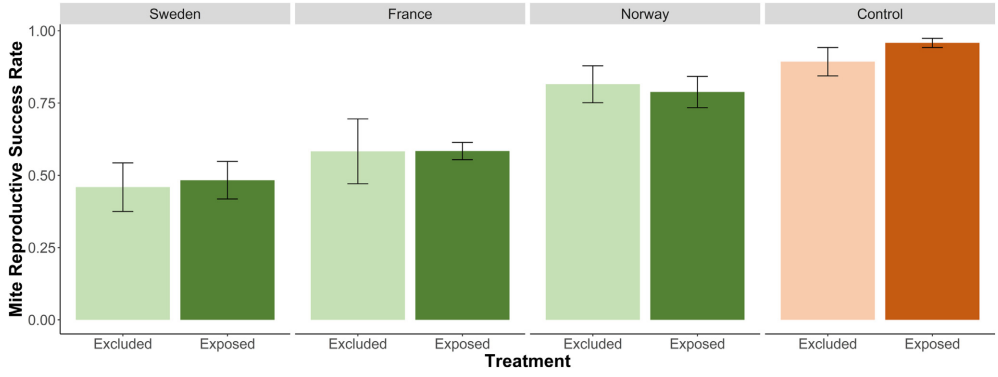

Fig. 2. The average rates of *Varroa destructor* mite reproductive success (means +/- SE) examined in four honey bee (*Apis mellifera)* populations (*n* indicates number of colonies) with error bars indicating standar error. Bars represent the three mite-resistant populations examined from: Sweden (n = 6), France (n = 5), and Norway (n = 3), and the mite-suspectable control group (n = 4). Within each population, treatment groups were differentiated between caged brood excluded from adult bees (light color) and brood exposed to adult bees (dark color).

age mite reproductive success rates were not different between the French and Swedish colonies (estimate = 0.121, df = 14, t.ratio = 1.57, P = 0.42; Fig. 2). Mite fecundity was also not affected by treatment (χ^2 = 0.806, df = 1, P = 0.37), but was significantly affected by the colony background (χ^2 = 31.11, df = 3, P < 0.001). The mite fecundity in the French and Swedish populations were similar to each other (estimate = 0.045, df = 14, t.ratio = 0.194, P = 0.997), but both were significantly different from the controls (Control-Sweden: estimate = 1.05, df = 14, t.ratio = 4.52, P = 0.002; Control-France: estimate = 1.01, df = 14, t.ratio = 4.00, P = 0.006), while the mites in the Norwegian colonies had similar fecundity rates to those in the control group (estimate = 0.38, df = 14, t. ratio – 1.41, P = 0.52).

Failed mite reproductive success, either due to the absence of a male mite, delayed egg laying, dead progeny or mite infertility was excluded from statistical analysis due to the small and uneven sample size (Table 2). Delayed egg laying was the most common reason for failed mite reproduction across all populations, while the absence of male mites occured more often in the French and Swedish colonies than in the Norwegian and control colonies (Fig. 3).

4. Discussion

The mite reproductive success rates and mite reproductive fecundity in this study were similarily low whether the parasitized brood was exposed to, or blocked off from, adult worker bees. This clearly demonstrates that *Varroa destructor* mite reproductive success can be suppressed by traits of the honey bee host brood, independent of adult worker behavioral traits.

With host-parasite relationships being particularly complex and intertwined, we do not exlude the potential for an additive effect of adult bee behavior on the expression of the SMR phenotype in any of these populations. However we believe these results eloquently reveal significant information regarding adaptations of host resistance and the SMR phenotype, in particular highlighting the role of host brood in *Varroa*-resistant honey bee populations.

The SMR phenotype has been widely considered to be an effect of the adult bee VSH behaviour (Harbo and Harris, 1999). The results of this study suggest that either VSH is not expressed to a significant degree in these colonies or that removal behaviors such as VSH do not specifically target the reproducing mites. A recent study examined the link between VSH and SMR, and found that the presence of mite offspring was not a crucial trigger for the VSH behaviour (Sprau et al., 2021).

The evolution of novel behaviors such as VSH is a complex and difficult process, even in the face of a strong natural selection such as high parasite load (Sokolowski, 2001). However, many honey bee mite-resistant breeding programs focus on behaviors such as VSH, but have had difficulty in producing sustainable mite resistance. Selecting for these behavioral traits is laborious and their genetic basis is not entirely understood, with one study only able to explain 10% of variance in the trait (VSH) measured with two quantitative trait loci (Tsuruda et al., 2012). Other studies looking at the genetic basis for VSH found different genes associated with the trait, implying that this a multi-loci complex, most likely involving many genes of small effect (Spötter et al., 2016; Scannapieco et al., 2017).

Frey et al. (2013) showed that the reproductive cycle of the mite is highly sensitive to changes in the cuticular pheremonal compound profiles of the brood. Honey bees use a variety of pheromonal compounds, functioning as complex releaser and primer signals, to regulate social organization in the colony (Nazzi and Le Conte, 2016). Some of these compounds are exploited by the

Table 2
The total number of mites (Varroa destructor) with failed reproduction presented for each population together with the number of failed reproductions due to the specific reasons observed and recorded.

Background	Total failed reproduction	Infertile mother	Delayed egg laying	Absence of male	Dead progeny
Sweden	160	43	59	56	2
France	72	19	33	20	0
Norway	34	10	21	3	0
Control	16	6	8	2	0

