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Use of *Apis mellifera* drone's olfactory sensitivity towards pathological odours as a selection trait in the breeding against *Varroa destructor*

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Abbreviations

9-ODA	(2E)-9-oxodecenoic acid
ABPV	Acute Bee Paralysis Virus
AL	Antennal lobe
csd	Complementary sex determiner
Cs	Conditioning stimulus
cAMP	Cyclic adenosine monophosphate
DNA	Deoxy ribonucleic acid
DWV	Deformed Wing Virus
EAG	Electroantennography
OR	G protein-coupled olfactory receptor
MNR	Mite non-reproduction
MB	Mushroom bodies
NMF	Natural daily mite fall
OBP	Odorant Binding Protein
PER	Proboscis extension response
QLT	Quantitative trait locus
RNA	Ribonucleic acid
RNAi	RNA interference
SMR	Suppressed mite reproduction
SNP	Single nucleotide polymorphism
Us	Unconditioned stimulus
VDV-1	Varroa destructor virus-1
VSH	Varroa-sensitive hygiene

1. Introduction

Honeycombs have been collected from nests by early hominids in the tropics starting 4 million years ago. Prehistoric bees and honey collection by *Homo sapiens* are depicted in cave paintings in present-day Spain dating from the late Pleistocene (Crane, 1999). Nowadays, the European honeybee (*Apis mellifera*) has significant economic importance as a honey producer and is an indispensable pollinator of crops. More than 35 of the 107 globally cultivated crops depend on it (Klein et al., 2007; Leonhardt et al., 2013). The annual market value of animal-pollinated crops amounts to 577 billion dollars (Potts et al., 2016). Moreover, beekeeping positively affects food security and sustainability on a planetary scale (Fikadu, 2020; Patel et al., 2021; Sillman et al., 2021).

In natural habitats, *A. mellifera* is the most frequent floral pollinator worldwide (Hung et al., 2018). During recent years, increased winter mortality of honeybee colonies has been recorded (Haber et al., 2019; Oberreiter and Brodschneider, 2020). The cause is a combination of various factors such as pathogens including parasites, pesticide use, poor nutrition, habitat loss (Becher et al., 2013; Neov et al., 2021). One of these factors – the mite *Varroa destructor* – plays a crucial role in honeybee colonies' health in Europe and North America (Genersch et al., 2010; Potts et al., 2016; Julie et al., 2021). *Varroa destructor* was brought to Europe in the 1970s from Asia, where it was a known parasite of the Asian honeybee *Apis cerana* (Oldroyd, 1999). Since then, the mite has spread worldwide, causing a spillover of honeybee viruses. *A. mellifera* populations are particularly affected by the Deformed Wing Virus (Highfield et al., 2009; Schroeder and Martin, 2012). It is still unclear whether honeybee viruses pose a threat to wild bees (Fürst et al., 2014; Loope et al., 2019; Gusachenko et al., 2020). So far, resistance breeding is considered the only long-term solution to the *Varroa*-problem (Guichard et al., 2020).

The following dissertation aims to develop a novel resistance breeding strategy against *V. destructor* using the drone's olfactory sensitivity. This thesis is structured in the following way:

- Chapter 2 introduces the two species – *A. mellifera* and *V. destructor*. Described aspects such as morphology, life cycle, pathogenesis, treatment management

and resistance breeding methods aim to create an accurate outline of the *Varroa*-problem. Furthermore, the experimental method – Proboscis extension response (PER) conditioning, used as the basis of the following work, is described.

- Chapter 3.1 (Publication I: "***Apis mellifera* worker bees selected for *Varroa*-sensitive hygiene show higher specific sensitivity and perception speed towards low concentrations of chemical cues emitted by the brood**") presents a comparison between the perception speed and perception ability of worker bees from control colonies and colonies bred for *Varroa*-sensitive hygiene (VSH) towards two types of odours – floral and the odour of *Varroa*-parasitised brood.
- Chapter 3.2 (Publication II: "**Suitability of drone olfactory sensitivity as a selection trait for *Varroa*-resistance in honeybees**") displays the use of *A. mellifera* drone's olfactory sensitivity towards odours connected to *V. destructor* parasitisation as a selection trait in the endeavour to create a novel resistance breeding method.
- Chapter 4 discusses the results of the work mentioned above and presents the conclusion and outlook.
- Chapter 5 summarises the work done in both German and English.

2. Literature

2.1. Biology of *Apis mellifera*

2.1.1. Systematics

Bees are believed to have arisen 100 - 120 million years ago in the Early Cretaceous (Danforth, 2007). Nowadays, around 25,000 species of bees are known. Their distribution is on every continent except Antarctica (DeWeerdt, 2015). Arid and semiarid regions such as the Mediterranean and southern Africa exhibit the greatest diversity of bees. The genus *Apis* is believed to have arisen around 35 million years ago. Ten species are classified in this genus (Raffiudin and Crozier, 2007):

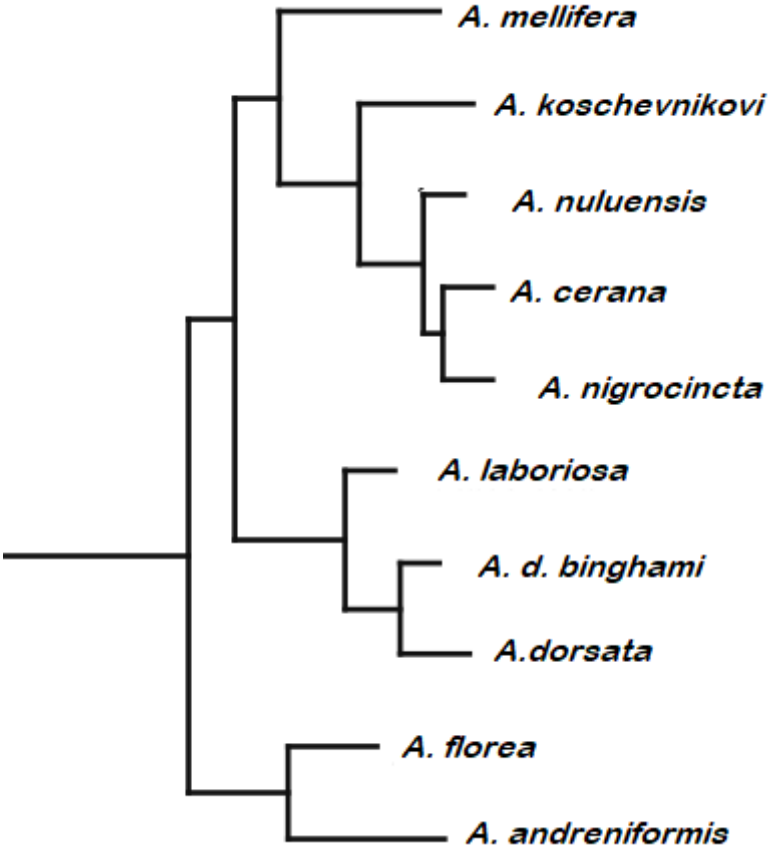

Figure 1 - Taxonomy of the genus Apis (modified from Raffiudin and Crozier, 2007)

The European, also Western, honeybee *Apis mellifera* is a relatively new eusocial species in this genus, compared to some of the older species such as *Apis dorsata* and *Apis florea* (Culliney, 1983). It is considered to be of African origin. The *A. mellifera* genome is the third insect genome that was fully sequenced. Nowadays, *A. mellifera*

acts as a model organism for social behaviour and studying human health issues (Weinstock et al., 2006).

2.1.2. Honeybee genetics

The honeybee is a haplodiploid organism. The number of chromosome sets determines the sex. Females are diploid and develop from a fertilized egg. Males, on the other hand, are derived from unfertilised eggs. As a result, they are haploid, and inherit a single set of chromosomes from the queen (Wilfert et al., 2007; Conlon et al., 2018). Diploid males have also been described (Beye et al., 2003). These males are sterile and are cannibalised by nursing bees right after hatching (Herrmann et al., 2005).

Queen bees naturally mate with multiple drones, so that the bee colony consists of a mixture of different patrilines. Due to the haploidy of the male sex (drones) in the honeybee, drones pass on their entire genome to their offspring, without the usual Mendelean sample variance. The female offspring, on the other hand, are derived from a representative selection of the genetic material of the mother and the father. Consequently, the offspring of a drone and a diploid mother share on average 75% of their genes (Blanchetot, 1991). Members within patrilines are 75% related, while members of different patrilines are only 25% related (Ratnieks, 1988; Blanchetot, 1991).

Because of the haploidy, no dominance interaction between alleles at the same locus is present in drones. The haploidy of the drones thus promotes genetic differentiation and the emergence of specialists within the honeybee colonies, which is of great advantage for the colony and the division of labour within (Ashby et al., 2016). Furthermore, drones are considered especially suitable for selection and breeding purposes as alleles are inherited by the offspring without change (Benatar et al., 1995; Lefebre et al., 2024). The use of this natural mechanism plays a central role in the work presented in the course of this dissertation.

2.1.3. Social structure and life cycle

The honeybee colony comprises a reproductive queen, 20,000-40,000 sterile female workers, and a few thousand drones (Stephen et al., 1969; Page and Peng, 2001).

The honeybee goes through four metamorphosis stages to reach its adult form – egg, larva, pupa, imago. Depending on the diet, a female larva can develop into a queen or a worker. This determination can take place up to the third day of larval development. Larvae, which are chosen to be queens, are fed with royal jelly by the nursing bees. Worker bees receive royal jelly for three days. Afterwards, it is mixed with beebread and honey. Through phytochemicals such as *p-coumaric acid* in the diet of larvae developing to worker bees, differential regulation of genes occurs, determining the cast and reducing the ovary size development in worker bees (Mao et al., 2015).

During the summer, the queen produces up to 1,000-2,000 eggs per day. In late autumn and winter, honeybee queens cease laying eggs due to low temperatures. The queen lays fertilised worker eggs in smaller cells or unfertilised drone eggs in larger cells (Snodgrass, 1956). The eggs are laid vertically at the cell's bottom (Stephen et al., 1969). They are oval and of a whitish colour. On the first day, the egg is upright in the cell. During the second day, it tilts to the side. On day three, it lies on the brood cell floor (DuPraw, 1961; Aupperle and Genersch, 2016).

On day three, a white, soft larva hatches from the egg. Its size is about 2 mm. It is covered in a chitinous cuticula. Because of the cuticula's inability to grow, the larva undergoes four moults during its larval stage (Nelson, 1924; Aupperle and Genersch, 2016). The larva then stretches and fills the length of the cell. The cell is capped at day eight. The larva spins a cocoon, and the metamorphosis begins.

The first three days after the cell capping are marked by the pre-pupal stadium. During this time, the larva transitions into a pre-pupa without moulting (Bertholf, 1925). The head, thorax, and abdomen are well pronounced. The pre-pupa retains the white colour and the softness of the larva. The fifth moult takes place, and a white pupa emerges (Bertholf, 1925). The pupal development undergoes three stages – white-eyed stage, red-eyed stage and brown-eyed stage (Aupperle and Genersch, 2016). After the final sixth moult, the wings expand, and the hair covering the body is exposed (Bertholf, 1925). The imago emerges from the cell at different times depending on sex and caste.

Queens. Queens live the longest – 1-3 years (Page and Peng, 2001). They weigh about 250 mg (Aupperle and Genersch, 2016) and need 16 days to develop from an egg to an adult. An elongated abdomen is typical for queens as they have working ovaries. A queen reaches sexual maturity 5-6 days after emergence (Ruttner, 1956).

During all her lifespan, the queen is taken care of by the workers. The queen exhibits smaller eyes and mouthparts than worker bees (Aupperle and Genersch, 2016).

Virgin queens make 1-3 mating flights and mate with several drones on one single flight (Woyke, 1955). The sperm is stored in the spermatheca, where it stays viable for 3-5 years (Page and Peng, 2001). The communication between the queen and workers in the colony is exercised through pheromones produced in the queen's mandibular glands (Winston, 1987; Pettis et al., 1997). A decrease in the produced queen mandibular pheromone, an injury or an exhausted sperm storage can lead to the queen's supersedure (Butler, 1946; Winston et al., 1990; Pettis et al., 1997; Tarpy et al., 2000). Usually, more than one queen is reared by the worker bees in special queen cells. The first queen to arise kills the other not yet hatched ones. Sometimes, the old and the new queen live together in the colony for a short period. The old queen either leaves the hive with a swarm or is killed by the new queen (Butler, 1946).

Worker bees. The worker's development lasts 21 days from egg to adult. Once emerged from the cell, the worker lives up to six weeks in the summer and about four months in the winter (Page and Peng, 2001). A worker bee weighs about 90 mg. Its abdomen is shorter than that of the queen. A clear labour-division in the colony can be observed (Wheeler, 1986). As the worker bees age, their physiology changes, so do the tasks they perform (Winston, 1987; Aupperle and Genersch, 2016). The newly emerged workers clean the nest (days 1-3) and feed the larvae (days 4-10). Middle-aged workers process food, guard the hive entrance and construct cells in the nest (days 11-20). The oldest worker bees engage in foraging behaviour (days 21-40) (Page and Peng, 2001; Aupperle and Genersch, 2016).

Once the temperature drops below 10°C, honeybees cease flying. Workers that hatch at the end of the summer – so-called winter bees – have a prolonged lifespan as they are crucial to the hive's survival until next spring. Winter bees exhibit higher fat body mass, allowing them to withstand unfavourable environmental conditions (Koubová et al., 2021). At the beginning of spring, winter bees are responsible for producing food for the new generation of worker bees.

Worker bees are sterile. In the case the queen is dead, and no new queen can be produced, workers can start laying unfertilised eggs. Only drones develop from those (Page and Erickson, 1988).

Drones. Drones are reared only in the swarming season because of their higher nutritional requirements (Winston 1987; Langowska and Zduniak 2020). Drones need 24 days to develop from an egg to an adult drone. Their size is about the same as the queens' and double the workers' size (Winston, 1987). The weight of a drone is 190 mg (Aupperle and Genersch, 2016). Drones have a shorter proboscis and no pollen baskets. After they emerge from the brood cell, drones are dependent on the nursing bees for the protein needed for their maturation (Szolderits and Crailsheim, 1993). Two weeks after their emergence, drones start their mating flights and gather at so-called drone congregation sites. Drones with a higher body mass at emergence tend to survive until maturation more often and produce more sperm (Czekońska et al., 2019). The drone's endophallus is ripped off during successful mating, leading to the drone's death (Woyke, 1955; Page and Peng, 2001).

The drone' average life span is 21-32 days (Fukuda and Ohtani, 1977; Page and Peng, 2001). Drone numbers in the colony are adjusted to the environmental conditions and food scarcity by expelling and killing them (so-called drone slaughter). This is a significant factor for their life expectancy (Fukuda and Ohtani, 1977; Page and Peng, 2001; Czekońska et al., 2019).

2.1.4. Sexual dimorphism in the honeybee olfactory system

Olfactory sensitivity and odour perception play a crucial role in the functioning of the honeybee eusocial construct (Rogers et al., 2013; Breed et al., 2015). Olfactory signals are first registered in the olfactory receptor neurons which are located in special cuticular structures (sensillae) on the honeybee antennae (Masson and Arnold, 1987; Sandoz et al., 2007). The most common sensilla is *sensilla placoidea* which plays a role in olfaction (Esslen and Kaissling, 1976). Other known sensillae are *s. basioconica*, *s. trichoidea*, *s. coeloconica*, *s. campaniforme*. While some of them fulfil a function connected to olfaction, others act as mechano- and temperature receptors (Esslen and Kaissling, 1976).

There is a well-pronounced sexual dimorphism between the antennae of worker bees and drones. Drone antennae have 11 segments and exhibit a higher number of sensory cells (~ 340,000) than worker bees (10 segments, ~ 65,000 sensory cells) (Esslen and Kaissling, 1976). On the drone's antennae only one sensillum type - *sensilla placoidea* - appears in large numbers. Other sensilla types are either missing

or diminished in numbers (Mariette et al., 2021). Drones' antennae are specialized in the perception of the queen pheromone (2E)-9-oxodecenoic acid (9-ODA). Worker's antennae, on the other hand, possess receptors connected to the distinction of floral odours, pheromone communication and cuticular hydrocarbon perception (Jain and Brockmann, 2020).