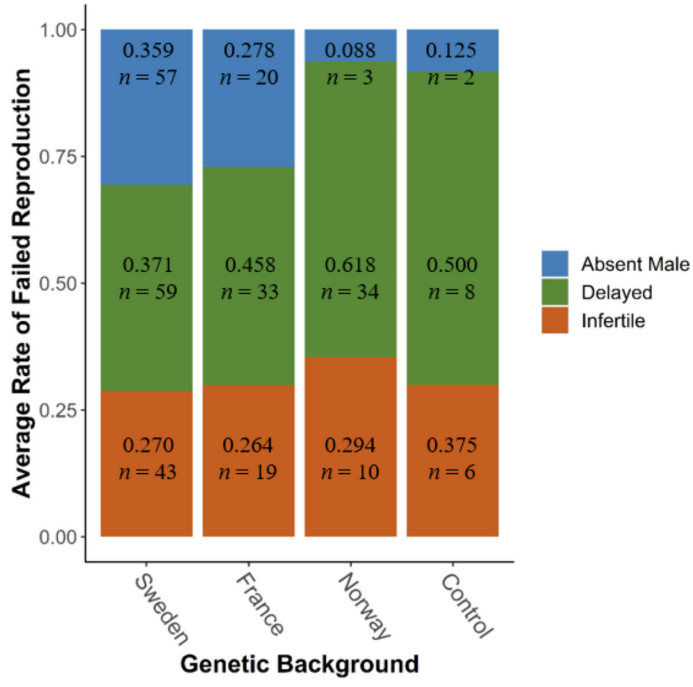

Fig. 3. Average rate of reasons for the failed *Varroa destructor* reproductive success in the three naturally adapted honey bee (*Apis* mellifera) populations and control group, exposed and exluded groups pooled. The recorded reasons are: i) absence of a male; ii) delayed egg laying as mite offspring were too young to successfully reproduce; and iii) infertility of the foundress.

mites, who use them to locate targets for feeding and reproduction. Fatty acid esters (FAE) such as methyl palmitate, ethyl palmitate, and methyl linolenate, are pheromones that signal adult nurse bees to cap the cells of developing bee larvae and have been shown to also attract mites to the brood cells (Nazzi and Le Conte, 2016). Small changes in brood volatile quantities or timing could therefore reduce the fitness of the parasites by interrupting their reproduction cycle. This could potentially be a simpler adaptive strategy for honey bee resistance as opposed to adult bee behaviors.

There have also been studies indiciating that brood developmental traits influence the SMR phenotype. Two ecdysone-related genes (*Cyp18a1* and *Phantom*) have been linked to mite resistance in the Swedish naturally adapted honey bee population using whole-genome sequencing for a quantitative trait locus analysis of reduced mite reproductive success (Conlon et al., 2018). These genes regulate important enzymes for pre-pupal development and metamorphosis by controlling steroid levels (Rewitz et al., 2010). Unusual concentrations of steroid compounds during the pre-pupal phase could make the age of the pupae appear suboptimal and the mother mite would suspend oogenesis (Frey et al., 2013; Conlon et al., 2018). Additionally, the *Ecdysone*-regulating gene *Mblk-1* has been linked with mite resistance in another honey bee population from Toulous, France (Conlon et al., 2019) and is responsible for both initiating metamorphosis in insects and initiating the reproduction in *Varroa* mites, once they acquire it from their host during feeding (Ureña et al., 2014; Cabrera et al., 2015; Mondet et al., 2018; Takayanagi-Kiya et al., 2017; Mondet et al., 2018).

Delayed egg laying was the most common reason for failed mite reproduction across all populations in this study, similar to a pan-European study assessing mite reproduction (Mondet et al., 2020). However, the absence of male mite offspring was significantly higher in the Swedish and French populations, which also have on average higher overall mite reproductive failure, compared with the Norwegian and control populations. The first egg laid by the mother mite develops into the male offspring (Donzé and Guerin, 1994). Adaptations by the honey bee brood that disrupt the oviposition or development of the male mite would need to occur early during the mite reproductive phase. Future research could investigate if differences in the brood pheromones that mites use to syncronize reproductive timing specifically influence ovipositioning and timing in relation to the first male egg (Frey et al., 2013). Previous research on the French and Swedish populations found that the most likely cause for failed reproductive success was delayed egg laying for the Swedish population and infertility for the French population (Locke et al., 2012). In this study there were no apparent differences between these population in the reasons for reproductive failure. This could be due to the different environmental conditions between this and earlier experiments, the minimal number of examined brood cells or colonies, or changes in the population phenotypes since last investigated. Recent studies have found that the *Varroa* mite has more genetic diversity than previously thought and therefore is potentially capable of adapting through a host-parasite evolutionary arms race. (Moro et al., 2020). Further research looking into how honeybees interrupt *Varroa* mite reproduction would be beneficial in understanding the

fluidity of this system, and what type of selection both the mites and honey bees are undergoing.

The differences between the French and Swedish mite-resistant honey bee populations and the mite-susceptible control population in this study mirror previous work and suggest the heritability and fixed genetic nature of the SMR phenotype in these naturally adapted mite-resistant populations (Locke et al., 2012; Locke, 2016b). The Norwegian honey bee population mite reproductive success rates were not significantly different from the mite-susceptible control population, in contrast with the French and Swedish populations which were significantly different from the control.

This contrasts previous work on the Norwegian population showing more dramatic differences in SMR between them and susceptible populations, when examined in Norway (Oddie et al., 2017). This could suggest that either Norwegian honey bees express mite-resistant phenotypes better in their local environment which they have adapted to, that they are specifically adapted for Norwegian mites that genetically differ from the mites they were exposed to in this study (Moro et al., 2020), or there has been a loss of the genetic heritability of the SMR phenotype in this population. Local adaptation has been shown to be important for colony survival when exposed to *Varroa* mite infections (Büchler et al., 2014; Meixner et al., 2015). Additionally, gene versus environment interaction studies have shown that mite-resistant populations do not necessarily maintain their resistant traits when moved to a new environment (Büchler et al., 2014; Meixner et al., 2015; Kovačić et al., 2020). This could mean that the Norwegian population has some factor that increases their SMR in Norway that is not present in Sweden. Further, while previous studies found that the mites showed little to no adaptation since their transition from *A. cerana* to *A. mellifera* (Kraus and Hunt, 1995; Solignac et al., 2005), a recent study has shown that it is possible for mite populations to change their reproductive strategies in resistant populations (Moro et al., 2021). They investigated an isolated artificially selected Dutch honey bee population that once displayed VSH (Panziera et al., 2017), but now shows no signs of VSH 4 years later. Genetic variation in mite genotypes exist in mite-resistant honey bee populations (Beaurepaire et al., 2019; Moro et al., 2020) which could potentially influence their reproductive success. However, this variation does not explain the differences in the SMR phenotype between the colonies examined in this study, since all the test colonies were managed in the same apiary, originally established from the same local bees and mites, where drifting of mites between colonies is expected (Frey and Rosenkranz, 2014; Nolan and Delaplane, 2017).

This study clearly distinguishes that adult bee behaviors are not involved in the expression of the SMR phenotype in these naturally adapted mite-resistant honey bee populations. Although we hypothesise that the reduced reproduction of mites is influenced by brood factors in these populations, there could still be factors that we have not examined, such as hive environment, that could be influencing mite reproduction. Brood transfer experiments could be used to identify such environmental effects and further studies testing the hypothesis that brood traits alone regulate the SMR phenotype are ongoing.

The distinction made in this study is an important first known step towards understanding the mechanisms behind SMR and more generally mite resistance, and opens the door for future research to discover more precisely what specific brood features are important for the SMR phenotype. A deeper understanding of the ecological interactions between *Varroa* mites and their hosts are also important for efforts in developing mite-resistant breeding programs. This could potentially simplify selection criteria evaluation methods, selection strategies, and help develop more efficient and sustainable efforts towards long-term genetic stock improvements for mite resistance in honey bees.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2023.04.001.

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Unique brood ester profile in a *Varroa destructor* resistant population of European honey bee (*Apis mellifera*)

Nicholas Scaramella✉, Robert Glinwood & Barbara Locke

Varroa destructor **is one of the greatest threats to** ***Apis mellifera*** **worldwide and if left untreated will kill a colony in less than three years. A Varroa-resistant population from Gotland, Sweden, has managed to survive for 25 years with little to no Varroa treatment by reducing the mite's reproductive success. The underlying mechanisms of this trait is currently not known, though previous research indicates that it is the honey bee brood, and not adult bee influence, that contributes to this phenotype. As the mite's own reproduction is synchronized with the brood's development though the interception of brood pheromones, it is possible that a change in pheromone profile would disrupt the mite's reproductive timing. To investigate this, we characterized the brood ester pheromone (BEP) profile of our resistant Gotland population compared to a non-resistant control. This was done by extracting and analyzing key cuticular compounds of the BEP using gas chromatography. A significant difference was found immediately after brood capping, indicating a divergence in their pheromonal production at this time point. This is an important step to understanding the mechanisms of the Gotland population's Varroa-resistance and contributes to our global understanding of** ***Varroa destructor*** **infestation and survival.**

Keywords *Apis mellifera*, *Varroa destructor*, Brood ester pheromones, BEP, Brood effects

The invasive ectoparasitic mite *Varroa destructor* (hereafter referred to as Varroa) is unarguably one of the largest threats to the European honey bee (*Apis mellifera*) causing colony death worldwide[1]. Varroa relies entirely on the honey bee for food and reproduction, which occurs mainly in the cells of developing brood[2]. When Varroa feeds on the honey bee fat bodies and hemolymph, a number of viruses are transmitted to the developing bee pupae, most notably *Deformed Wing Virus* (DWV)[3,4]. DWV causes reduced body weight, a shorter lifespan, and malformed wings resulting in flightless adult bees that cannot contribute to colony functions[5–7]. With exponentially increasing mite infestation vectoring viruses in the brood, a virus epidemic eventually occurs leading to a dwindling adult bee population and ultimately colony mortality within 1–2 years if the mite infestation is not controlled by beekeepers[8–10]. The best defence beekeepers have against high Varroa infestation, and to avoid a virus epidemic, is to use chemical treatments such as synthetic pyrethroids or organic acids such as oxalic or formic acid applied to the hive. These treatments unfortunately can also reduce bee health, and Varroa can develop resistance towards some of these treatments[11,12]. An alternative method towards mitigating the harmful consequences of Varroa infestation is through Varroa resistance selective breeding programs. Several programs, usually focusing on adult bee behaviours that target the mite, such grooming behaviour, hygienic behaviour, and more specifically Varroa Sensitive Hygiene (VSH), where adult bees selectively remove Varroa parasitized brood[13,14], have had some success in increasing the frequencies of these behaviours but producing long-term stable Varroa resistance has been challenging[15]. A deeper understanding of the complex host-parasite relationship and interactions between Varroa and honey bees is necessary in order to improve the efficacy of Varroa resistance selective breeding programs and increase honey bee resistant stock on a large scale[16–18].

In the Baltic sea, on the island of Gotland, Sweden, there is a population of honey bees that have survived with Varroa infestation with little to no chemical treatment since 1999[19]. This population exhibits naturally adapted Varroa-resistant phenotypes, specifically the ability to reduce mite reproductive success rates. Only around 50% of the mother mites in the Gotland Varroa-resistant population are able to produce viable offspring at a given occasion[20,21] compared to non-resistant regularly managed honey bee colonies, where mother mites have reproductive success rates over 80%[20,21]. While it is still unclear how the bees reduce the mite reproduction,

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it is clearly a genetic feature of the bee population, rather than a reduction of virulence on the side of the mite[22,23] and has been a stable trait in the population observed over multiple occasions since it was first reported in 2011[20,24,25]. Recent research shows that the reduced mite reproduction, in this and other naturally adapted mite resistant honey bee populations, appears to be due to characteristics of the honey bee brood, as opposed to Varroa resistance behaviours of adult worker bees[24], which are often the focus in Varroa resistance breeding programs. This population therefore, provides a unique opportunity to study the natural relationship and interactions between Varroa mites and European honey bees.

Chemical signaling is a major method of communication between developing brood and attending nurse bees within a honey bee colony[26,27]. In particular, a cocktail of different volatile compounds have been identified in the brood ester pheromone (BEP) profile of the brood that are used to communicate information to adult bees such as the brood's caste or age[27–29]. Originally ten BEP compounds have been identified in brood communication: fatty acid methyl (FAME) & ethyl esters (FAEE) of palmitate, linoleate, stearate, oleate, and linolenate acids, with E-β-Ocimene, a terpene, discovered later[28,30]. These BEP compounds are known to cause changes in the behavior and biology of the receiving nurse bees depending on the timing and amount of BEP's produced[26,31]. The effects of the specific BEP compounds can vary and range from methyl palmitate & methyl linolenate initiating capping to ethyl palmitate & methyl linolenate preventing ovary development in worker bees, among other effects[27,29,32,33].