When odorant molecules come in contact with the antennae, they are usually transported to the membrane of the receptor neurons through diffusion or with the help of odorant binding proteins. Once the odour molecule has bound to the receptor, a cellular transduction cascade involving the second messenger cyclic adenosine monophosphate (cAMP) is activated. As a result, the receptor membrane depolarises (Sandoz et al., 2007).

The signal is transmitted via the antennal nerve to the antennal lobe (AL) which acts as the primary olfactory centre in the insect brain (Szyszka et al., 2005; Sandoz et al., 2007). The anatomical and functional units of the AL are the glomeruli. These spheroid neuropile units enable the synaptic interaction between different types of neurons (Sandoz et al., 2007). The drone's AL consists of 103 glomeruli, four of which are hypertrophied, known as macroglomeruli (Arnold et al., 1985). These macroglomeruli are responsible for the processing of female pheromones such as 9-ODA. Worker bees possess 165 normal glomeruli in their ALs which process both floral signals and social pheromones (Sandoz et al., 2007).

The information from the AL is delivered to the higher brain areas - the lateral horn and the mushroom bodies (MB) – through five antenno-cerebral tracts consisting of axons of projection neurons (Szyszka et al., 2005). The role of the lateral horn most probably consists in the separation of axons of the pheromone from plant sensitive neurons as shown in *Drosophila spp*. (Jefferis et al., 2007). The MB comprise densely packed highly odour specific neurons - the Kenyon cells (Sandoz et al., 2007). The role of this centre is connected to olfactory learning and memory (Sandoz et al., 2007).

2.2. Biology of *Varroa destructor*

2.2.1. Systematics

Varroa destructor is a representant of the class *Arachnida*, subclass *Acari*, order Mesostigmata. In the Genus *Varroa,* at least four species are known: *Varroa jacobsoni,*

Varroa underwoodi, Varroa rindereri and Varroa destructor. The virulence of the above-described species of *Varroa*-mites differ toward *A. mellifera* (Rosenkranz et al., 2010). Until the year 2000, *V. jacobsoni* was considered responsible for causing symptoms in infested honeybees (Anderson and Trueman, 2000). As it was later discovered, only *V. destructor* causes substantial damage to the honeybee colony and research based on *V. jacobsoni* before the year 2000 was in most cases referring to *V. destructor* (Rosenkranz et al., 2010).

Initially only found with the Asian honeybee (*A. cerana*), *V. destructor* experienced a host shift when the European honeybee was brought to Asia for beekeeping purposes (Oldroyd, 1999). The *Varroa*-mite was introduced to Europe in the 1970s and the Americas in the 1980s (Wantuch and Tarpy, 2009). Currently, there are three known haplotypes of *V. destructor* – C (China), K (Korea) and J (Japan) – each of them consisting of multiple haplogroups and variants (Lin et al., 2021). The host shift from *A. cerana* to *A. mellifera* occurred at least twice. The K haplotype is more widespread because of the global honeybee trade (Anderson and Trueman, 2000; Lin et al., 2021) and shows higher reproduction rates than the Japanese haplotype (Anderson and Trueman 2000; Roberts, Anderson, and Tay 2015; Evans and Cook 2018). The relatively high density of honeybee colonies in some regions in Europe favoured the spreading. In 2008 the sister species of *V. destructor* – *V. jacobsoni* – was observed parasitising the European honeybee in Papua New Guinea for the first time, thus posing another potential threat to worldwide beekeeping (Roberts et al., 2015). So far, only some isolated islands are granted the *Varroa*-free status. Australia, which managed to keep its honeybee population *Varroa*-free since the first introduction of the mite in Europe in the 1970s, registered a *V. destructor*-infestation at over a hundred sites in 2022 (Chapman et al., 2023).

2.2.2. Morphology

Varroa-mites exhibit a pronounced sexual dimorphism (Ifantidis, 1983). While the females are 1.1 mm long and 1.6 mm wide, males are only 0.7 mm long and 0.9 mm wide (Chauhan et al., 2021). The body of both sexes is divided into a small gnathosoma and a larger idiosoma. The males are of whitish-transparent colour and a pear-shaped form and stay smaller than the females during their whole life cycle. Females are reddish-brown, with a dorsoventrally flattened ellipsoid form. The dorsal and ventral

shields of the *Varroa*-female are sclerotised. The legs of the female are strong and relatively short. A sensory pit organ for the perception of volatiles is located on the tarsi of the first leg pair. It consists of nine internal sensilla with nine longer hair sensilla that encircle the organ (Rosenkranz et al., 2010). Different kinds of hair cover the *Varroa*-female's whole body, some with chemo- or mechanoreceptive functions (Rosenkranz et al., 2010). The palptarsus also exhibits chemoreceptive sensilla, which allow the perception of a broad range of odours (Liu and Peng, 1990).

Once *V. destructor* finds a host, it uses a chemical camouflage to make itself undetectable. Exchanging cuticular hydrocarbons is a mechanism described in invertebrates such as termites (Vauchot et al., 1997). Some invertebrates' cuticula can absorb the "odour" of their cohabitants, creating a sense of belonging to the group. In the case of *V. destructor*, this mechanism is used for chemical camouflage. For the mite to mimic the host's odour, direct contact between the mite and the host's cuticular lipid layer is needed. Even dead mites exhibit the ability to absorb their host's odour (Kather et al., 2015). Honeybees of different age exhibit differences in their cuticular profile. Whereas mites collected from adult honeybees present higher levels of alkenes in their cuticula, mites found on pupae show higher methylalkene levels. If the mites change their hosts from adult to pupae or vice versa, their cuticular profile is adjusted to resemble that of the new host in the first few hours after transition (Kather et al., 2015; Le Conte et al., 2015).

The mite's ontogenetic stage also plays a role in forming the cuticular profile. Immature mites display a strong resemblance to the pupal cuticula and faecal material, which could be explained by the more intensive contact between the developing mite with the pupa and its faeces. On the other hand, mature mites exhibit bigger differences to the healthy pupa’s cuticular profile – a possible reason for their better detection through nursing bees (Martin et al., 2002).

2.2.3. Host finding and life cycle

Varroa destructor is an ectoparasitic mite, and all developmental stages parasitise on honeybees. Hence, it relies on the honeybee for its survival. Up to this date, a broad spectrum of publications exploring mite behaviour and host finding strategies exist; still, the key factors have not yet been identified.

Varroa destructor has no visible eyes or ears; nevertheless, it can perceive light stimuli and vibration (Kirchner, 1993), but none of those is considered crucial to the mite's host finding ability. On the other hand, semiochemicals play an essential role in host acquisition (Pernal et al., 2005). The mite's ability to recognise the age/function of the worker bee is an important survival mechanism. While newly emerged honeybees and foragers are considered least attractive to *V. destructor*, middle-aged honeybees and fifth instar larvae are most luring (Kraus et al., 1986; Kuenen and Calderone, 1997; Pernal et al., 2005; Rosenkranz et al., 2010; Xie et al., 2016). If the mites are experimentally removed from their host, they will move towards a newly emerged worker bee provided there is no other choice (Pernal et al., 2005). This behaviour may pose a survival mechanism considering that *Varroa*-mites can only survive a limited time away from their host.

Different compounds have been identified to act as *V. destructor* attractants – methyl-palmitate, ethyl palmitate and methyl linolenate. Of these, methyl palmitate displays the highest mite attraction levels (Le Conte et al., 1989). These esters act as brood pheromones and elicit capping behaviour (Le Conte et al., 1994). During the pre-capping stage, these esters' levels increase (Boot et al., 1992), guiding the mite to its new host. Drone larvae produce more brood pheromones, thus posing a more prominent attraction to the mites (Le Conte et al., 1989; Calderone and Lin, 2001; Rosenkranz et al., 2010).

The female mite goes through two life stages – reproductive and phoretic. During the former, the female mite enters an open brood cell with a pupation-ready larva just before it is sealed (Rosenkranz et al., 2010; Evans and Cook, 2018). Worker brood is infested 15-20 h before capping and drone brood 40-50 h before capping (Boot et al., 1992). Specific cues in the mite's surroundings act as a trigger for the oogenesis. The developmental stage of the host larva is one of these cues (Frey et al., 2013).

Garrido and Rosenkranz (2004) observed the activation of the mite's oogenesis when a honeybee larval extract consisting of the polar fraction of the cuticula was presented to them. Frey et al. (2013) confirmed these findings with an in-hive assay. In order to identify whether different stages of larval development can stimulate or interrupt the reproduction of *Varroa*-females, the mites were introduced in the capped cell at different times. When the mites were transferred into the brood cells later than 18 h (worker brood) or 36 h (drone brood) after capping, their reproduction rate decreased

significantly compared to the control group. Chemical analysis showed a decline in the levels of three fatty acid ethyl esters in the larval cuticula during this period - ethyl palmitate, ethyl oleate and ethyl stearate. The decline rendered *Varroa*-mites unable to reproduce. These fluctuations in the host's "odour" might act as a stop sign for the mite's reproduction. Frey et al. (2013) also proved that once the oogenesis has begun, it can be interrupted by a change of the host milieu, i.e., by moving the mite in another cell.

Once inside the capped brood cell, the *Varroa*-mite lays the first egg around 60 h after capping. It remains unfertilised and develops into a male (Ifantidis, 1983; Piou et al., 2016). The following up to 5 diploid eggs are laid in a 30-hour interval. They are fertilised and develop into females. The male pairs with its sisters and dies before the honeybee emerges. When the honeybee emerges from the brood cell, the same foundress *Varroa*-mite can slip into another brood cell and undergo another reproduction cycle. Up to three reproduction cycles are possible in one summer (Martin and Kemp 1997).

Newly moulted females pose a bigger attraction to males than older mites. The younger the female mite, the more potent the sex pheromones it emits (Häußermann et al., 2015; Evans and Cook, 2018). The sensory pit organ of the male mite plays a crucial role in reproduction. Häußermann et al. (2015) conducted a bioassay with two control groups (non-varnished male mites, male mites with varnished idiosoma) and a group of males with varnished front legs. The three groups were tested on their reproductive behaviour in the presence of newly moulted females. While the two controls showed copulation attempts, the males with varnished front legs showed no such, confirming the sensory pit organ's vital role for the reproduction of the *Varroa*-mite.

Not all daughter-mites mate during the reproductive cycle. Some of them leave the brood cell as virgins. No mating is needed to produce male offspring. If a virgin mite enters a new brood cell, a haploid egg is laid, and the virgin mite mates with its son. This allows the female mite to successfully reproduce in the next reproductive cycle (Häußermann et al., 2020).

Once the reproduction has taken place, and the honeybee has hatched, the *Varroa*-mite and her female offspring enter the phoretic state while searching for a new brood cell to enter. The phoretic stage duration can vary depending on the amount of brood and the colony's strength. The mite hides under the honeybee's abdominal sternites,

where it can only be reached by other honeybees with great difficulty (Nazzi and Le Conte, 2016). Mites usually transfer onto older nursing bees in order to be transported to the next brood cell.

In the Asian honeybee (*A. cerana*), *V. destructor* reproduces solely in the drone brood. In the European honeybee, the mite infests both drone and worker brood (Boecking and Spivak, 1999; Dillier et al., 2006). The duration of the post-capping period acts as a limiting factor to the reproduction of the *Varroa*-mite (Rosenkranz et al., 2010; Oddie et al., 2018b). Drone brood has the longest one (14 days), followed by workers (12 days) and queen brood (8 days) (Fuchs, 1992). Drone brood shows the highest infestation rates in the colony – 8-10 times higher than the worker bee brood (Calderone et al., 2002; Calderone and Kuenen, 2009). One of the reasons for this may be that drone brood is more frequently tended by the nursing bees. This increases the likelihood of the drone larvae being infested with mites. (Rosenkranz et al., 2010). Another possible explanation is the higher quantities of brood pheromones produced by drone brood during the longer development period (Calderone et al., 2002). Cell size, the distance between cell rim and larva, and the brood cell shape play a role in the mite's orientation. The smaller the distance between the larva and the cell rim, the higher the infestation frequency (Goetz and Koeniger, 1993; Calderone et al., 2002). Moreover, repeatedly used brood cells with many cocoon debris show a significantly higher mite count than new brood frames (Piccirillo and De Jong, 2004). While drone cells are preferred, some substances in the hive, such as royal jelly, act as a repellent against *Varroa*-mites, explaining the low infestation rates of queen brood (Calderone et al., 2002; Nazzi et al., 2009).

2.2.4. Population dynamics of *Varroa destructor*

In a temperate climate, the *Varroa*-population in the colony increases gradually and reaches its maximum in autumn. Following the stop of brood rearing, *Varroa*-numbers in the colony decrease in winter (Vidal-Naquet, 2015; Fig. 2).

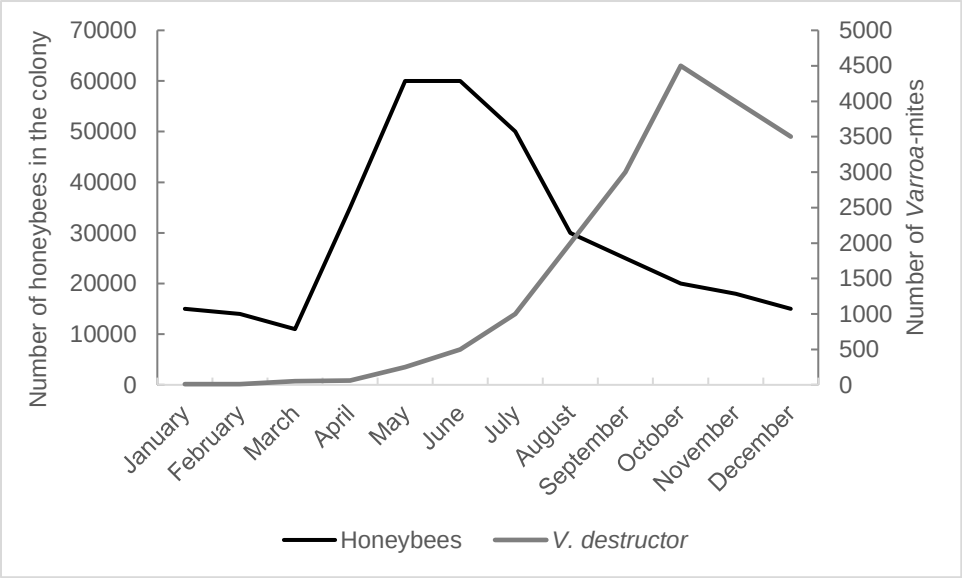

Figure 2 - Annual population dynamics of *Varroa destructor* (adapted from Vidal-Naquet, 2015).

Rising temperatures due to climate change in recent years have been described as a major factor in the increase of *Varroa*-numbers in autumn. Higher temperatures in March-May and October can lead to the elongation of the brood-rearing period in honeybee colonies, leading to a higher brood availability in autumn (Smoliński et al., 2021). As a result, the *Varroa*-strain on the colony increases.

2.2.5. Pathogenesis

Until 2019 scientists considered that *V. destructor* damages host honeybees by consuming their haemolymph (Kanbar and Engels, 2005; Annoscia et al., 2019). Ramsey et al. (2019) discovered that the *Varroa*-mite feeds on the fat body tissue (the equivalent of the mammal liver regarding metabolism) of the infested larvae and adult honeybees, thus weakening them. Consequently, honeybees lose weight and show reduced flight performance. Additionally, the lifespan of the infested honeybees is considerably decreased. Infested honeybees also have a significantly lower learning capacity than healthy honeybees and often do not return after a flight. *Varroa destructor* also decreases the honeybees' water regulation capacity by feeding on them. As a result, honeybees are more prone to stress (Annoscia et al., 2012). Through contact with honeybees from other hives during foraging, a ubiquitous spreading of the mite is assumed to occur (Rosenkranz et al., 2010). *Varroa destructor* displays high

agility and can even infest foraging honeybees from flowers during nectar collection (Peck et al., 2016).

Varroa destructor negatively affects larval provisioning at the colony level, leading to a decreased weight of pupae. Lower protein content has also been described in *Varroa*-infested pupae, which might cause their decreased growth (Aronstein et al., 2012).