These BEP compounds can be classified as kairomones, instead of pheromones, when they are intercepted by unintended target organisms such as ectoparasites like Varroa. The same BEP compounds that the brood produce to communicate with worker bees, such as methyl linoleate and ethyl palmitate, are intercepted by Varroa as signals on the timing for brood cell invasion[34–38]. Variation in BEP profiles exists between the different castes within a honey bee colony, and this affects the ability of Varroa to exploit them. For example, Varroa is often more attracted to drone brood as they produce a larger quantity of BEP compounds over a longer period of time compared to worker brood[39,40]. The BEP profile is also a major factor in the large reduction in Varroa infestation of queen cells as queen brood produce larger amounts of methyl oleate, which is a Varroa repellent[37].

Mite reproduction is tightly synchronized to brood development, with mite oogenesis linked to certain BEP volatiles produced by the brood at specific times[41,42].The first 12 h post capping of the brood cell are critical for mite reproductive success. A disruption in the BEP communication between the developing pupae and the mite during this time can cause the invading mite (foundress) to reabsorb any eggs that she has started to produce[41,43]. Therefore, even slight alterations in the brood's BEP profile could break the kairomone-timing network and lead to reduction in successful mite reproduction.

The aim of this study was to characterize the BEP profile of developing brood in the Varroa resistant population from Gotland, Sweden over time and identify any possible alterations of the BEP profile that could explain the reduced mite reproductive success observed in this population. This was approached by comparing the timing and quantity of cuticle volatiles produced by brood collected from the Varroa resistant honey bee population on Gotland, Sweden (hereafter referred to as resistant honey bees) with a control population of non-resistant honey bees. Cuticular volatiles were chemically extracted at biologically relevant time points during the early stages of the post-capping period when mite reproduction is initiated and were identified and quantified using gas chromatography (GC). We hypothesized that if a change in the BEP profile is responsible for the reduced mite reproduction phenotype in this resistant honey bee population, we would see a significant difference in the timing or quantity of the BEP profile produced between the two populations.

Methods

Twelve experimental honey bee colonies were established during June of 2021 from splitting six non-resistant honey bee colonies equally. Non-resistant colonies were purchased from a private beekeeper on Åland, Finland. Half of the colonies kept their original non-resistant queens and became the control group for this experiment, while the other six colonies were given mated queens obtained from the mite-resistant population located on Gotland, Sweden[20]. The colonies were given a minimum of four weeks to allow a replacement of the brood so that any larvae in the colony at the time of sampling were known to be produced by the resistant or non-resistant, control group queen. All experimental colonies were located in a single apiary at the Swedish University of Agricultural Sciences (GPS Coordinates: 59° 48′ 55.60596″, 17° 39′ 54.39866″) and managed with normal beekeeping practices with the exception that no Varroa control treatment was performed.

Eight-day old larvae were checked hourly to capture the time point when the brood cell was being capped. Using a transparent acetate sheet overlay on the frame of brood, cells that were newly capped were marked out as the 0 h for our experiment. Individual pupae were extracted from their brood cells over a time-series at 00, 06, 12, 18, 24, and 36 h post capping using the transparent acetate sheet overlay to identify the post-capping age of individual brood cells.

To extract the BEP volatile compounds, frames were removed from their respective hives and transferred to a designated indoor workspace. Using forceps, the cell capping was opened, and the developing pupae was careful removed. The pupae were placed on filter paper (Munktell's Swedish Filter Paper; No. 8, 9 cm) to ensure that their cuticle had not been punctured during removal and to locate any Varroa infestation. Pupae with a cuticle puncture or Varroa infestation were excluded from the experiment. Forceps were flamed between each colony and time point to minimize cross contamination of volatiles compounds.

Each chemical sample contained 4 pupae pooled together for each time point per colony. The pupae were submerged in 2 ml (1.25g) of n-pentane for 10 min, following the procedure detailed in Frey et al.[41]. Chemical extracts were stored in glass vials (Thermo Scientific 1.1 ml screw top tapered glass vials) and immediately put in a – 20 °C freezer before being transferred to a − 80 °C freezer.

For sample concentration, vials were removed from the freezer and left at room temperature for 5 min to thaw completely. They were then agitated for 15 s to homogenize the mixture before being concentrated to 100 µl under a gentle nitrogen flow.

For tentative identification of target compounds, samples were analyzed by gas chromatography-mass spectrometry (GC/MS) on an Agilent 7890N (Agilent Technologies) GC coupled to an Agilent 5975C mass selective detector (electron impact 70 eV). The GC was equipped with an HP-1 column (100% dimethyl polysiloxane, 50 m, 0.32 mm i.d. and 0.52 µm film thickness, J&W Scientific, USA), and fitted with a cold on column inlet. The GC temperature program was 30°C/4 min, 5°C/min to 150°C/0.1 min, 10°C/min to 250°C/15 min, using helium as carrier with a flow rate of 1.3 ml/min. BEPs present in the samples were identified by comparison against a commercially available library (NIST 08) and by comparison of mass spectra and retention indices with commercially available authentic standards (Sigma-Aldrich, Sweden). Based on the above analyses, the following compounds were selected for quantification: FAMEs methyl palmitate (Methyl hexadecanoate/MP), methyl linoleate (methyl (9Z,12Z)-octadeca-9,12-dienoate/ML) and methyl stearate (Methyl octadecanoate/MS), FAEEs ethyl palmitate (Ethyl hexadecanoate/EP), ethyl linoleate ((9Z,12Z,15Z)-Ethyl octadeca-9,12,15-trienoate/EL), ethyl stearate (Ethyl octadecanoate/ES), and the monoterpene, (*E*)-β-ocimene (EO).

For quantification, the concentrated samples (2 µl injections) were analyzed by gas chromatography (GC) on an Agilent 6890N with a flame ionization detector equipped with an HP-1 column (100% dimethylpolysiloxane, 50 m, 0.32 mm i.d., 0.52 µm film thickness, J&W Scientific, Folsom, CA), with hydrogen as carrier gas and fitted with a cold on column inlet.