Honeybees hatched in late summer – so called winter bees - are essential for the survival of the colony during winter. Their role is to regulate the hive's temperature during the cold months and take care of the queen. In spring, winter bees nurse the new generation of summer bees. An infestation with *V. destructor* during the pupal stage leads to a significantly lower vitellogenin level in the haemolymph. Vitellogenin acts as a storage protein and is used by nursing bees to produce brood food (Amdam et al., 2003). Hence decreased protein levels in the haemolymph might lead to impairment of the winter bees and the inability to produce food for the new generation of honeybees in spring. In different studies, both Amdam et al. (2004) and Zaobidna et al. (2017) observed a down-regulated immune system in younger worker bees infested with *V. destructor* during their pupal stage. As a result, hives are more susceptible to *Nosema* spp., Deformed Wing Virus (DWV), Acute Bee Paralysis Virus (ABPV) and American foulbrood. If the hive is not treated during the late summer against *V. destructor*, the winter bees' lifespan decreases, making the colony more likely to collapse (Amdam et al., 2004).

2.2.6. Deformed Wing Virus (DWV)

Varroa destructor not only weakens the individual honeybee and thus the colony but also acts as a vector and reservoir for various viruses (Berényi et al., 2006; Martin et al., 2012; Woodford et al., 2022). Of these, the DWV plays the most important role as a secondary pathogen in the increased loss of honeybee colonies worldwide (Schöning et al., 2012). DWV is a positive-stranded RNA virus of the genus Iflavirus with three major structural proteins (Lanzi et al., 2006). Currently, there are three known genotypes – DWV A, DWV B (also known as *Varroa destructor* virus-1: VDV-1) and DWV C (Ongus et al., 2004; Lanzi et al., 2006; Mordecai et al., 2016; Posada-Florez et al., 2019). In recent years, the prevalence of the highly virulent genotype B has been observed in different countries, amongst others Italy, Germany, and the UK (Norton et al., 2020; Paxton et al., 2022).

The virus demonstrates the ability to replicate in the honeybee and the mite host (Bowen-Walker et al., 1999; Ongus et al., 2004; Yue and Genersch, 2005; Yue et al., 2007; Gisder et al., 2009). Since the spread of *V. destructor*, DWV has become ubiquitous in most *A. mellifera* populations (Grozinger and Flenniken, 2019; Beaurepaire et al., 2020; Paxton et al., 2022). DWV has been detected not only in the European honeybee but also in the Asian honeybee and wild populations of bumblebees (*Bombus terrestris, Bombus pascuorum)* and other arthropods (Genersch et al., 2006; Fürst et al., 2014; Nanetti et al., 2021). It is not yet clear whether DWV also plays a role in the decline of wild pollinator populations (Fürst et al., 2014).

Not every mite acts as a carrier. Even when 100% of the mites are infected with DWV, not all parasitised pupae develop deformities (Yue and Genersch, 2005). DWV can be transmitted vertically – through the queen's eggs or the drone's sperm (Yue et al., 2007) – or horizontally – through larval food (Yue and Genersch, 2005; Ryabov et al., 2014). Both lead to a covert infection. However, if the pupae are infested with *V. destructor*, the virus is transmitted directly into the haemolymph. Due to the slow development of the DWV, the pupae survive the infection. After hatching, some young honeybees show morphological changes such as paralysis of the legs and wings and a shortening of the abdomen. Even without visible changes, infected honeybees have a 50-57 % lower lifespan (Schroeder and Martin, 2012), tend to fly shorter distances than non-infected honeybees (Wells et al., 2016), show disorientation and learning difficulties. The virus amplifies the effect of the *Varroa*-infestation on the colonies (Yang and Cox-Foster, 2005; Zaobidna et al., 2017). A monitoring study performed in Switzerland in 2007/2008 displayed a reduction in honeybees' life expectancy and higher winter mortality when *V. destructor* and DWV are present (Dainat et al., 2012). *Varroa*-mites that spend more time on the host honeybees carry higher DWV loads (Piou et al., 2016). By combating *V. destructor*, the burden of DWV decreases at the same time.

2.2.7. Winter losses

To give an idea of the scale of the *Varroa*-problem, this section focuses on the pathology of *V. destructor* and the consequences of an infestation by summarising some figures. Starting in 2004, the German bee monitoring project analysed different factors for winter colony losses and named *V. destructor* the leading cause of winter

losses in Germany during the period 2004-2008 (Genersch et al., 2010). In Southern Spain, the mite and DWV were considered the main factor for colony weakening during a two-year survey in 2015-2017 (Barroso-Arévalo et al., 2019).

In Ontario, Canada, *V. destructor* accounted for more than 85% of the total cases of colony mortality (27.2%) in the years 2007 and 2008 (Guzman-Novoa et al., 2010). In Luxembourg, 26% winter colony losses were registered in the winter of 2014/2015 (Beyer et al., 2018). Due to *V. destructor* in Austria, the winter colony losses amounted to 15.2% in 2018/2019 (Oberreiter and Brodschneider, 2020). In New Zealand, the colony losses attributed to the mite infestation have risen from 16.9% in 2017 to 38.9% in 2021 (Stahlmann-Brown et al., 2022).

2.2.8 Diagnosis of a *V. destructor* infestation

Various techniques for assessing mite numbers in the colony exist. One of the most accurate predictions is made through examination of debris in the hive. A sticky board is used to monitor the natural daily mite fall (NMF) (Bienkowska and Konopacka, 2001a, 2001b; Vidal-Naquet, 2015). It is placed on the bottom board of the hive for three days. A grit is used to keep the honeybees from removing the fallen mites.

Another alternative diagnostic method is to collect mites from adult honeybees via the "sugar shake" method. Live honeybees are sprinkled with powdered sugar. The sugar clogs the mites' tarsal pads and causes them to fall off the honeybees (Macedo et al., 2002; Gregorc and Sampson, 2019). While the method has a relatively high mite recovery rate, it is more effortful than the debris examination.

A significant correlation between colony mortality and *Varroa*-count has been observed (Liebig, 2001). Depending on the month, the critical *Varroa*-infestation threshold varies (Tab. 1). Liebig (2001) assumed that 7% infested winter bees was the critical infestation threshold for the survival of honeybee colonies. A more recent study conducted by Genersch et al. (2010) suggested that the threshold lies even lower. Other authors considered the highest acceptable maximum threshold before overwintering to be 50 *Varroa*-mites (Noireterre, 2011; Vidal-Naquet, 2015). In the summer, an infestation of more than 30% of the honeybees is likely to lead to the colony not surviving until next spring (Fries et al., 2003; Rosenkranz et al., 2010).

Colonies with an NMF>4 mites per day in August-September should be treated without delay.

NMF/day	Low	Medium	High
April – June	<2	4-8	>8
July	4-8	6-10	>10
August – September	<4	>4	

Table 1 - NMF according to Vidal-Naquet (2015)

2.2.9 Treatment

Various strategies are used to combat *Varroa*-mite infestations. These include using veterinary medicinal products and biological methods, such as removing capped drone brood in spring (Vidal-Naquet, 2015). Four different active ingredient groups are approved as veterinary medicinal products for the treatment of a *Varroa*-infestation in Germany – organic acids, pyrethroids, triazapentadiens and essential oils (Emmerich, 2019; Deutscher Imkerbund e.V., 2022).

One of the most popular therapy concepts amongst beekeepers in Germany comprises the removal of drone brood in spring, followed by the treatment with formic acid after the last honey harvesting and one treatment with oxalic acid in late autumn/winter. The goal of these treatments is not to eradicate the infestation, but to reduce mite numbers below the threshold at which mites would cause significant damage to the colony. Currently, none of the approved veterinary medicinal products can achieve a long-lasting effect. Moreover, the colonies' survival and well-being depend on the beekeeper’s knowledge and the effectiveness of disease control (Jacques et al., 2017). The treatments are time-consuming, not sustainable and none of the above-mentioned methods is considered 100% efficient against *V. destructor* (Vidal-Naquet, 2015). Mutations in the *Varroa*-population worldwide have also led to resistance against some of the most commonly used acaricides (Rodríguez-Dehaibes et al., 2005; Pohorecka and Bober, 2007; González-Cabrera et al., 2016; Hernandez-Rodriguez et al., 2021). Some of the products can also leave residues in honey and other bee products (Bogdanov et al., 1999; Bogdanov, 2006).

The inability to control the *Varroa*-infestation in the long-term using standard veterinary medicinal products has made the development of alternative strategies necessary. One very promising aspect is the use of natural resistance traits in the honeybee for maintaining good colony health (Pérez-Sato et al., 2009; Rosenkranz et al., 2010).

2.3. Natural defence mechanisms against *V. destructor*

2.3.1. Hygienic behaviour

Like all other insects, honeybees have natural protection mechanisms against diseases, one of which is the so-called hygienic behaviour. This includes the uncapping and removal of the infected, parasite-infested, or dead brood (Rothenbuhler, 1964). In the case of *Varroa destructor*, hygienic behaviour is known under the term *Varroa*-sensitive hygiene – VSH (Harris, 2007).

Previous studies have shown a tendency of nursing bees to use olfactory stimuli emitted from the infested brood cells to remove affected larvae or pupae (Martin et al., 2002; Swanson et al., 2009; Schöning et al., 2012; Chakroborty et al., 2015; Wagoner et al., 2018, 2020). This ability to efficiently detect and eliminate sick or diseased brood is a trait that is particularly pronounced in so-called hygienic bee colonies (Gramacho and Spivak, 2003). Non-hygienic colonies leave the diseased brood longer or untouched in the brood cells, which leads to the spread and propagation of the disease (Gilliam et al., 1988; Spivak and Gilliam, 1998). As hygienic behaviour is a complex polygenic trait, the difference between hygienic and non-hygienic bee colonies lies in the expression of a set of different genes that are mostly responsible for odour sensitivity (Parker et al., 2012; Hu et al., 2016; Guarna et al., 2017; McAfee et al., 2017). There is evidence of a positive selection among the genes for hygienic behaviour within the honeybee genus, with hygienic behaviour acting as a trait contributing to fitness (Harpur et al., 2019).

Two groups of proteins in the olfactory sensilla of honeybees and other insects are considered responsible for the olfaction – Odorant Binding Proteins (OBPs) and G protein-coupled olfactory receptors (ORs) (Forêt and Maleszka, 2006; Guarna et al., 2015; Spötter et al., 2016). Guarna et al. (2015) named OBP 16 and 18 the proteins most highly correlated with hygienic behaviour, with OBP 18 having the highest affinity to oleic acid – a well-known necromone in many insects. The honeybee brain also

shows differences in the expression of genes coding for enzymes (cytochrome P450 pathway) connected to detoxification and degradation of odorants and other chemicals (Boutin et al., 2015). The over-expression of cytochrome P450 in non-hygienic honeybees is thought to cause increased pheromone degradation speed, thus removing the stimulus before the activation of hygienic behaviour (Boutin et al., 2015). Highly hygienic bees also differ from honeybees with low hygienic behaviour through the molecular processes in their brains (Galizia and Sachse, 2009). Synaptic proteins in the mushroom bodies – a pair of structures that play an essential role in olfactory learning and memory – are highly expressed in hygienic bees compared to non-hygienic bees, which may lead to faster signal transmission and higher cerebral activity (Hu et al., 2016). Moreover, high octopamine expression in the honeybee brain plays a vital role in detecting low concentrations of diseased brood odours (Spivak et al., 2003). This divergence in gene products and neuronal pathways between hygienic and non-hygienic colonies could provide a possible clue to understanding different levels of hygienic behaviour (Le Conte et al., 2011; Gempe et al., 2016; Harpur et al., 2019).

When in distress, capped brood changes its cuticular profile, sending a signal to the nursing bees (Wagoner et al., 2018). The same chemical substances emitted by the diseased brood have been found on the cell caps (Martin et al. 2002), which seem to function as a possible "interface" between worker bees and brood. During the execution of hygienic behaviour, worker bees display specific movements such as touching the inspected and the neighbouring cell caps with the antennae, moving the head in a particular way, visiting the empty cells next to the affected cell. Through this behaviour, honeybees can detect the affected cell's exact location without opening multiple cells separately (Bienefeld et al., 2015).

One behavioural mechanism part of VSH, known as brood cell recapping, is also considered beneficial for colonies in the management of diseases and has been observed in stocks resistant to *V. destructor* (Hawkins and Martin, 2021). By opening and resealing the cell cap, workers can inspect the brood and assess its condition, or even remove mites without sacrificing the brood. Furthermore, multiple recappings can have a negative effect on mite reproduction by interrupting the egg-laying cycle (Oddie et al., 2021). While some scientists define it as part of VSH (Martin et al., 2020), others consider it an independent trait (Oddie et al., 2018a).

Grooming is a behavioural mechanism in many vertebrates and arthropods used for removing dust, dirt and ectoparasites (Boecking and Spivak, 1999; Aumeier, 2001). In the case of *V. destructor*, grooming effectiveness plays an important role for the defence against the *Varroa*-mite (Boecking and Spivak, 1999; Anderson and Trueman, 2000). By using their mandibles, workers damage mites located on nestmates causing the mites to fall to the bottom of the hive. Both self-grooming (auto-grooming) and social grooming (allo-grooming) can be observed (Boecking and Spivak, 1999). Colonies surviving a *V. destructor* infestation are described as having a more superior grooming behaviour than susceptible colonies (Dadoun et al., 2020).

2.3.2. Defensive mechanisms of *Apis cerana*

In comparison with *A. mellifera*, *A. cerana* shows more pronounced hygienic behaviour towards *V. destructor*. Drone brood in *A. cerana* colonies is capped by a thick wax layer, preventing weakened parasitised drones from coming out of the cell. Worker bees do not remove drone brood from the cells. Sometimes, they create an additional wax layer, closing the drone cell's central pore and entombing the mites inside the brood cell (Boecking and Spivak, 1999). In an experiment conducted by Lin et al. (2016), the Asian honeybee was significantly faster and more efficient in discovering and removing freeze-killed brood than the European honeybee. Its grooming behaviour is also considered more effective than that of its European counterpart (Büchler et al., 1992). Additionally, during a nationwide screening in China in 2012, Asian honeybees had a significantly lower DWV prevalence than European honeybees. The reason behind these findings is presumably the higher resistance of *A. cerana* against *V. destructor*, resulting in low infestation rates (Li et al., 2012).

2.3.3. Host adaptations in *A. mellifera* populations

Some *A. mellifera* populations have naturally adapted to the mite infestation and can survive without treatment (Locke, 2016b; Dadoun et al., 2020; Le Conte et al., 2020; Luis et al., 2022). The mechanisms that provide such adaptations vary and are not yet fully understood.

Host tolerance describes the ability to limit the impact of the parasite on the host, host resistance is the ability to reduce the fitness of the parasite (Locke et al., 2012; Schmid-

Hempel, 2021). Tolerance and resistance often correlate with each other in the colony and host adaptations to *V. destructor* are often derived from a combination of resistance and tolerance mechanisms (Locke et al., 2012; Mondet et al., 2020; Schmid-Hempel, 2021).

The East African lowland honeybee (*Apis mellifera scutellata*) displays a higher resistance towards the *Varroa*-mite parasitisation than other European honeybee subspecies. It exhibits tolerance mechanisms such as a more aggressive grooming behaviour towards mites and heightened hygienic behaviour as well as resistance mechanisms such as low mite fertility in the worker brood cells and a high percentage of unmated daughter mites. The number of laid eggs is also lower than in *A. mellifera* colonies of European origin (Nganso et al., 2017, 2018).

Resistant honeybee populations in Gotland, Sweden and Avignon, France show a reduction in the reproductive success of the *Varroa*-mite (Locke, 2016b). In Norway, one described honeybee population displays a shortened post-capping period compared to susceptible honeybee colonies in the region. Reducing the length of the post-capping period has a negative effect on the mite's reproductive success (Oddie et al., 2018b). Honeybee populations in Cuba show high mite removal levels, high recapping behaviour and low mite reproduction levels. This is the biggest *A. mellifera* population that has developed a natural resistance against *V. destructor* and has not been treated for over twenty years (Luis et al., 2022).

Environmental effects are considered a potential distortion for the expression of host adaptations (Le Conte et al., 2020). When colonies, tolerant/resistant to *V. destructor*, are removed from their native area, their survival rates do not differ from those of nonselected colonies, indicating the existence of genotype-environment interactions (Meixner et al., 2015). Generational change can also pose as a distortion for the inheritance of beneficial traits (Odemer, 2020).