The GC temperature program was 40°C for 1 min, 10°C/min to 280°C and held at 280°C for 10 min.

The amount of each target compound was calculated relative to the FID response to commercially available authentic standards (Merck, Sweden; 1 µl injection of 5 ng/µl standard solution).

Statistical analysis

Statistical analyses were performed using R version 4.0.1 and R Studio Version 1.3.959 using the R packages "lme4" (version 1.1.35.1), "car" (v. 3.1.2), "moments" (v. 0.14.1), "glmmTMB" (v. 1.1.8), "DHARMa" (v. 0.4.6), "performance" (v. 0.11.0), "RVAideMemoire" (v. 0.9.83.7), "emmeans" (v. 1.10.0), "effects" (v. 4.2.2), and "bestNormalize" (v. 1.9.1)[44,45] with all graphs made using the R package "tidyverse" (version 2.0.0).

A generalized mixed effect model was used to compare BEP differences between backgrounds. Individual models were used for each compound analyzed as well as the combined FAME and FAEE's results. The BEP compound quantities were used as a response variable, with background and time points used as fixed variables, and hive origin as a random variable. Zero inflation adjustment was performed on all models. Combined FAME, Methyl & Ethyl Linoloate, and (E)-β-Ocimene were square root transformed while combined FAEE, Methyl & Ethyl Palmitate, and Methyl & Ethyl Stearate were arcsign transformed to improve model fit. An estimated marginal means (emmeans) post-hoc pairwise comparison of the generalized mixed effect model was done for comparing the effect of background on compound levels within each time point, as well as with one step forward in time (i.e. 00H vs 006H).

Results

For all BEPs measured, we found lower amounts in the resistant population at almost all time points compared to the control population (Fig. 1, Table 1). While only the 00H time point was significantly different between our populations across all BEPs, we see a clear trend that lower amounts of BEP were produced by the resistant population compared to the control population in 38 out of 46 comparisons (83%). The main exception to this trend appears to be the production of methyl stearate at the 12H time point, which interestingly is also the compound with the most dramatic difference at the 00H time point ($p < 0.001$; Fig. 1).

Colony background was a significant factor for all chemicals, excluding EO ($p < 0.005$), indicating that the resistant Gotland bees have a unique overall BEP profile in the first 36 h post-capping when compared to the non-resistant population, characterized by the overall lower BEP production throughout. Time was also a significant factor for MP ($p = 0.047$), ML ($p < 0.005$), MS ($p < 0.005$), ES ($p < 0.005$), and EO ($p < 0.005$) with our non-resistant population having a much higher production of the stated chemicals at 0H before a significant decrease at the 6H mark, with the exception of EO which had a steady increase over time before decreasing at the 36H mark (Fig. 1, Table 1, Supplemental Table S1). The interaction between background and time was significant only for MS ($p < 0.005$) and ES ($p < 0.005$), with EL falling just short of significant ($p = 0.055$) (Fig. 1, Table 1) (Fig. 1, Supplemental Table S1).

The most significant differences between the populations occurred at 00H, with lower amounts of all BEP analyzed in the resistant population ($p = 0.0029$ (MP); 0.0049 (EP); 0.0008 (ML); 0.0038 (EL); < 0.0001 (MS); 0.0009 (ES)) (Fig. 1, Supplemental Table S1). This is continued by a non-significant trend throughout all time points of less compounds produced by the resistant colonies. For the non-resistant population there is a significant drop for ML ($p = 0.0003$), EL ($p = 0.0006$), MS ($p = 0.0018$), and ES ($p = 0.0003$) between 00 and 06H, returning to non-significant differences at further time points (Fig. 1, Supplemental Table S1). One exception is EO, where we instead see a steady increase until a significant drop in both populations at 36H.

Discussion

This study demonstrates biologically important differences in brood ester pheromones (BEP's) in a unique Varroa resistant population, compared to non-resistant control population, produced at time points during pupal development that are fundamentally relevant to disrupting Varroa mite reproduction. Specifically, a significant difference was observed between the two populations at the 00H time point just after the larvae are capped in their cells for pupation. Overall lower amounts of BEP were produced in the resistant population,