2.4. Resistance Breeding Methods

Understanding traits that influence the honeybee’s resilience towards *V. destructor* and creating an efficient breeding strategy is considered vital for maintaining honeybee health and keeping pollination services upright. Selective breeding is considered the only long-term and sustainable solution to the *Varroa*-problem (Harbo and Harris,

1999; Pérez-Sato et al., 2009; Rosenkranz et al., 2010; Danka et al., 2011; Locke, 2016a; Wagoner et al., 2018). Current selection programs aim at amplifying single resistance traits (Guichard et al., 2020). Some of the methods used to measure the colony's resilience towards *V. destructor* are not directly related to the detection and removal of infested brood cells but are, nevertheless, considered to be positively correlated with the colony's survival ability. Some of the classical methods used in resistance breeding as well as some novel methods are presented below.

Pin-killed assay. The evaluation of hygienic behaviour can be achieved through the pin-killed assay. During the pin-killed assay, a comb with capped brood is removed from the hive. Subsequently, a rhomboid frame is placed on the brood cells, and the edges are marked. Fifty brood cells are pierced with an entomological pin, row after row. Cell 51 is also marked so the area can be identified. The brood frame is placed back in the hive. After 5-17 h, the number of opened brood cells is counted and subtracted from 50 (Büchler et al., 2013). When all test colonies have removed more than 50%, the test has the highest explanatory power. The pin-killed assay should be repeated two or three times during the main brood season (Büchler et al., 2013).

Freeze-killed brood assay. Another widely known assay is the freeze-killed brood assay. It consists of cutting a comb section with sealed brood (approximately 100 cells) and freezing it at -20°C for 24 h (Büchler et al., 2013). The section is reinserted in the comb and placed inside the hive. In less than 24 h, the comb is removed, and the number of capped and uncapped brood cells is counted. The test should be repeated at least twice for representative results. Hygienic colonies remove >95% during the first 24 h of the experiment (Büchler et al., 2013). Recent studies argue that the freeze-killed brood assay is not suitable for predicting VSH as it shows a correlation neither to the percentage of infested pupae or honeybees in the colony nor to the number of mites present in brood cells (Leclercq et al., 2018).

Mite non-reproduction. Mite non-reproduction (MNR) - also formerly known as Suppressed mite reproduction (SMR) - describes the number of viable mated offspring per mother mite found in worker brood. Infested brood cells are usually opened at day 9 of the honeybee's development. Foundress mites which exhibit normal reproduction at this point share the brood cell with at least one daughter mite and one male (Büchler et al., 2017; Morin and Giovenazzo, 2023). Mites with no offspring are considered non-reproductive.

Many factors contribute to MNR by hindering the reproduction of the foundress mite – low mite fecundity when entering the cell, brood influence on the reproduction of the mite, as well as the influence of adult honeybees through recapping and VSH (Harbo and Harris, 2005; Ibrahim and Spivak, 2006; Rinderer et al., 2010; Eynard et al., 2020; Scaramella et al., 2023). A study conducted by Oddie et al. (2021) displayed an association between lower mite reproductive success and high recapping activity in the test colonies.

MNR measurements can give information on *Varroa*-resistance in honeybee colonies on a large scale. However, no precise protocol for the evaluation of the data has been established worldwide up to date, thus making it difficult to compare published results (Eynard et al., 2020).

Infra-red camera observation of *Varroa*-infested brood cells. A more recent method for the observation of VSH in different colonies is the infra-red camera observation described by Bienefeld et al. (2015). Honeybees bred for VSH remove significantly more infested pupae in the first five days after cell-capping than nonselected colonies (Harris, 2007). While both honeybees from colonies bred for VSH and non-hygienic colonies can detect the signals emitted from parasitised pupae, only honeybees initiating VSH are able to distinguish between healthy and parasitised pupae (Mondet et al., 2021).

This method allows examining individual honeybees from more than one origin performing VSH. An observation hive, consisting of an observation unit, a supporting unit and a camera, is used. The supporting unit comprising unmarked worker bees maintains suitable humidity and temperature in the test hive. The observation unit comprises a brood frame with open and sealed brood placed in a framed cage - one side made out of glass, the other out of mesh gauze. Thirty-five capped brood cells are artificially infested with one *Varroa*-mite each. For the evaluation of VSH, workers are marked with numbered chips on their dorsal thorax and placed in the observation unit. A queen is also placed in the observation unit to simulate normal hive conditions (Bienefeld et al., 2015). Marked worker bees can communicate with honeybees from the supporting unit through the mesh gauze but cannot mix. The marked honeybees are recorded through the glass side of the cage for a total of seven days (Bienefeld et al., 2015). VSH can accurately be observed in the video-recordings.

Due to the high time investment, this long-term observation is more suitable for molecular follow-up studies than for more extensive breeding programmes (Bienefeld et al., 2015).

Marker-assisted selection. This breeding method is based on the mapping of regions of deoxy ribonucleic acid (DNA) – so-called quantitative trait loci (QTLs) – that influence the engagement in hygienic behaviour and account for a high percentage of phenotypic variation in the population (Oxley et al., 2010; Sainsbury et al., 2022). By identifying single nucleotide polymorphisms (SNPs) in these regions, an association with the desirable trait can be studied (Dekkers and Hospital, 2002; Sainsbury et al., 2022; Lefebre et al., 2024).

Protein markers can also be used for the identification of hygienic traits (Guarna et al., 2015, 2017). Selection can be based not only on the hygienic behaviour of the adult worker but on the honeybee pupae as well. Some pupae exhibit a mutation in their *ecdysone* biosynthesis pathway, which inhibits the mite's reproduction in the capped cell (Conlon et al., 2019). If the brood cell conditions are not optimal, the mite will halt its reproduction (Frey et al., 2013).

Symbiont-mediated RNAi. Another novel method of improving honeybee health is the up-regulation of the honeybee immune system and limiting pathogen damage trough a symbiont-mediated RNAi approach (Leonard et al., 2020). A symbiotic bacterium in the honeybee gut - *Snodgrassella alvi* - can be engineered to elicit eukaryotic RNA interference (RNAi) immune responses and kill *V. destructor* and the viruses carried by the mite (Leonard et al., 2020).

Symbiont-mediated RNAi can also be used to study gene function via gene knockdown (Lariviere et al., 2023). Through the reduction of the expression of chosen genes, their function and effects on the honeybee phenotype can be examined.

2.5 Differential conditioning

As mentioned in subsection 2.3.1, olfactory sensitivity plays a vital role in the localization of diseased or parasitised brood. One non-invasive method for examining the honeybee’s olfactory sensitivity is via classical conditioning. One particular reflex – the proboscis extension reflex (PER), is used as a foundation for the conditioning. By stimulating the antennae of honeybees with a sugar solution, the PER is elicited as

part of the honeybee's feeding behaviour (Menzel, 1996). The proboscis is automatically stretched to enable drinking.

Conditioning experiments on honeybees were first described in the 60s by Takeda (1961). Takeda (1961) observed the ability of the honeybee to form a connection between an olfactory stimulus and the PER when the honeybee was allowed to drink from the sugar solution following the presentation of the stimulus. Since then, countless experiments studying the learning ability and memory of honeybee have been conducted (Bitterman et al., 1983; Ray and Ferneyhough, 1997; Frost et al., 2012; Matsumoto et al., 2012). Furthermore, PER conditioning has been described as a suitable test for studying pesticides' adverse effects (Herbert et al., 2014; Goñalons and Farina, 2015) and the sensitivity towards odours connected to VSH (Masterman et al., 2000; Chakroborty et al., 2015).

The PER conditioning comprises two stimuli – conditioning stimulus (Cs) and unconditioned stimulus (Us). A sugar solution is used as the Us as it evokes a natural reaction – stretching of the proboscis. The Cs is a neutral stimulus – an odour – which elicits no initial response. Through multiple stimuli presentation, a connection between both stimuli is formed (Bitterman et al., 1983; Menzel et al., 2001). When differential conditioning is performed, two odours are presented – Cs+ and Cs-. While the Cs+ is rewarded with the sugar solution, the Cs- is not (Matsumoto et al., 2012).

Honeybees can retain a lifelong association of the Cs with the Us after only three trials (Menzel, 1969). The mushroom bodies (MB) in the insect brain are responsible for olfactory learning. During the PER conditioning, both gustatory and olfactory stimuli overlap for a short period of time. The signals are transmitted to the Kenyon cells in the MB simultaneously, allowing the establishment of a connection (Heisenberg, 1998; Fahrbach, 2006; Van Nest, 2018).

As part of the conditioning preparation, the honeybees are harnessed in small metal tubes and left to adapt to the environment (Bitterman et al., 1983). The animals are trained in groups, usually up to 20 honeybees. The Cs can be delivered either through an airstream or on a piece of paper (Scheiner et al., 2013). During six seconds, the Cs+ is presented. In the last three seconds of the Cs+, the Us is presented (Bitterman et al., 1983). Through the overlapping of Cs+ and Us, a connection is formed. Six or eight trials with Cs+ and Cs- are usually performed in order to establish an association

between Cs+ and Us. The most widely used trial order is Cs+, Cs-, Cs-, Cs+, Cs-, Cs+, Cs+, Cs- (Bitterman et al., 1983).

During the experiment, the honeybees have a fixed order. The first honeybee undergoes the second trial only after all the honeybees have completed the first trial. After all trials are completed, the honeybees are fed with the sugar solution and left to regenerate before the outcome testing (Matsumoto et al., 2012). The outcome is tested through the presentation of Cs- and Cs+ without the Us. If the honeybees are conditioned successfully, they will show a response to the Cs+ but not to the Cs-.

2.6 Study Objectives

As outlined above, selection breeding against *V. destructor* faces many challenges. Breeding efforts with the goal of enhancing resistance traits have been ongoing for more than three decades, but to date no breeding strategy has reached a broad-scale host-parasite balance. Due to the strong environmental dependence of resistance traits, the reproductive and genetic peculiarities of the honeybee, as well the small-scale structure of the German beekeeping community, very labour-intensive traditional breeding programmes for this species are in need of transformation. A better understanding of the inheritance mechanisms of VSH as well as the creation of a novel easily implemented breeding strategy is necessary.

To reach this goal, the following aspects were considered:

- Can PER conditioning detect differences in odour sensitivity between control and selection line worker bees to different odours? If so, can differences be observed in the perception of brood odours in connection with the *Varroa*-parasitisation?
- Can drones perceive the odours emitted by brood parasitised by *Varroa destructor*? If so, can this odour sensitivity enhance the colonies' VSH and be utilised to create a breeding strategy?
- Can the PER conditioning be used as a selection tool for drones?

3 Research publications in journals with peer-review

3.1 Publication I

Title: *Apis mellifera* worker bees selected for *Varroa*-sensitive hygiene show higher specific sensitivity and perception speed towards low concentrations of chemical cues emitted by the brood

Authors: Ivelina Ivanova, Kaspar Bienefeld

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RESEARCH

Apis mellifera Worker Bees Selected for *Varroa*-sensitive Hygiene Show Higher Specific Sensitivity and Perception Speed Towards Low Concentrations of Chemical Cues Emitted by the Brood

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Abstract *Varroa*-sensitive hygiene (VSH) is highly influenced by the worker bee's olfactory ability. Workers bred for VSH and non-selected control line workers were tested for differences in their speed and perception ability when presented with highly diluted stimuli. Four different substances (citral – dilution 1:1300, linalool dilution 1:1300, *Varroa*-parasitized brood extract, isopropanol) were used as tactile stimuli for differential conditioning with the proboscis extension response (PER). Discrimination ability and generalization were assessed. In a second set of conditioning experiments differences in sensitivity to the highly diluted citral and the *Varroa*-parasitized brood extract as reinforced stimuli (Cs+) were explored between workers from both lines. The worker bees were classified into three groups (***Time points***) depending on how long before they started correctly extending their proboscis to the Cs +, and results were examined separately for each of the two stimuli and group. While the VSH-selected line exhibited a significantly higher perception ability for the parasitized brood extract than the non-selected line, the two lines showed no differences when conditioned with the floral stimulus citral as Cs +. Furthermore, the VSH-selected line displayed a significantly higher number of worker bees that perceived the complex bouquet of the *Varroa*-parasitized brood extract at the earliest time grouping (***Time point 1***). The odds of perception at the earliest possible time point were 2.6-times higher for the VSH-selected line. Although no comparison was made between healthy and parasitized brood, the results indicate an enhanced specific sensitivity in VSH-selected workers towards chemical cues emitted by the brood, which might play a role in the detection of *Varroa destructor*.

Keywords *Varroa*-sensitive hygiene · Olfactory sensitivity · Resistance breeding · *Apis mellifera*

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Introduction

The European honey bee, *Apis mellifera*, is one of the most important agricultural pollinators worldwide.

However, since the parasitic mite *Varroa destructor* shifted hosts from the Asian honey bee *Apis cerana* to the European honey bee, a global increase in colony loses has been observed for the latter species (Genersch et al. 2010; Dietemann et al. 2012; Martin et al. 2012). While some populations appear to be *Varroa*-resistant (Locke 2016; Oddie et al. 2017, 2018), most of the honey bee colonies are still dependent on the *Varroa*-treatment administered by beekeepers (Jacques et al. 2017). However, recent breeding efforts to create bees with enhanced *Varroa*-sensitive hygiene (VSH) — a specialized type of hygienic behavior comprising the targeting and removal of *Varroa*-infested brood — have improved bee colonies' survival in the face of parasitization (Mondet et al. 2020).

Varroa destructor induces a shift in the cuticular hydrocarbon profile of parasitized brood (Nazzi et al. 2004; Wagoner et al. 2019; Mondet et al. 2021) which is detected through the cell cap by nursing bees. Compounds such as tricosan-2-one, pentacosan-2-one, tetracosyl acetate, heptacosan-2-one, hexacosyl acetate and nonacosan-2-one have been detected in extracts of parasitized pupae (Mondet et al. 2021). Furthermore, (Z)-pentadec-6-ene and (Z)-10-tritiacontene, the non-volatile oleic acid, as well as the increase of brood ester pheromone are also able to elicit a hygienic response (Nazzi et al. 2004; Mondet et al. 2016; Wagoner et al. 2020) and are associated with *Varroa*-parasitization (Wagoner et al. 2021). This odor change acts as a signal for the worker bees and a trigger for VSH (Harbo and Harris 2005; Wagoner et al. 2018). Subsequently, the brood cells are uncapped and the diseased pupae removed (Martin et al. 2002; Swanson et al. 2009). Mondet et al. (2021) observed that while all worker bees can perceive the compounds typical for a *V. destructor* parasitization at the level of the antennae, only those bees performing VSH can differentiate between these compounds and the odor of unparasitized healthy brood. Moreover, worker bees from colonies bred for VSH are more likely to uncap infested cells with more than one foundress mite (Kim et al. 2018) and brood severely affected by transmitted viruses (Schöning et al. 2012).

The early detection of parasitized brood and the subsequent removal of the mites has been identified as being significantly genetically influenced (Spötter et al. 2012, 2016; Guarna et al. 2015). The differential expression of genes for the olfactory and sensory activity determines the perception ability and olfactory sensitivity of the single worker bee (Boutin et al. 2015; Hu et al. 2016; Gempe et al. 2016). Under laboratory conditions, olfactory ability can be tested with the help of differential conditioning using the proboscis extension response (PER). First described by Takeda in 1961, this method lies at the center of assessing olfactory discrimination abilities in bees (Takeda 1961; Bitterman et al. 1983; Giurfa and Malun 2004; Giurfa 2008; Matsumoto et al. 2012; Smith and Burden 2014). Through a series of trials, a bee learns to differentiate between two odors: Cs + (reinforced with a reward) and Cs- (unreinforced, or novel odor). In order to feed on the reward sugar solution, the bee displays a behavioral change by extending its mouthparts, or proboscis.

PER conditioning can provide valuable information on the differences in perception ability towards various chemicals in lines bred for enhanced hygienic behavior including VSH and non-selected lines. Masterman et al. (2000) observed significantly better discrimination ability in hygienic bees when exposed to the odor of healthy and chalkbrood infested brood compared to non-hygienic bees. Flower odors, on the other hand, were perceived equally well by both groups of bees. Compared to a chalkbrood infection where the brood dies, the parasitization with *V. destructor* causes amongst others immunosuppression without killing the brood (Rosenkranz et al. 2010; Vidal-Naquet 2015). While chalkbrood mummies elicit a strong stimulus leading to their removal, the changes in the brood during a *V. destructor* parasitization are likely more subtle, therefore more difficult to sense. A study conducted by Chakroborty et al. (2015) using PER conditioning tested VSH-selected and non-selected worker bees with the odor of healthy and *Varroa*-parasitized pupae. The study did not deliver conclusive results whether VSH colonies are endowed with better odor discrimination abilities than the non-hygienic colonies. During the experiment, the colonies bred for enhanced hygienic behavior towards *V. destructor* exhibited only small differences in odor discrimination ability towards the *Varroa*-infested brood compared the non-hygienic worker bees. These observations on odor sensitivity may be accounted for by the small numbers of tested individuals ($N_{hygienic} = 54$, $N_{control} = 42$). The method of presentation (olfactometer) might also play an important factor, considering that some of the

compounds extracted from *Varroa*-parasitized brood are non-volatile and therefore cannot be presented through an air stream (Nazzi et al. 2004).

Here we describe a complementary study aimed at observing the perception ability of worker bees to different stimuli by using learning as a marker for sensitivity. While the PER response does not measure the sensitivity of an individual bee, but the learning behavior to a stimulus, if the stimulus is not detected during a tactile or volatile presentation, there is no learning success even with large differences in learning ability. We therefore defined higher sensitivity as a faster and generally higher perception of the presented stimulus. Bienefeld et al. (2015) displayed that learning does not play a role in hygienic behavior. Rather, hygienic behavior is an instinctive reaction to abnormal cues, with olfactory sensitivity playing a central role (Schöning et al. 2012; Mondet et al. 2015, 2021; Wagoner et al. 2021). We hypothesized that conditioning using the PER can be utilized as a method for quantifying olfactory sensitivity for the use in *Varroa*-resistance breeding (Ivanova and Bienefeld 2021). Workers from two origins (a VSH-selected line and a non-selected line) were presented with two highly diluted extracts — citral (essential oil, well known for its use in conditioning experiments (Vareschi 1971; Nagaraja and Bruckner 2013) as well as a minor component of the Nasonov pheromone (Shearer and Boch 1966)) and an extract of *Varroa*-parasitized brood. In order to better define the differences in the perception ability of each group, we used a larger sample size and a lower concentration of *Varroa*-parasitized brood extract rather than live parasitized pupae as used in the experiments of Chakroborty et al. (2015). A tactile presentation of the extract was chosen as means of delivering the stimuli. We further hypothesized that the undiluted odors used in Chakroborty et al.'s (2015) experiments pose an easy task for the test subjects and provide information on overall odor perception ability but give no feedback on olfactory sensitivity.

The following questions formed the basis for the performed experiments: Is there a difference in the perception ability of worker bees bred for VSH and the non-selected line worker bees, when presented with highly diluted stimuli? Does the perception speed between the two lines differ? Are the differences in the perception ability a result of an overall higher olfactory sensitivity or a specific sensitivity towards cues which are likely to cause VSH?

Materials and Methods

Colonies

Worker bees from a total of sixteen colonies participated in two PER-conditioning experiments. The colonies were situated at one of the Institute for Bee Research Hohen Neuendorf's own locations in Brandenburg, Germany (coordinates: 52.66943; 13.39455). Each colony was used only once. Half of the colonies originated from the institute's VSH-selection program (Bienefeld et al. 2001), while the other half was not selected for VSH but shared a similar genetic background.

The institute's VSH-selection program comprises video-observation of recognition and uncapping of *Varroa*-parasitized brood through individual workers in a standardized observation unit using a sample of 40–50 worker bees/mother (Bienefeld et al. 2015). The main selection criterion for the mother queens and father colonies (sperm donors) is the relative proportion of worker bee offspring that has started uncapping *Varroa*-parasitized brood during a 6-day video-observation. Details on the development of this line will be available in a separate publication.

During the preparations of the conditioning experiments, 50 workers from each colony were gathered as they emerged from the brood cells and marked with numbered plates on the dorsal thorax. Subsequently, they were fostered in a hive with a virgin queen until they were tested. A brood frame with sealed brood was placed in the hive to stimulate nursing behavior. The marked worker bees were tested at an age spanning from 3 to 11 days, with an average age of 6 days, as this time range corresponds with glandular development of the hypopharyngeal glands and nursing behavior as stated by Page and Peng (2001).

Extract Preparation

The *Varroa*-parasitized brood extract was created as presented by Ivanova and Bienefeld (2021). Twenty newly capped prepupae (9–10 days old) were artificially infested with four *Varroa*-mites each. Since the distress signal emitted by the brood, rather than the number of mites in the brood cell, is perceived by the nursing bees, we wanted to produce an extract that mimicked the changes in the brood's cuticular hydrocarbon profile (Bauer et al. 2018; Mondet et al. 2021).

By using four mites per prepupa, we ensured that even if mites were damaged during artificial infestation, a sufficiently strong stress factor for the brood would still be present.

The cell caps were cut open on one side and the mites inserted with the help of a moistened brush. The cell caps were subsequently closed. Afterwards, the brood frame was introduced to the hive it came from for two hours, in order for the incisions in the cell caps to be completely resealed by workers (Ivanova and Bienefeld 2021). The brood frame was incubated for four days in an incubator at 35 °C. Subsequently, fifteen parasitized pupae were extracted from the brood cells without being damaged and were soaked in 4 ml isopropanol for 10 min. The supernatant was decanted in 2 ml glass vials with PVC lids (Ivanova and Bienefeld 2021). Between the conditioning experiments, the extract was stored at -20 °C. Five microliters of the extract contained 0.02 brood equivalents.

Both floral stimuli – citral and linalool – were diluted in isopropanol. One microliter of the floral compound was combined with 1299 µl isopropanol using a micropipette. The extracts were stored in vials at -20 °C between the conditioning experiments.

PER-conditioning Experiment

To find a suitable concentration of citral and linalool, a series of preliminary tests using differential conditioning were carried out. Dilutions of up to 1:1500 were tested. The preliminary tests were performed the same way as the main experiment, described in the remaining part of this subsection. The dilution of 1:1300 (equivalent to a concentration of 0.69 µg/µL for citral and 0.66 µg/µL for linalool) was chosen as only one third of the workers exhibited a behavioral response when presented with the diluted extract. Higher dilutions were deemed unsuitable for the experiment as they would provide insufficient data for the analysis.

For the differential conditioning (referred to as main experiment from now on), two stimulus combinations were used:

– citral (dilution 1:1300) 5 µl as Cs + and linalool (dilution 1:1300) 5 µl as Cs-
– Extract from *Varroa*-parasitized brood 5 µl as Cs + and the solvent isopropanol 5 µl as Cs-

As all the extracts contained isopropanol as a solvent, they were left to dry after being applied on the filter paper (including isopropanol as Cs-). This was done to ensure that the stimuli would not be overlayed by the smell of the solvent. A total of 15 bees from each colony were conditioned per stimulus combination for a total of 240 worker bees tested from each origin (VSH-selected and non-selected line). Each bee was conditioned using only one of the two stimulus combinations.

Parallel to the main conditioning experiment, a reversed differential conditioning was performed to assess potential differences in the salience of the stimuli used throughout the experiment. The stimulus combinations used in the main experiment were swapped:

– Linalool (dilution 1:1300) 5 µl as Cs + and citral (dilution 1:1300) 5 µl as Cs-
– Isopropanol 5 µl as Cs + and the extract from *Varroa*-parasitized brood 5 µl as Cs-.

The reversed conditioning was performed as described for the main experiment. Twenty workers were tested per stimulus combination and subsequently compared to the same number of workers conditioned with citral and *Varroa*-parasitized brood extract as Cs +.

During the main experiment, a total of 120 workers were conditioned per stimulus combination (citral as Cs +, *Varroa*-parasitized brood extract as Cs +) (Table 1). The bees were tested in groups of ten. Each group comprised individuals from different colonies. Before conditioning, worker bees were gathered from the brood frame in the test hive. The bees were shortly cooled down at -20 °C until they stopped moving. Subsequently, they were strapped in small metal tubes using paraffin tape so that the body was immobilized without the movement of the head and mouthparts being constricted. The worker bees were placed in an incubator (34 °C) to regain their physiological temperature after the cooling. The willingness of the worker bees to stretch their proboscis was examined by presenting them with a 50% sugar solution on a toothpick. One of the worker's antennae was touched with a drop of the sugar solution which resulted in extension of the proboscis. Those workers that did not respond were not included in the conditioning.

Table 1 Distribution of colonies participating in main conditioning experiment

Origin	Colonies (N)	Participating worker bees (N)		Worker bees that completed the conditioning (N)		Discarded worker bees (N)	
		Citral	*Varroa*-parasitized brood extract	Citral	*Varroa*-parasitized brood extract	Citral	*Varroa*-parasitized brood extract
VSH-selected line	8	133	134	120	120	13	14
Non-selected line	8	141	128	120	120	21	8

Displayed are the two lines with the corresponding number of worker bees which were conditioned per stimulus combination (citral/linalool or *Varroa*-parasitized brood extract/isopropanol). Worker bees that stretched their proboscis at the first presentation of the reinforced stimulus Cs + or stopped responding to the sucrose solution during the conditioning were discarded. Their numbers are shown in the last two columns

As the solvent isopropanol was present in both Cs +and Cs-, only workers who were able to perceive the brood components, would sense the difference between both stimuli (Ivanova and Bienefeld 2021). The presentation of stimuli was conducted using pieces of filter paper and tweezers. The bees' antennae were touched three times with a piece of untreated filter paper before the start of the experiment. This was done to avoid the extension of the proboscis due to a mechanical irritation rather than a response to the presented stimulus. During the stimuli presentation, both antennae were touched with the filter paper. The direct contact ensured the perception of both volatile and the non-volatile compounds, which are usually emitted by the distressed parasitized brood (Mondet et al. 2016; McAfee et al. 2018; Wagoner et al. 2020). A conditioned stimulus Cs +, the extract of *Varroa*-parasitized brood or citral, was paired with a 50% sugar solution (unconditioned stimulus Us). Additionally, an unreinforced stimulus, isopropanol or linalool, was presented without a reward (Cs-). The conditioning consisted of six trials in the following order: Cs +, Cs-, Cs-, Cs +, Cs +, Cs-. The intertrial-interval was between 4–5 min. Each worker bee was given 20 s to acclimate to the experimental surroundings, before a six second presentation of the stimulus. The presentation of the reward overlapped the last three seconds of the Cs +. The Us was presented by touching a drop of sucrose to the antennae without contaminating the filter paper carrying the Cs +. After completing the six trials, each worker bee was tested for its conditioning outcome through the presentation of the two stimuli (Cs +und Cs-) without the reward (unrewarded tests). If the conditioning was successful, the workers stretched their mouthparts to the presentation of the Cs +but not to the Cs-.

The experiments with the two odor combinations were swapped each day in order to exclude daytime biases. An exhaust system was used to remove any residual odors during the experiment. The toothpicks used for the presentation of the sugar reward were replaced before the beginning of each trial to avoid the accumulation of sugar. While wooden toothpicks give off a wooden odor, we assumed that the interference with the conditioning performance would be minimal as they were only used for the presentation of the reward. If workers were to form an association between the wooden odors and the sugar solution, they would not extend their proboscis when presented only with the Cs + during the unrewarded tests.

Worker bees which extended their proboscis during the first conditioning trial were excluded from the experiment as they were considered "spontaneous responders" which might have had prior contact with the stimuli used during the experiment or exhibit a heightened appetitive motivation that triggered PER to neutral stimuli (Matsumoto et al. 2012). Worker bees that stopped responding to the Us during the course of the conditioning were also excluded as they would also not shown any response during the unrewarded trials.

Statistical Analysis

Reversed Differential Conditioning

The acquisition during the reversed differential conditioning experiment was analyzed and compared to the stimuli as presented in the main experiment using a Chi-Square test (two-sided) with an alpha level of 0.05. The exact significance was reported.

Perception Speed and Conditioning Outcome

Using the data from the main experiment's conditioning trials, the worker bees' perception speed was analyzed with the help of a generalized linear mixed model with a logit function (GLMM) in SPSS V.25. The alpha level was set at 0.05. All reported p-values were two-sided. The parameters "VSH-selected line/non-selected line" and age during the experiment (< 6 days, 6–7 days, > 7 days) were set as fixed factors in the GLMM (see suppl. Tab. S1). The age groups were chosen in such a matter, so that the number of workers in each age group was similar. The colony effect was set as a random factor. In order to display the differences in the worker bees' perception speed to the two highly diluted stimuli, three time points were defined. The extension of the proboscis to the Cs + before the presentation of the reward was considered a correct answer. For the Cs- a correct answer was defined as "no proboscis extension".

- ***Time point 1***: Worker bees gave correct answers starting from trial No. 4 (Cs +, Cs-, Cs-, **Cs +, Cs +, Cs-**) and during the unrewarded trials (**Cs+, Cs-**)
- ***Time point 2***: Worker bees gave correct answers starting from trial No. 5 (Cs +, Cs-, Cs-, Cs +, **Cs +, Cs-**) and during the unrewarded trials (**Cs+, Cs-**)
- ***Time point 3***: Worker bees gave non-consecutive correct answers during the trials. The unrewarded tests were also correctly answered. (Cs + , Cs-, Cs-, **Cs +** , Cs + , **Cs-**) (**Cs + , Cs-**)

It was assumed that workers who made mistakes during the last three trials of the conditioning possessed an inferior discrimination ability than individuals that perceived the conditioning stimulus at ***Time point 1***. Furthermore, to estimate the conditioning success of the two lines (VSH-selected/non-selected line) while taking the perception speed into account, a Kaplan–Meier estimator with a Log Rank function was performed. The significance level was set at 0.05 (two-sided).

Worker bees which showed no reaction during the conditioning and gave a positive answer only during the unrewarded tests, were not considered successful, as it was unsure whether the response occurred coincidentally. A proboscis extension was recorded as "1", no behavioral response was documented as "0".

Results

Floral Stimuli

Reversed Differential Conditioning

Compared to linalool as Cs + , workers tested with citral as Cs + exhibited a significantly higher proboscis extension frequency during the conditioning trials. Workers tended to generalize more at trials 2–3 when citral was used as Cs + and exhibited significantly higher number of proboscis extensions during trials three to six (Fig. 1 and suppl. Tab. S2). The generalization was stronger at the beginning of the conditioning and decreased with each trial. At trial six a slight decrease (40%) in proboscis extension frequency was observed when workers were presented with the Cs- (linalool) than at trial three (45%) (Fig. 1).

During the unrewarded tests no significant difference in the proboscis extension frequency for citral and linalool was observed (**unrewarded Cs +:** $\chi 2(1;\ N = 40) = 0.91;\ p = 0.53$; **unrewarded Cs-**: $\chi 2(1; N = 40) = 2.9; p = 0.49$). Workers tested with citral exhibited higher numbers of proboscis extensions during the unrewarded tests although the difference was not significant. With regard to the results, citral and linalool were considered perceptually similar with citral posing a more potent stimulus at the chosen dilution.

Perception Speed and Conditioning Outcome for Both Origins (Citral as Cs + , Linalool as Cs-)

During the main conditioning experiment with the flower substances, no significant differences in stimulus perception between the two lines were observed. The VSH-selected line exhibited a slightly higher percentage of worker bees that perceived the difference between the two stimuli (citral as Cs + and linalool as Cs-) at the earliest time point (***Time point 1***) than the non-selected line (VSH-selected line: 15%; non-selected line: 10.8%) (see Fig. 2, suppl. Tab. S3). The relative increase of stimulus perception by the VSH-selected line was 39% (equivalent to 5 worker bees more than the non-selected line). Worker bees that perceived citral at one of the later conditioning time points, also showed no origin-related differences in perception (VSH-selected line/non-selected line). The perception ability of the tested

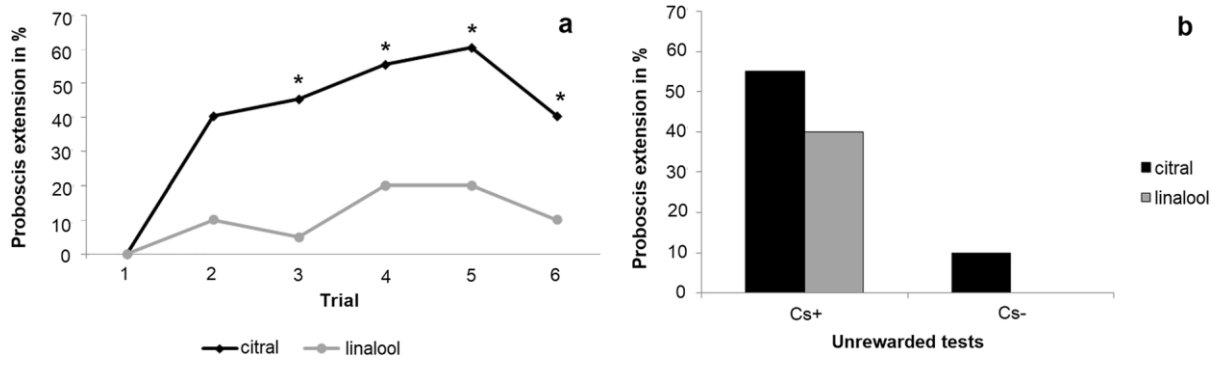

Fig. 1 Acquisition curves and unrewarded tests for conditioning with citral as Cs + (black line) and linalool as Cs + (grey line). **(a)** Proboscis extension frequencies for citral as Cs + / linalool as Cs- and linalool as Cs + / citral as Cs- are shown in percent for each trial. Significant differences are marked with an asterisk. The alpha-level is set at 0.05. Per conditioning experiment (citral as Cs + / linalool as Cs- and linalool as Cs + / citral as Cs-) the same number of worker bees were used (N = 20). **(b)** Proboscis extension frequencies during the unrewarded tests are shown in percent. No significant differences were observed

workers did not differ with age (see suppl. Tabs. S4 and S5). The colony effect also had no significant influence on stimulus detection (see suppl. Fig. S F1).