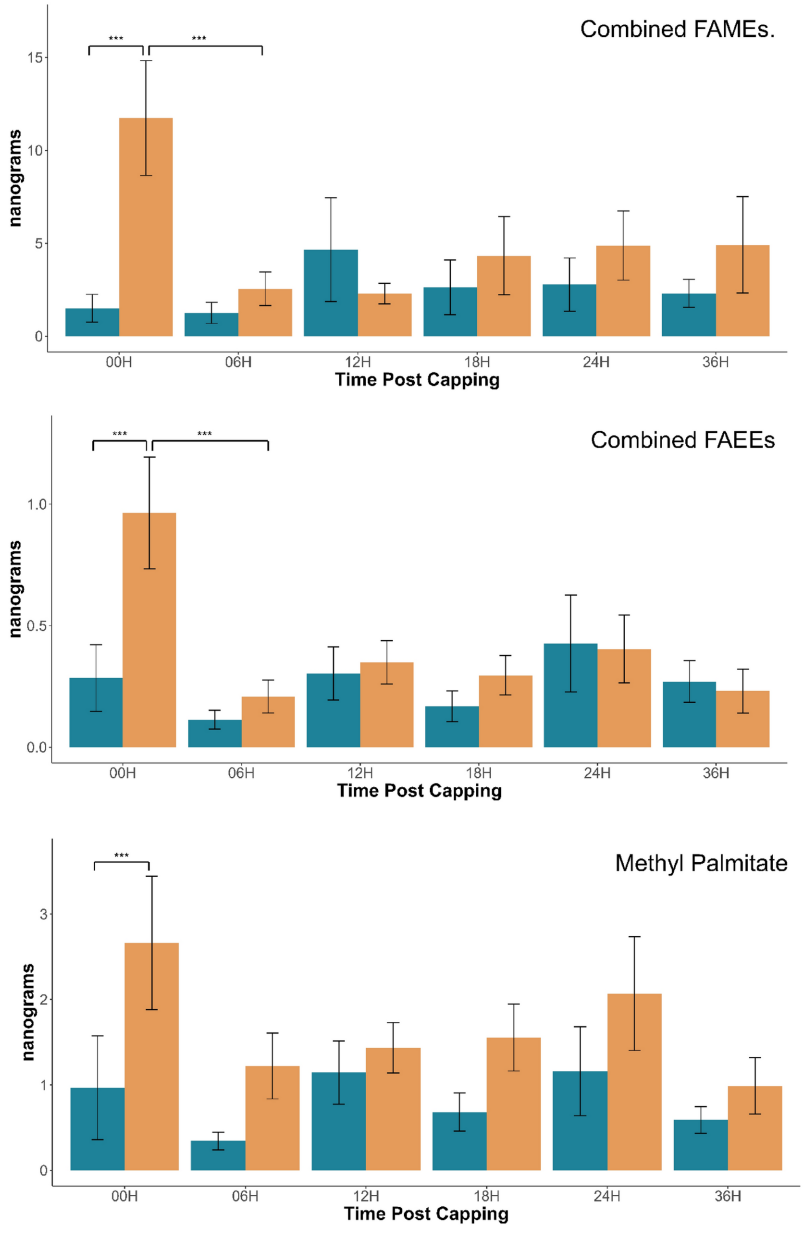

Fig. 1. Amount of named compound present on the cuticle of *Apis mellifera* worker brood 00, 06, 12, 18, 24, and 36 h after cell capping. Blue = Resistant, Orange = Non-Resistant. n = 18 (Resistant 00H, 06H, 12H, 18H, 36H; Non-Resistant 00H); n = 17 (Resistant 24H; Non-Resistant 06H, 12H, 18H, 24H, 36H) p value of significant differences added. Errors bars representing standard error used.

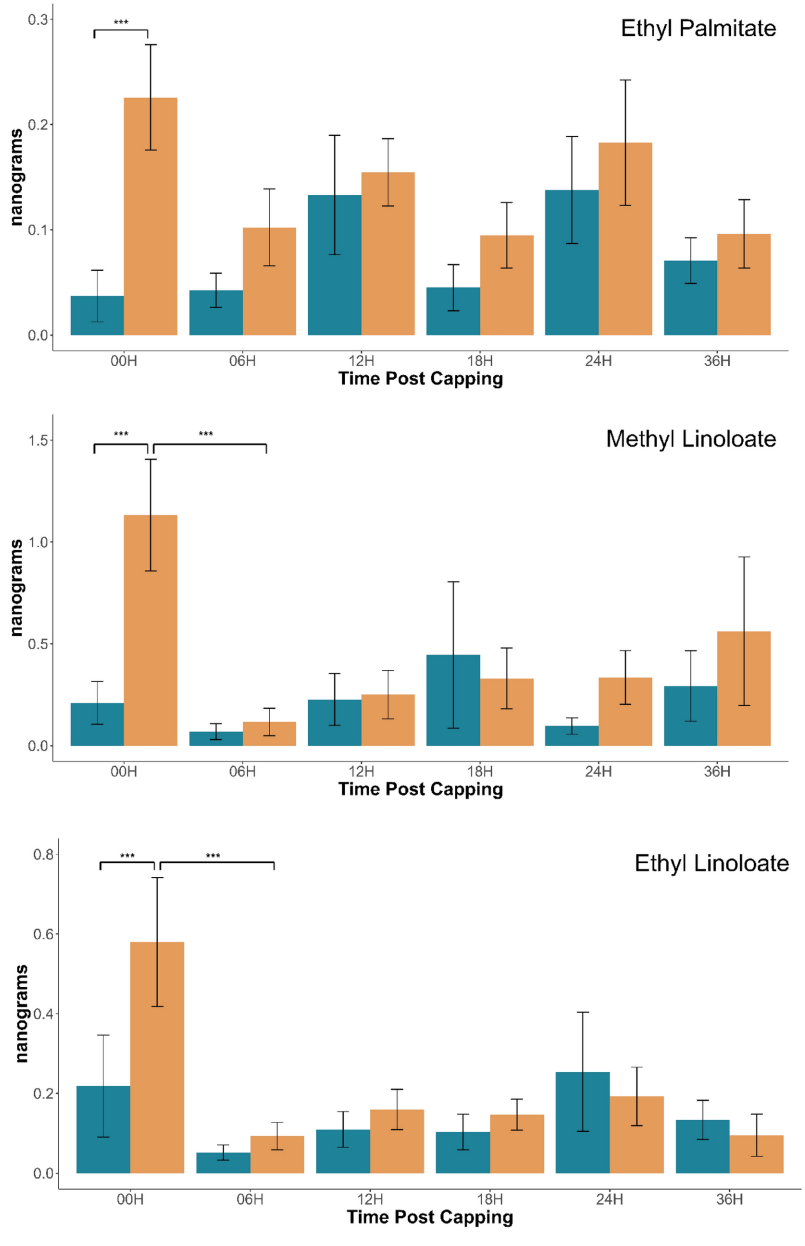

Figure 1. (continued)