A Kaplan–Meier curve was created to display the perception ability of the participating worker bees (see Fig. 3). The overall conditioning outcome of the two groups (VSH-selected line and non-selected line) shown during the unrewarded tests with citral as Cs + and linalool as Cs- exhibited no statistically significant difference (Kaplan–Meier estimator, Long rank test, $\chi^2(1; N = 240) = 0.60$, $p = 0.438$).

Varroa-parasitized Brood Extract

Reversed Differential Conditioning

Worker bees in the reversed differential conditioning experiment with isopropanol as Cs + discriminated well between the solvent isopropanol and the extract of *Varroa*-parasitized brood. No differences were found in the ability to discriminate between substances, regardless of which substance (brood extract or solvent) was chosen as the conditioning stimulus Cs + (Fig. 4 and suppl. Table S7). The unrewarded

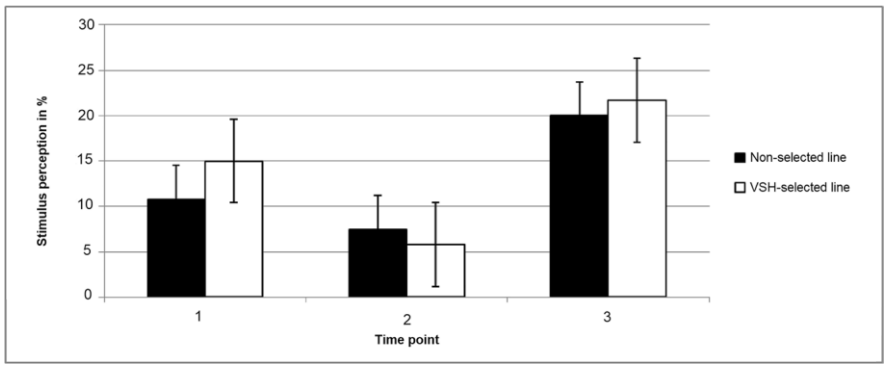

Fig. 2 Number of workers, that are able to perceive the Cs + (citral, dilution 1:1300), in their corresponding speed group. Displayed are the two lines – VSH-selected line (white) and the non-selected line (black). The columns show the number of workers which successfully perceived the Cs + at one of the three time points. Each worker is listed in only one group. Standard error is displayed for each time point and group

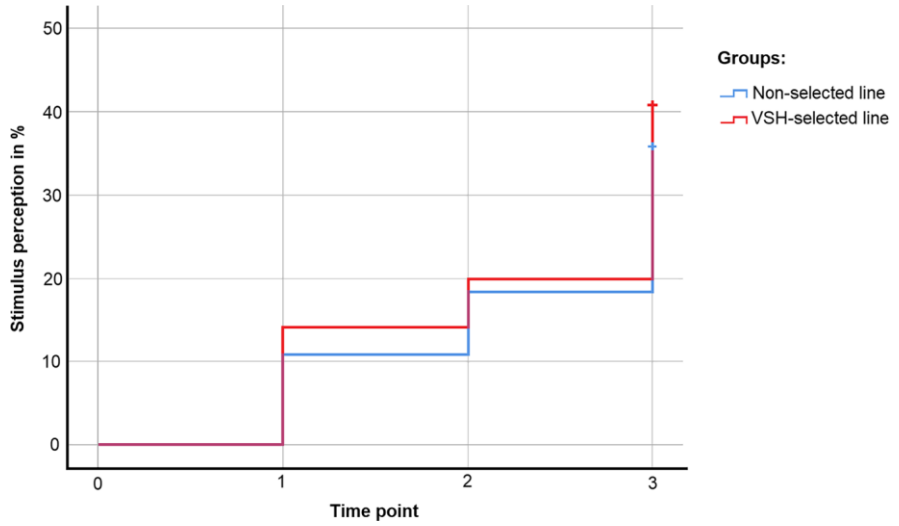

Fig. 3 Kaplan–Meier perception curve for the flower odors. Citral (dilution 1:1300) was used as Cs + and linalool (dilution 1:1300) as Cs-. The cross at the end of each line represents the end of the conditioning for all subjects of the corresponding group. The three vertical lines represent ***Time points 1, 2*** and ***3***. The collective perception of the bees in each group is displayed on the y-axis in percent. The two groups are presented separately: VSH-selected line ($N = 120$; red color), non-selected line ($N = 120$; blue color)

tests also did not provide significant differences in proboscis extension responses (**unrewarded Cs + :** $\chi2(1;\ N = 40) = 0.10;\ p = 1.0$; **unrewarded Cs-**: $\chi2(1; N = 40) = 0.37;\ p = 1.0$) between the two substances. These results led us to believe that none of the two substances posed as a stronger conditioning stimulus for workers during the experiment.

Perception Speed and Conditioning Outcome of Both Origins (Varroa-Parasitized Brood Extract as Cs + , Isopropanol as Cs-)

At the earliest possible time point, ***Time point 1***, 10% more VSH-selected line bees (12 workers) perceived the *Varroa*-parasitized-brood extract than the

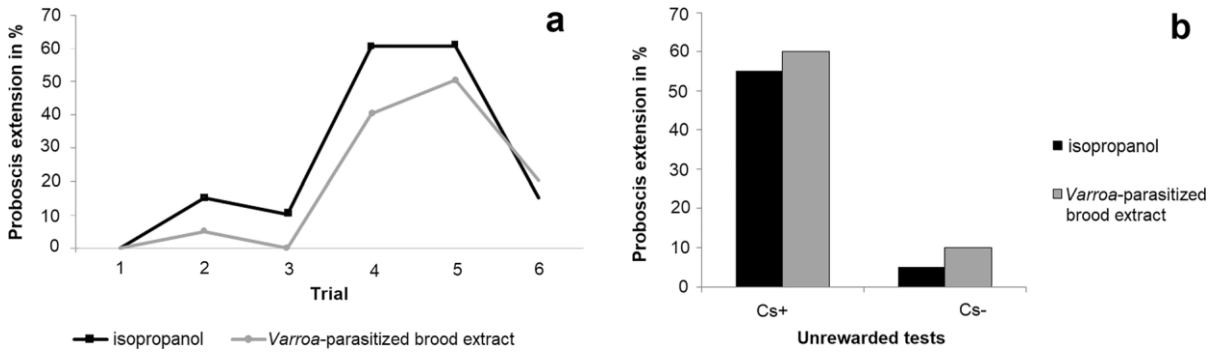

Fig. 4 Acquisition curves and unrewarded tests for conditioning with isopropanol as Cs + (black line) and *Varroa*-parasitized brood extract as Cs + (grey line). **(a)** Proboscis extension frequencies for isopropanol as Cs + / *Varroa*-parasitized brood extract as Cs- and *Varroa*-parasitized brood extract as Cs + / isopropanol as Cs- are shown in percent for each trial. The alpha-level is set at 0.05. Per conditioning experiment (isopropanol as Cs + / *Varroa*-parasitized brood extract as Cs- and *Varroa*-parasitized brood extract as Cs + / isopropanol as Cs-) the same number of worker bees were used ($N = 20$). No significant differences were observed. **(b)** Proboscis extension frequencies during the unrewarded tests are shown in percent. The alpha-level is set at 0.05. No significant differences were observed

non-selected line (see Fig. 5, suppl. Tab. S8). This percentage difference corresponds to 133% relative increase of the VSH-selected line's response rate. The differences were statistically significant (GLMM, $p = 0.027$; CI: 0.11; 1.77). Moreover, the VSH-selected line had 2.6 times higher odds of perceiving the Cs + at ***Time point 1*** than the non-selected line (OR = 2.6; CI: 1.12; 5.89) (see suppl. Tab. S9).

Worker bees from both the VSH-selected and non-selected lines that perceived the *Varroa*-parasitized brood extract at ***Time points 2*** and ***3*** performed similarly (see suppl. Tabs. S10 and S11). Again, there was no difference between the three age groups in terms of the ability of the worker bees' perception of the extract. Similar to the conditioning with citral and linalool, the colony effect also had no significant influence on stimulus detection (see suppl. Fig. S F2).

The VSH-selected and non-selected lines exhibited a difference in their overall conditioning outcome during the unrewarded tests. The VSH-selected line displayed a higher percentage of worker bees (34%) which were able to perceive the extract of *Varroa*-parasitized brood (see Fig. 6) than the non-selected line (23%). The difference was significant (Kaplan–Meier estimator, Long rank test, $\chi^2(1; N = 240) = 3.97$, $p = 0.046$).

Discussion

In the course of this work two sets of experiments were carried out. The salience of the substances used throughout the experiments was assessed using a reversed differential conditioning with groups of 20 workers per stimulus combination. Furthermore, during the main experiment, a total of 240 workers – 120 from the VSH-selected line and 120 from the non-selected line – were conditioned per stimulus combination.

Because the PER response does not measure the sensitivity of an individual bee per se, but the learning behavior to a stimulus, we hypothesized that there would be no learning success even with large differences in learning ability, if the stimulus is not recognized. As sensitivity towards different chemical cues plays a central role in hygienic behavior (Schöning et al. 2012; Mondet et al. 2015, 2021; Wagoner et al. 2021), the learning behavior of the worker bees was used to determine whether low concentrations of the stimuli are perceived at all and thus could be regarded as marker for perception ability and olfactory sensitivity.

All three research questions could be answered. Although the chosen experimental setup did not examine the ability of workers to differentiate between healthy and parasitized brood, it displayed workers' ability to perceive the complex chemical bouquet of *Varroa*-parasitized brood cues at a very low concentration. No difference in salience was observed between the extract of *Varroa*-parasitized brood and the solvent isopropanol during the reversed differential conditioning, further strengthening this observation.

The results of the main experiment additionally indicated an enhanced specific sensitivity in the VSH-selected line towards chemical cues emitted by the

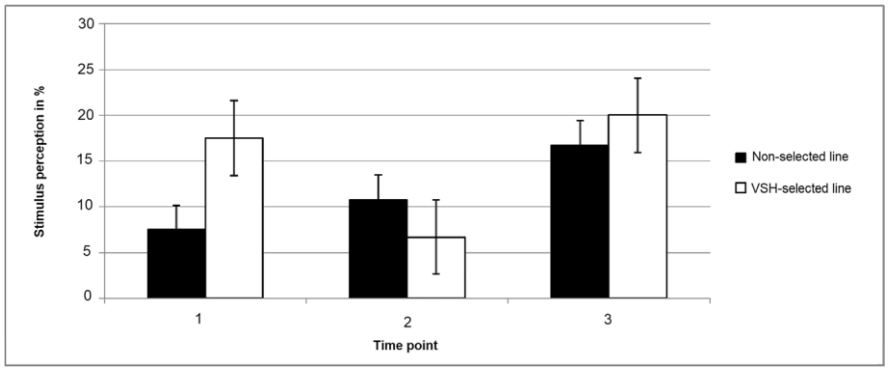

Fig. 5 Number of worker bees, that are able to perceive the Cs + (*Varroa*-parasitized-brood extract), shown in their corresponding speed group. Displayed are the two lines – VSH-selected line (white) and the non-selected line (black). The columns show the number of workers which successfully perceived the Cs + at one of the three time points. Each worker is listed in only one group. Standard error is displayed for each time point and group

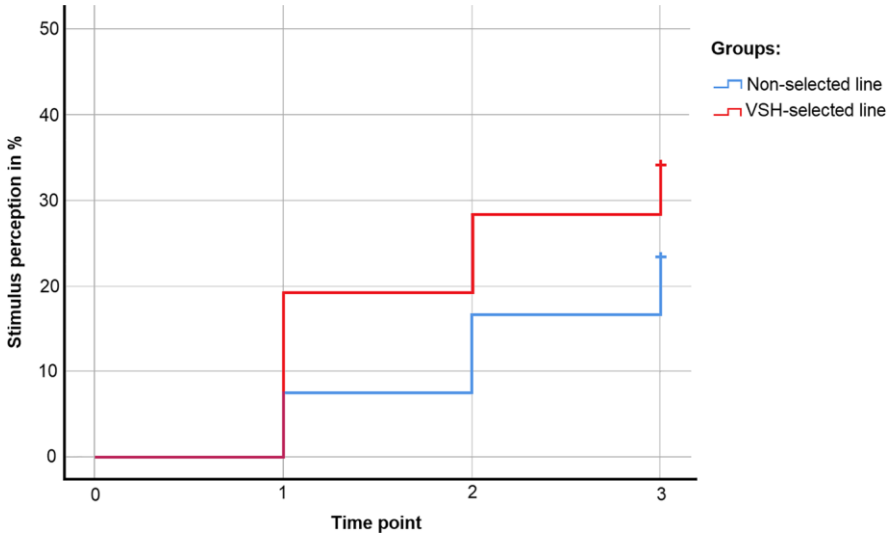

Fig. 6 Kaplan–Meier perception curve for the *Varroa*-parasitized-brood extract. The *Varroa*-parasitized-brood extract was used as Cs + , the solvent isopropanol as Cs-. The cross at the end of each line represents the end of the conditioning for all subjects of the corresponding group. The three vertical lines represent ***Time points 1, 2*** and ***3***. The cumulative perception of each line is displayed on the y-axis in percent. The two groups are displayed separately: VSH-selected line (N = 120; red color), non-selected line (N = 120; blue color)

brood, which can play a role in detection of *Varroa*-parasitization. When tested with a low concentration of the *Varroa*-parasitized brood extract, workers selected for VSH exhibited a significantly higher perception ability and a higher percentage of stimulus recognition (Cs +) at the earliest possible time point compared to the non-selected line.

Unlike the observations from the conditioning with the *Varroa*-parasitized brood extract and isopropanol, citral and linalool exhibited significant differences in the proboscis extension frequencies during most trials of the reversed conditioning. Citral posed as more salient compared to linalool at the dilution (1:1300) used in the course of this work. During the main experiment, the highly diluted floral extract citral was perceived equally well by both lines. The speed of perception for citral was also comparable for the two lines.

Perception Ability of Worker Bees Towards Flower Substances

Odor detection is an important part of food and host selection in invertebrates and mammals (Visser 1986; Masson and Mustaparta 1990; Firestein 2001). The ability to form an association and gather experience from previous foraging decisions is a result of long-lasting natural selection in the honey bee, as foraging behavior acts as a major determinant for the survival of both the individual and the colony (Kramer 2001; Page et al. 2006). Olfactory generalization is considered crucial for foragers' ability to find suitable food sources with varying volatile release (Sandoz et al. 2001). This ability allows animals to extend a behavior from a particular stimulus to another, novel stimulus, which is perceived similarly enough (Shepard 1987). Especially molecules with a similar carbon length and chemical group are subjected to high generalization (Sandoz 2011).