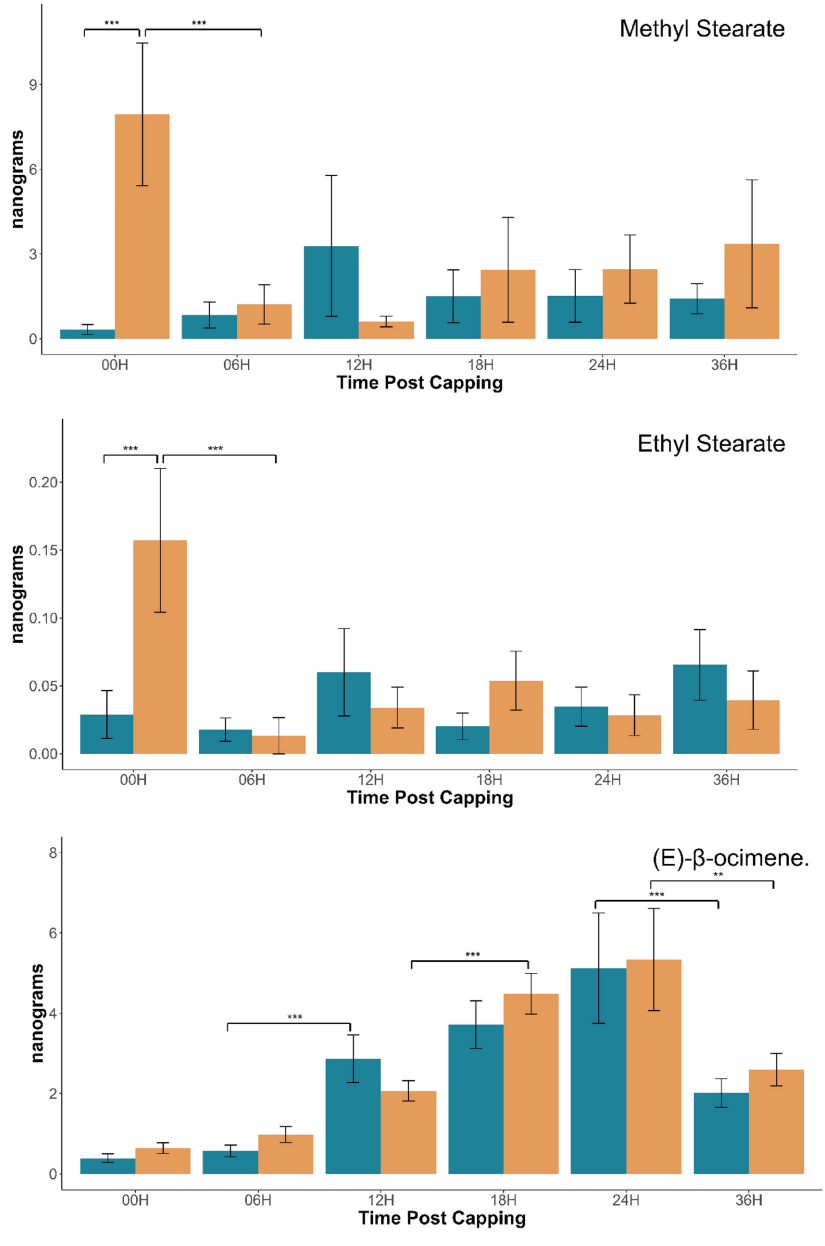

Figure 1. (continued)

	Chi Sq	df	p
A. Combined FAMEs			
Intercept	123.499	1	**< 0.005**
Background	19.476	1	**< 0.005**
Time	21.884	5	**< 0.005**
Back*Time	12.420	5	**0.029**
B. Combined FAEE			
Intercept	72.768	1	**< 0.005**
Background	14.519	1	**< 0.005**
Time	19.291	5	**< 0.005**
Back*Time	10.578	5	0.060
C. Methyl Palmitate			
Intercept	59.015	1	**< 0.005**
Background	14.562	1	**< 0.005**
Time	11.203	5	**0.047**
Back*Time	6.219	5	0.285
D. Ethyl Palmitate			
Intercept	38.849	1	**< 0.005**
Background	13.512	1	**< 0.005**
Time	10.535	5	0.061
Back*Time	7.165	5	0.208
E. Methyl Linoloate			
Intercept	59.205	1	**< 0.005**
Background	15.806	1	**< 0.005**
Time	22.693	5	**< 0.005**
Back*Time	10.024	5	0.075
F. Ethyl Linoloate			
Intercept	72.124	1	**< 0.005**
Background	12.658	1	**< 0.005**
Time	24.873	5	0.061
Back*Time	10.839	5	0.055
G. Methyl Stearate			
Intercept	84.552	1	**< 0.005**
Background	25.176	1	**< 0.005**
Time	28.960	5	**< 0.005**
Back*Time	21.992	5	**< 0.005**
H. Ethyl Stearate			
Intercept	43.436	1	**< 0.005**
Background	15.532	1	**< 0.005**
Time	25.677	5	**< 0.005**
Back*Time	16.508	5	**< 0.005**
I. (E)-β-Ocimene			
Intercept	84.552	1	**< 0.005**
Background	25.176	1	0.267
Time	28.960	5	**< 0.005**
Back*Time	21.992	5	0.581

Table 1. Results of generalized mixed effect model. Chemical in table title used as response variable. Background and time used as fixed variable. Hive was used as a random variable. Zero inflation adjustment was performed on all models. FAME, Methyl & Ethyl Linoloate, E-Ocimene were square root transformed to improve model fit. FAEE, Methyl & Ethyl Palmitate, Methyl & Ethyl Stearate were arcsign transformed to improve model fit. Significant values in bold. FAME & FAEE values calculated by adding all Methyl (FAME) or Ethyl (FAEE) values.

which suggests a type of chemical camouflage, more specifically what we have termed chemical whispering to disrupt or interfere with the initiation of mite reproduction. Many of these compounds have been found to be Varroa attractants as well as to initiate mite reproduction[34–38]. By reducing the overall BEP produced, the signal may be more difficult for the mite to intercept, while still being recognizable by adults with increased sensitivity to chemical recognition[46].

Frey et al.[41] found that if pupal BEP volatiles were artificially added to brood cells 24 h after capping (well after the 12 h critical period), there was a significant increase in mite reproduction. While the exact chemicals used by the mite to initiate oogenesis are still unknown, there is clear evidence that Varroa use BEP compounds as instigators for reproduction[37,38,41]. Frey further found a decline in BEP production around the critical 12 h post capping time point, suggesting these BEPs as possible candidates as Varroa reproductive kairomones. While the authors were not able to say that the two occurrences are linked, FAEE's may be involved in the initial activation of Varroa reproduction[41]. This could be a possible explanation to the observed lower amounts of BEP in our resistant populations.