The ability to distinguish between different odorants is also dependent on stimulus concentration (Getz and Smith 1991; Wright 2004). During our preliminary tests, we observed a great decrease in the behavioral responses to both substances (citral and linalool) when using a dilution of more than 1:1300 (equivalent to a concentration of 0.69 µg/µL solution) for the differential conditioning. Only a third of the worker bees used in the conditioning discriminated between stimuli of this particular dilution by touching the filter paper with the antennae or sensing the emitted odor via molecules in the air, therefore we chose not to dilute our probe any further in order to gather sufficient data on the differences in discrimination

ability between the two lines. Nevertheless, from the data gathered during the preliminary tests, we suspected that the conditioning threshold for citral using a tactile presentation lies in the range of 1:1500 (~ 0.6 µg/µL).

In the course of the reversed differential conditioning, we observed a generalization between citral and linalool when citral was rewarded. This was not the case when linalool was used as Cs + . One possible reason for the generalization could be the similar carbon chain length of their molecular structures – $C_{10}H_{16}O$ (citral) and $C_{10}H_{18}O$ (linalool). Another reason could be the fact that citral not only plays a role as a flower odor but is also a compound found in secretions of the Nasonov gland (Butler and Calam 1969; Getz and Smith 1991). Social pheromones are described as producing higher generalization as general odors which would suggest that biological value influences generalization (Sandoz et al. 2001). This could explain the overall higher proboscis extension response frequency when citral was used as Cs + . Shearer and Boch (1966) described citral as a minor compound of the Nasonov pheromone that increases the attractiveness of geraniol – one of the major components – when both are presented together. On its own, citral was far less attractive than geraniol or the Nasonov pheromone itself (Shearer and Boch 1966; Williams et al. 1981). It could therefore be argued that citral's biological value is given only as part of the mixture and the experimental results display merely a difference in attractiveness between two floral substances.

When both VSH-selected line worker bees and non-selected line worker bees were trained with the highly diluted floral compounds during the main experiment, they exhibited a similar olfactory sensitivity and discrimination ability. The VSH-selected line showed a 14% relative improvement of perception. Nevertheless, this difference was not significant. Unlike previous studies like those by Masterman et al. (2000) and Chakroborty et al. (2015), where undiluted odors were used to observe differences in perception, we hypothesized that high concentrations pose an easy task for the test subjects and provide information on overall perception ability but give no feedback on olfactory sensitivity. Such information can only be displayed by using concentrations near the threshold limit for eliciting a behavioral response (Laska 2000). We can now add to the previous studies' conclusions and confirm that the olfactory sensitivity and discrimination ability of hygienic and non-hygienic lines does not significantly differ when low concentrations near the perception threshold of citral and linalool are used.

Perception Ability of Worker Bees Towards the *Varroa*-parasitized Brood Extract

During the conditioning with a low concentration of the *Varroa*-parasitized-brood extract, a different picture than with the flower extracts was observed. The VSH-selected line exhibited a significantly stronger tendency of perceiving the complex bouquet of *Varroa*-parasitized brood than the non-selected line, with a relative improvement of 133% in perception.

Previous studies proved an important step in describing the improvements in hygienic behavior caused by breeding efforts (Ivanova and Bienefeld 2021). Masterman et al. (2001, 2000) described a better performance of hygienic lines when conditioned to the strong stimulus of chalkbrood diseased brood. Chakroborty et al. (2015) used live pupae parasitized by *V. destructor* to assess differences in the perception ability of worker bees from VSH-selected and non-selected lines and described a "slightly better performance" of the VSH bees as well. This tendency of VSH workers to better perceive the cues connected to a *V. destructor* parasitization are likely a result of proteome differences in the central nervous system and the antennae of worker bees (Mondet et al. 2015; Hu et al. 2016). Mondet et al. (2015) conducted a differential gene expression on the antennae of bees selected for VSH and non-VSH. Genes connected to defense responses were over-expressed in VSH-bees' antennae (Mondet et al. 2015). In the mushroom bodies, proteins connected to neuronal sensitivity by activation of synaptic vesicles and calcium channels were upregulated in VSH workers (Hu et al. 2016). Moreover, hygienic bees were shown to have lower stimulus thresholds for olfactory and behavioral responses than non-hygienic bees (Masterman et al. 2001). Boutin et al. (2015) suspected that non-hygienic bees have an over-expression of cytochrome P450, an enzyme that participates in the degradation of odorant pheromones. An over-expression could lead to the removal of stimuli before the hygienic behavior can be initiated and thus influence the worker bee's olfactory capability.

Compared to Masterman et al. (2001) and Chakroborty et al. (2015), the brood extract during our conditioning experiments contained only a fraction of the stimulus intensity (for comparison: 0.02 brood equivalents versus a whole parasitized pupa) so as to approach the perception threshold of worker bees as much as possible while still eliciting a behavioral response. Furthermore, we used a tactile presentation which is closer to the natural perception of *Varroa*-parasitized brood in the colony, compared to the air stream presentation used by Chakroborty et al. (2015). While it is possible that tested workers might have been exposed to higher concentrations of the extract through the direct contact with the filter paper compared to the amounts of the extract delivered only via an air stream, we hypothesized that the very low concentration of the extract would nevertheless provide a more difficult task for the workers than previously done by Chakroborty et al. (2015). Our aim was to mimic reality as closely as possible, considering that worker bees in the colony must recognize subtle brood distress signals, superimposed by the odors of neighboring cells, through the closed cell caps. While discrimination between the extract of *Varroa*-parasitized brood and the cuticular profile of healthy brood was not tested in the course of this work, the results from the main conditioning experiment (*Varroa*-parasitized brood extract as Cs +) nevertheless showed that workers can perceive the low concentration of *Varroa*-parasitized brood extract and clearly distinguish it from the solvent. This observation was strengthened by the fact, that no difference in salience was present between extract and the solvent isopropanol during the reversed conditioning. The differences in discriminatory ability between the two lines made during the main experiment are therefore not due to contrasts in stimulus intensity but a result of selection breeding.

Speed of Perception

Both lines (VSH-selected and non-selected line) showed similar numbers of worker bees with a positive conditioning outcome for the highly diluted citral. Although the VSH-selected line exhibited a 39% relative improvement of perception at ***Time point 1*** compared to the non-selected line, the difference was non-significant.

During the experiment with the *Varroa*-parasitized brood extract, both lines displayed worker bees which are capable of early perception. While most of the worker bees from both lines exhibited similar discriminatory ability and perceived the extract at ***Time point 3***, only a third of all workers displayed superior olfactory sensitivity. At the earliest possible time point (***Time point 1***), VSH-selected line showed 2.6 times higher odds of perceiving the *Varroa*-parasitized brood extract than the non-selected line, complementing a relative improvement of perception of 133%. A possible explanation for the different numbers of worker bees exhibiting fast perception in both groups is the aforementioned difference in stimulus threshold. While both hygienic and non-hygienic hives exercise hygienic behavior, the latter remove diseased brood less efficiently (Arathi et al. 2000; Arathi and Spivak 2001). VSH-selected bees might be responding to stimuli faster thanks to a difference in the expression of genes compared to non-hygienic bees (Navajas et al. 2008; le Conte et al. 2011; Hu et al. 2016; Gempe et al. 2016).

It could be argued that the observed results are caused by a higher "sucrose responsiveness" of the VSH line and not by a superior olfactory sensitivity. However, in our experiment the workers from both lines (hygienic and non-hygienic) displayed similar reactions to sucrose during the conditioning, suggesting that the enhanced ability of hygienic bees to perceive diseased brood cues during conditioning experiments is independent from the bees' sucrose responsiveness. Rather, it appears that the VSH breeding efforts produce higher olfactory sensitivity in worker bees, leading to the higher sensitivity to low concentrations of the *Varroa*-parasitized brood extract as observed. Learning performance and speed were no selection criteria for the creation of the VSH-selected line. The selection criterion used was the reaction to parasitized brood, whose chemical profile is known to deviate from that of healthy brood (Mondet et al. 2021). These observations are consistent with Goode et al. (2006), who suggested that the high sensitivity towards pathological cues does lead to a quicker and more efficient detection and removal of parasitized brood.

Lapidge et al. (2002) suggested that hygienic behavior is a quantitative trait whose differential expression leads to variations in each hive's performance and even between bees in the same hive.

Indeed, Gramacho and Spivak (2003) observed differences in the olfactory sensitivity in bees from the same hive and of the same age that were performing hygienic behavior. During their PER conditioning experiment, worker bees that initiated uncapping behavior exhibited greater olfactory sensitivity than bees which were engaged only in removing the brood. This variation is likely a consequence of the queen mating with several drones from different colonies. In contrast, in our experiments the VSH-selected line was created exclusively through artificial insemination with sperm from several drones coming from one colony, resulting in less gene dispersion within the colonies.

With the results of our experiments in mind, we anticipate that the worker bees with the highest perception speed towards the extract of *Varroa*-parasitized brood at ***Time point 1***, could, in fact, be the most sensitive ones and most likely to elicit uncapping behavior. The difference in the number of workers with a higher olfactory sensitivity is most probably a result of the *Varroa*-resistance breeding efforts. More studies are needed to further strengthen this hypothesis. This could be done by testing worker bees with a high perception speed towards the extract of *Varroa*-parasitized brood for their uncapping activity on a brood frame, artificially infested with *V. destructor*. As workers with faster perception for the *Varroa*-parasitized brood extract were also present in the non-selected line but in smaller numbers, we expect to exhibit a difference in the uncapping activity between the two origins.

Enhanced Specific Olfactory Sensitivity

The results from our experiments suggest breeding efforts can enhance bees' olfactory sensitivity and discrimination ability to chemical cues emitted from the brood in connection to a *V. destructor* parasitization. The VSH-selected line displayed specific higher sensitivity towards the extract of *Varroa*-parasitized brood compared to the non-selected line. This specific sensitivity was characterized by a faster (at the earliest time point) and generally higher perception of the complex chemical blend emitted by the brood, even in small quantities and low concentrations. The sensitivity to flower extracts was comparable to that of the non-selected line.

Specialization to ecologically relevant stimuli has been observed in countless species. It supplies the nervous system with valuable information, allowing animals to respond appropriately to a given situation (Hansson and Stensmyr 2011). Olfaction plays an important role in most insects (Dethier 1947), and changes in the olfactory system can enhance the fitness and breeding success (Hansson and Stensmyr 2011). In *Drosophila sechellia*, for instance, increased numbers of one type of olfactory sensillum allow the fly to specialize in one type of fruit that is toxic to other drosophilids (Hansson and Stensmyr 2011; Linz et al. 2013). Mosquitoes of the *Culex* taxa possess high selectivity and sensitivity towards nonanal—a semiochemical characteristic for birds and humans — allowing the insects to detect their hosts from a long range (Syed and Leal 2009). For honey bees, the ability to detect disease-specific cues is a vital part of hygienic behavior and social immunity. Workers exhibiting higher olfactory sensitivity to abnormal brood initiate its removal thus prolonging the survival of the colony (Gramacho and Spivak 2003).

While the development of resistant honey bee populations based on increased VSH can occur naturally (Panziera et al. 2017), breeding efforts have also been shown to successfully increase hygienic behavior (Pérez-Sato et al. 2009). Indeed, enhanced hygienic behavior is correlated with various changes to the proteome of the olfactory system (Parker et al. 2012), particularly the expression of different proteins such as Odorant binding protein, VAMP, Calcyclin Binding Protein, which are connected to signal transduction in the antennae (Guarna et al. 2015). As Gempe et al. (2016) describe, an over-represented signal transduction can be seen in the brain of highly hygienic bees. Furthermore, bees tolerant to *V. destructor* also display an up-regulation of genes connected to neuronal excitability (Navajas et al. 2008). These genes might participate in the increase of responsiveness to environmental stimuli and lead to engagement in hygienic behavior (Navajas et al. 2008). While the PER conditioning can be used to estimate for differences in discrimination ability and sensitivity, we suspect that the measured differences may not adequately reflect the complete potential of bees with respect to their hygienic behavior to *Varroa*-parasitized brood. One reason for this can be the stress caused by the conditioning itself. Furthermore,

cues other than those emitted from the brood – like thermal cues – could play a supplementary role in the decision to uncap a brood cell (Bauer et al. 2018). In an extensive experiment conducted at the Institute for Bee Research Hohen Neuendorf, the worker bees of the VSH-selected line started uncapping 8-times more *Varroa*-parasitized cells than bees of the non-selected control line (Bienefeld, in preparation). Previous research further displayed strong maternal and additive genetic effects for the manifestation of VSH (Ivanova and Bienefeld 2021). The results shown by the worker bees in this experiment demonstrate enhancements of the VSH-selected line's specific olfactory sensitivity towards cues emitted by the brood caused by resistance breeding.

Conclusion

Our findings further deepen the knowledge of VSH and provide valuable information on the effects of breeding for *Varroa*-resistance. The difference in perception speed shown by the VSH-selected line during the PER conditioning experiment is most likely based on a lower stimulus threshold for olfactory and behavioral responses. However, more research is needed to optimize the methodology of assessing sensitivity to the relevant stimuli and to determine whether other influencing variables are further drivers of hygienic behavior towards sick brood, beyond characteristics that control olfactory sensitivity.

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Author Contributions K.B. and I.I. designed the study. I.I. conducted the experiments, analyzed the data and wrote the paper. KB supervised the study and assisted with the interpretation of results and writing of the manuscript.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of Interests The authors declare no conflict of interests.

Competing Interests The authors declare no competing interests.

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3.2. Publication II

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Suitability of drone olfactory sensitivity as a selection trait for *Varroa*-resistance in honeybees

Ivelina Ivanova[1,2*] & Kaspar Bienefeld[1]

The most effective strategy against brood diseases, such as those stemming from infestation by the mite *Varroa destructor,* is the early detection and removal of sick brood. Recent findings suggest that genes associated with worker bee olfactory perception play a central role in *Varroa*-sensitive hygiene (VSH). In this study, the odour sensitivity of *Apis mellifera* drones was examined through proboscis extension response (PER) conditioning. Individuals sensitive/insensitive to the two *Varroa*-parasitised-brood odours (*extract-low* and *extract-high*) were used for breeding. Twentyone queens from a VSH-selected line (*SelQ*) and nineteen queens from a nonselected line (*ConQ*) were single-drone-inseminated with sperm from drones that showed either sensitivity (*SenD+*) or insensitivity (*SenD−*) to the two extracts. Individual VSH behaviour in a total of 5072 offspring of these combinations (*SelQ* × *SenD+*, *SelQ* × *SenD−*, *ConQ* × *SenD+*, *ConQ* × *SenD−*) was subsequently observed in a specially designed observation unit with infrared light. The results from the video observation were also separately examined, considering the genetic origin (VSH-selected or nonselected line) of the participating queens and drones. While the drone PER conditioning results were not significantly reflected in the VSH results of the respective offspring, the genetic origin of the participating queens/ drones was crucial for VSH manifestation.

The ectoparasitic mite *Varroa destructor* plays a dominant role in colony losses of the European honeybee *Apis mellifera*[1–3]. Currently, available treatments for *Varroa*-infested colonies such as pyrethroids and formic acid are not only labour intensive but also leave residues in honeybee products[4,5]. In addition, studies have shown an alarming tendency of increasing mite resistance against miticides[6–8]. While current treatment methods provide only temporary benefits, breeding colonies resistant to *V. destructor* is considered the only long-term solution[9,10].

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The antennae of bees play an essential role in perceiving their environment and communication within the hive[11,12]. Amid this process, both olfactory and tactile stimuli are perceived and processed. One of the natural defence mechanisms of honeybees that has proven effective against *V. destructor* is hygienic behaviour. Mechanisms similar to the hygienic behaviour of honeybees have also been observed in other social insects[13–15]. In honeybees, hygienic behaviour consists of detecting, uncapping, and removing damaged brood[16–18]. This particular behaviour directed towards *V. destructor* has received different names during the years[19–23]. Among others, the term "suppression of mite reproduction" (SMR) was created by Harbo and Harris[15] to describe the lack of viable progeny of the mite observed in resistant colonies during their experiments. Subsequently, SMR was renamed *Varroa*-sensitive hygiene (VSH), as the observed suppression was found to be the result of removing reproductive mites and not of inhibiting reproduction of *V. destructor* in resistant colonies[21,24,25].

Research has shown that selective breeding can improve the colonies' performance regarding their hygienic behaviour against *V. destructor*[9,21,26,27]. VSH is assumed to be based on the differential expression of genes responsible for the olfactory system and perception[28–32]. Mondet et al.[33] presented evidence that all worker bees can detect *Varroa*-parasitisation-specific compounds, but only bees performing VSH can distinguish those from the healthy brood odour.