Previous research with high resolution QTL analysis on the resistant honey bee population on Gotland found three genes relating to the Varroa resistance phenotype of reduced mite reproduction; Phantom, Cyp18a1, and Mblk-1[47]. While these genes are not directly linked to BEP production they are significant for brood health and development by initiating metamorphosis and molting through the ecdysone biosynthesis pathway[47–53]. This means they are all active during the critical mite reproduction time-period. However, the authors note that much of the resistant phenotype variation still remains unexplained by these genes[47]. Gene expression analysis of the BEP biosynthesis pathways, as performed by Qin et al. may be beneficial to understand not only the possible genetic components of the observed differences, but also the mechanisms that create differences in the final BEP products we observed in this study.

In order for a pheromone change to persist in a population there must not only be a different pheromonal profile created by the signaller (in this case, the brood), but also for it to be received and interpreted correctly by the receiver (in this case, the nurse bees). Theoretical modelling of the coevolution between signallers and receivers has two major predictions, that (1) the selective pressure of receivers should be greater than those on the signaller[54] and (2) when chemical communication is under strong selective pressure, natural selection should favour receivers that are able to detect a wide range of novel compounds as well as novel ratios of compounds[55]. Based on these predictions, if a signaler alters their BEP profile, a receiver theoretically should be able to adapt and correctly interpret the new signal, particularly in a system with strong selective pressure. Varroa represents a strong selective pressure towards its host with an exponential population growth rate and by vectoring viruses that lead to colony death. It is therefore not unlikely that adaptations on chemical communication in this population could have resulted in a short timeframe. While these arguments of "receiver advantage" can also be applied to Varroa's receiving of kairomones, this may be compounded with its lower genetic diversity and high occurrence of inbreeding compared to the honey bee[56–61].

In classic host-parasite co-evolution theory, the parasite is usually viewed as having the "advantage" in an arms race due to their shorter generation times and larger population size leading to more rapid adaptations than their host[62–64]. A rare advantage that the honey bee brood may have over Varroa however is that the unintended receiver of an olfactory signal, like the intended received, must be able to detect the specific compounds of the signal as well as be able to interpret them correctly[65,66]. While we know that Varroa possess the receptors necessary to intercept the broods signals[67], it has also been suggested that with minor changes in the emitter's genetics, new pheromone compounds and blends can be produced[55,68–72]. If this genetic variation pre-existed in the population, then the evolutionary response to parasitism may occur quite rapidly, in some cases only taking a handful of generations[73–75]. The possibility of rapid changes in pheromonal signals, as mentioned above, could result in the mite having increased difficulty in adapting to the shifting signals.

In predator–prey systems, where kairomones are intercepted by predator species, there are examples of prey changing their pheromonal composition to camouflage themselves over a relatively short ecological time frame[2]. Over just three years bark beetles (*Ips pini*) altered the blend of their pheromonal compounds between the preferences of two predators, as well as incorporating a synergistic compound that increased the receptiveness of conspecifics with no additional reaction from predators[2]. Similarly, the honey bee population on Gotland has been naturally exposed to uncontrolled Varroa infestation and displayed unique resistance phenotypes after only a short time. In parasite and parasitoid systems we can also see a reliance on cuticular kairomones by the parasite/parasitoids for information related to reproductive conditions that if disrupted may increase difficulties in finding hosts or spatial/temporal optimums[76–81]. This would reduce reproductive success and may be similar to what we are seeing in our own host/parasite system.

Further research is needed to determine how the differences in BEP between our resistant and non-resistant populations observed in this study have an effect on Varroa reproduction, and if there are trade-offs on the overall health and survival of these resistant colonies with these differences in BEP for the communication between brood and adult. Across other studies there is large variation in quantities, timings, and ratios of BEP profiles, making comparisons between different experiments difficult and raising questions on what a typical BEP profile is, or if one even exists[31,35,38,41,82]. While our study was designed to reduce temporal, spatial and methodological variation, we cannot be sure that the BEP profiles in the non-resistant honeybee can be considered standard. A large-scale study looking at brood BEP profiles across time, space, and genetics with standardized methodology would help to better understand what should be considered typical or atypical and would help when comparing populations in vastly different environments as well as to create a better understanding of brood development and capping signals. Further, in this experiment, only non-infested brood were collected in order determine a baseline BEP profile of our populations without interference of Varroa presence. An important consideration for future work would be to examine the plasticity of the production of BEP in response to Varroa mite infestation to determine if, and how, BEP profiles differ when larvae are infested. A behavioural assay on Varroa mite

choice for host selection, using similar methods to either Pernal et al.[83] or Li et al.[84], would also help provide an understanding of the mite's reproductive preferences. Coating live or dummy larvae with increased levels of the BEPs found to be reduced in the resistance larvae of this study, would help to characterize their role in both Varroa mite host selection and Varroa reproductive success. Finally, gene expression and proteomic analysis would contribute to a more complete understanding of what is happening with the brood during these critical time points of mite reproduction.

In conclusion, this study has demonstrated clear differences of BEP production at biologically relevant time points in the brood of a Varroa resistant honeybee population compared with a non-resistant population and provides a strategic foundation for future research looking at honey bee adaptations towards Varroa mite infestation and the evolution of this unique host-parasite system.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author (nicholas.scaramella@slu.se) on reasonable request. The dataset will also be stored in the Swedish National Data Service repository, [https://doi.org/https://doi.org/10.5878/h2hc-h513].

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Author contributions

N.S., B.L., and R.G. designed the experiments. N.S. collected samples, extracted volatiles, performed the chemical analysis, performed statistical analysis, wrote the main manuscript text. BL provided financial funding, and project oversite. RG interpreted gas chromatography data. All authors reviewed the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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The parasitic mite Varroa destructor threatens honey bees, yet some European colonies persist untreated, offering insight into natural resistance. This thesis examines three such populations, focusing on suppressed mite reproduction (SMR) and chemical communication. Brood traits, particularly altered pheromone profiles in Gotland bees, played a key role. Even when treated for varroa, Gotland colonies maintained low mite fertility, suggesting SMR is partly constitutive. These findings highlight mechanisms that may inform sustainable strategies for enhancing resistance in managed bees.

Nicholas Scaramella received his PhD education from the Department of Ecology, SLU, Uppsala. He obtained his Bsc degree from Humboldt State University, U.S.A. and his Msc from Uppsala University, Sweden.

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