Signals from the damaged brood are present on the cell cap[34]. VSH bees use their olfaction to perceive these cues emitted by the infested pupae and thereby target the most compromised brood cells containing multiple mature females and higher numbers of mite progeny[35,36]. Through typical movements with the head, the worker bees can localize the damaged brood very accurately[37]. By uncapping and removing the diseased brood, VSH bees diminish the mite's spread in the colony[38]. In some cases, instead of removing the parasitised brood, workers open and recap parasitised brood cells multiple times. This behaviour disrupts mite reproduction without sacrificing the developing brood[39,40].

Differences in the odour discrimination abilities of hygienic and nonhygienic colonies have also been observed under laboratory conditions[41,42]. Masterman et al.[41] used differential conditioning with two odour combinations—geraniol/1-hexanal and odour of healthy pupae/odour of chalkbrood infested pupae—to examine the discrimination

abilities of worker bees from hygienic and nonhygienic lines. While there was no significant difference between the two genetic lines when presented with flower odours, Masterman et al.[41] observed discrepancies in the perception of the brood odour. The hygienic line discriminated better between the two brood odours during the conditioning process than did the nonhygienic line. The authors suspected a genetically induced increased specific odour sensitivity to pathogens in the hygienic line, which would allow worker bees to remove sick individuals from the population more efficiently. Masterman et al.[41] used conditioning with the so-called proboscis extension response (PER).

The PER is a biological reflex that occurs in different species of insects due to antennal stimulation[43]. Honeybees usually exhibit this behaviour while foraging or during trophallaxis. PER is easily replicated under laboratory conditions. Based on Pavlovian classical conditioning, conditioning using PER was first introduced by K. Takeda in 1961 and has been used as a foundation for many olfactory experiments ever since[43–48]. Among others, the PER conditioning is widely applied for observing the learning ability of individual honeybees[46,48], the odour sensitivity connected to VSH[33,42] and the adverse effects of pesticides on honeybee behaviour[49,50]. The subject learns to associate a conditioned stimulus (CS)—usually an odour—with an unconditioned stimulus (US) such as a sugar solution[51]. The odour presentation leads to the extension of the mouthparts (proboscis), as a reward is expected. Through varying concentrations of the odour substance, the individual animal's odour sensitivity and perception threshold can be determined[52–54].

While current breeding strategies concentrate on worker bees and their ability to recognize mite-infested cells, our focus lies in identifying the drone's role as a genetic carrier for the manifestation of VSH. Because of drones' impressive ability to detect the queen from a distance during mating flights using olfactory cues[55], we speculated that the use of individually tested drones could be a very efficient approach to significantly improve the genetic progress in developing *Varroa*-resistance. Drones are haploid, and all their genetic material is completely passed on to the offspring without the Mendelian sampling effect. Having this in mind, we used PER conditioning to noninvasively evaluate the drone's odour sensitivity towards an extract of *Varroa*-parasitised brood. Unlike other brood diseases, such as chalkbrood, which cause more extensive damage to the brood, the signals emitted from the *Varroa*-parasitised brood are much weaker[31]. The perception of the subtle stimulus caused by the parasitisation

with *V. destructor* is therefore suitable for selecting for a better resistance not only against *V. destructor* but also against most brood diseases.

To observe whether brood odour sensitivity would be reflected in the VSH of the F1 generation, queens underwent a one-drone insemination, and the offspring of the tested drones (worker bees) was observed in a unit with infrared light for its ability to detect and remove artificially *Varroa*-infested brood.

Results

Drone conditioning of the two lines regarding different odour concentrations. The selection of drones for artificial insemination was performed through two conditioning experiments using different concentrations of a *Varroa*-parasitised pupae extract—*extract-low* and *extract-high*. The solvent used for the creation of the extract was used as CS−. During the conditioning, the drones had to differentiate between the *Varroa*-parasitised brood odour and the solvent control. The conditioning consisted of six trials (CS+, CS−, CS−, CS+, CS+, CS−) and was followed by an unrewarded presentation of both stimuli (CS+ and CS−). Drones from a line selected for VSH and drones from a nonselected line were conditioned with one of the two extracts (*high* and *low*).

Before the start of the main experiment, preliminary tests were conducted in order to determine which odour concentrations were suitable for our experimental design. An important criterion for the decision was to obtain a sufficient number of successfully conditioned drones for the sperm extraction. After a series of preliminary tests, the concentrations of *extracts high* and *low* were deemed suitable for the official experiment. A third of the drones (30%) managed to perceive *extract-low.* For *extract-high*, that number was ~ 60%.

During the main experiment drones conditioned with both extracts exhibited an increase in the behavioural reactions (proboscis extension) when the CS+ was paired with the reward. This was not the case with CS−. The responses to CS− remained almost constant (Figs. 1, 2).

The number of drones successfully conditioned to *extract-low* and *extract-high* was 39% and 46%, respectively. During the main experiment, the drones excluded for not

responding to the sugar stimulus amounted to 22% for *extract-low* and 16% for *extract-high.*

To evaluate whether the origin of the drones participating in the conditioning played a role in the conditioning outcome, the results of the two lines were analysed separately using a Generalized linear mixed model (GLMM). The drones from the nonselected line were set as reference group in the model and the stimulus effect (CS+ or CS−) was also included. The temperature during the conditioning and the drone's mother were used as random factors in the model.

The GLMM model showed no statistically significant difference between the conditioning results of the VSH-selected and nonselected line drones. These findings applied to both *extract-low* (GLMM, p = 0.36; CI − 0.80; 0.29) and *extract-high* (GLMM, p=0.14; CI: −0.14; 0.96). The stimulus effect proved to be significant (*extract-low*: GLMM, p<0.001; CI 2.34; 3.75 and *extract-high*: GLMM, p<0.001; CI 2.32; 3.54), showing a conditioning success only for the rewarded stimulus CS+ but not for the unrewarded CS− (Figs. 1, 2). The temperature during

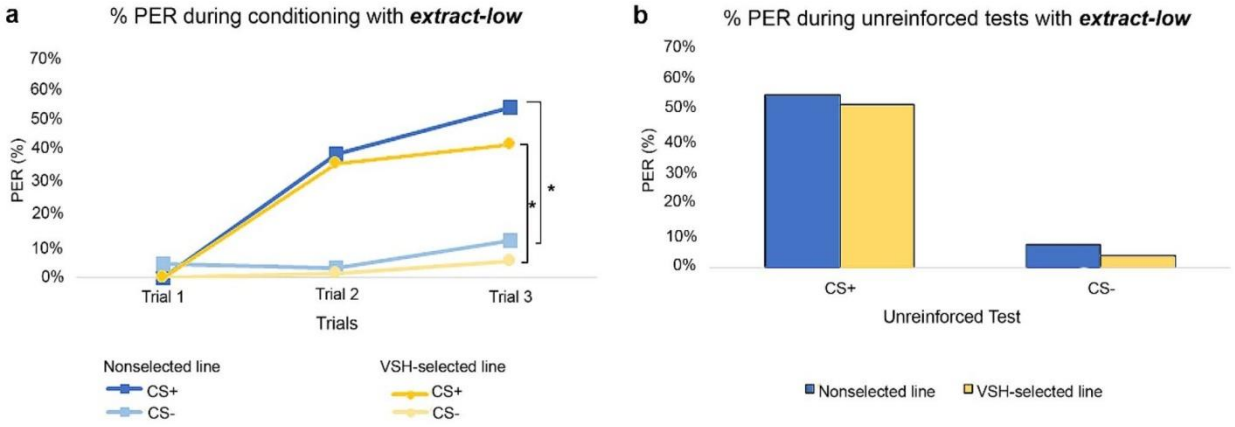

Figure 1. Drone performance during PER-conditioning experiment with *extract-low*. Acquisition (**a**) and results from the unreinforced tests (**b**) are shown for both stimuli (CS+ and CS−) and origins (nonselected line, VSH-selected line). The curves display the behavioural reaction—proboscis extension—for the reinforced (CS+) and the non-reinforced (CS−) stimulus. The bars show the behavioural reaction during the unreinforced tests with both stimuli. A total of 223 drones were tested using *extract-low*. The stimulus effect (reinforced, nonreinforced) was significant— (*) p < 0.001.

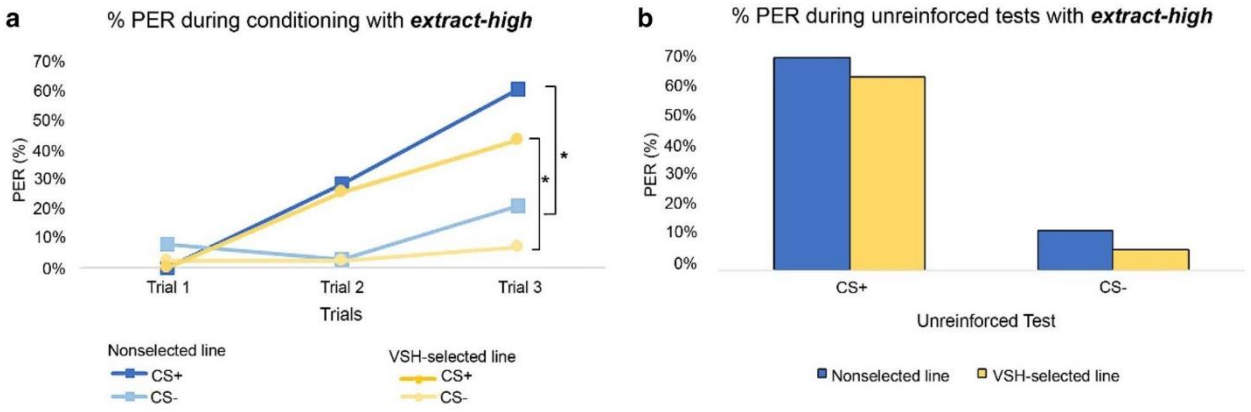

Figure 2. Drone performance during PER-conditioning experiment with extract-high. Acquisition (**a**) and results from the unreinforced tests (**b**) are shown for both stimuli (CS+ and CS−) and origins (nonselected line, VSH-selected line). The curves display the behavioural reaction—proboscis extension—for the reinforced (CS+) and the non-reinforced (CS−) stimulus. The bars show the behavioural reaction during the unreinforced tests with both stimuli. A total of 202 drones were tested using extract-high. The stimulus effect (reinforced, nonreinforced) was significant— (*) $p < 0.001$.

the conditioning and the drone's mother had no significant effect on the conditioning results for either *extract-high* or *extract-low*.

Mating design. Depending on the conditioning results, the drones were divided into two groups— "*Varroa*-parasitised-brood-odour sensitive" (*SenD+*) and "*Varroa*-parasitised-brood-odour insensitive" (*SenD−*). "Sensitive" drones responded to the CS+ but not the CS− during the last two trials and during the unrewarded tests with *extract-low*—Trials: CS+, CS−, CS−, CS+, CS+, CS−; Unrewarded test: CS+, CS−. The "insensitive" drones responded to the US throughout the experiment with *extract-high* but showed no positive responses to the CS+ during the last two trials, and the unrewarded tests indicated a negative conditioning outcome.

Queens from both a VSH-selected line and a nonselected line were one-drone inseminated with sperm from the "sensitive" or "insensitive" drones. Four groups were created during the one-drone insemination depending on the queen's affiliation with the VSH-selected line (*SelQ*) or nonselected line (*ConQ*)[56] and the drone's olfactory sensitivity towards the *Varroa*-parasitised-brood odour.

The groups were created without regards to the genetic origin of the drones. Because drones from both origins were tested during the experiment, each group consisted of queens inseminated with sperm from drones from both lines (Suppl. Table 1).

Group	Queens	Offspring	Beginner		Helper	
	N	N	N	%	N	%
SelQ × *SenD+*	12	1382	47	3.4	92	6.7
SelQ × *SenD−*	9	850	66	7.8	95	11.2
ConQ × *SenD+*	8	1273	18	1.4	32	2.5
ConQ × *SenD−*	11	1567	39	2.5	58	3.7

Table 1. Grouping considering queen's origin and drone's olfactory sensitivity. Summary of the number (N) of inseminated queens per group and their offspring (worker bees). Displayed are furthermore the number of beginner and helper bees in each group (N) and the corresponding equivalent in percent per group (%).

Of the 87 *Varroa*-parasitised-brood-odour sensitive drones that qualified for insemination, only 26 were used for the insemination of queens since the rest did not have sperm. Of those 26, only 20 queens produced enough offspring to participate further in the experiment.

Of the 48 *Varroa*-parasitised-brood-odour insensitive drones, 22 were used for insemination. Of those, 20 had enough offspring to participate in the experiment.

Video observation. The offspring (worker bees) of the one-drone inseminated queens was marked with numbered plates on the dorsal thorax and its VSH towards an artificially *Varroa*-mite-infested brood frame was recorded during six days in an infrared video observation unit. The video observation was performed three times (courses) with different bees during the experiment.

For the evaluation of the video recording two activities were of importance. Beginner bees were the first to open a mite infested cell. Helper bees enlarged the hole in the cell cap created by the beginner. If the cell was resealed, the next beginner and helper bees were noted.

VSH of groups considering drones' olfactory sensitivity in PER conditioning experiment. The new generation of worker bees was divided into four groups considering their mother's origin (VSH-selected line *SelQ* or nonselected line *ConQ*) and their father's odour sensitivity—*SenD−* (*Varroa*-parasitised-brood-odour insensitive drone) or *SenD+* (*Varroa*-parasitised-brood-odour sensitive drone). The data was analysed using a Generalised linear mixed model with group *ConQ* × *SenD−* as a reference. The course of observation and the drones' origin (VSH-selected or nonselected line) were considered as factors in the analysis. The queen mother's

affiliation to one of the two lines (VSH-selected or nonselected line) was also included as a random factor in the model.

Group *SelQ* × *SenD*− exhibited the highest number of VSH-active bees in the two categories—beginner (7.8%) and helper (11.2%). Compared to the reference group, these results were statistically significant—beginner (GLMM, $p < 0.001$; CI 0.84; 1.64) and helper (GLMM, $p < 0.001$; CI 0.85; 1.54). The odds of *SelQ* × *SenD*− uncapping a parasitised cell were 3.5 times higher than that of the reference group (GLMM, OR=3.46; CI 2.32; 5.15).

Group *SelQ* × *SenD*− was followed by group *SelQ* × *SenD*+ (beginner: 3.4%, helper: 6.7%) (Table 1). Group *SelQ* × *SenD*+ displayed slightly but not significantly higher uncapping activity than the reference group (GLMM, $p = 0.225$; CI − 0.16; 0.68). The odds of this group initiating the uncapping of a parasitised cell were similar to those of the reference group.

Group *ConQ* × *SenD*+ did not perform better than the reference group in any of the activities (see Suppl. Tables 2 and 3). In fact, the reference group exhibited more beginner (2.5%) and helper bees (3.7%) than the *ConQ* × *SenD*+ group (beginner: 1.4%, helper: 2.5%) (Fig. 3).

The origin of the queen mothers had significant effect on the beginner bees' activity—GLMM, $p < 0.001$ (CI: 0.62; 1.47). The origin of the father drone played a significant effect on the helper bees' activity with VSH-selected line drones producing more active offspring—GLMM, $p < 0.001$; CI 0.39; 0.95.

The three observation courses also exhibited differences in the number of active beginner and helper bees. The worker bees scored significantly higher in their beginner actions in courses two (GLMM, $p = 0.017$; CI 0.09; 0.97) and three (GLMM, $p < 0.001$; CI 0.58; 1.36) than the reference in course one. Course three also exhibited the highest results for helper activity (GLMM, $p = 0.02$; CI 0.05; 0.57).

VSH in groups considering the genetic origin. In the second evaluation step, the genetic origin of the queens (*SelQ, ConQ*) and drones (*SelD, ConD*) was used to restructure the aforementioned groups. The colonies participating in the experiment were divided into four new groups (Table 2 and Suppl. Table 4)—*ConQ* × *ConD*, *ConQ* × *SelD*, *SelQ* × *ConD*, *SelQ* × *SelD*. Group *ConQ* × *ConD* was used as reference group.

When comparing the groups' beginner and helper activities to those of the reference group *ConQ* × *ConD*, a significant increase from the nonselected to VSH-selected line was observed. The pairing of queens from the VSH-selected line (*SelQ)* with drones from the VSH-selected line (*SelD*) delivered the highest number of active beginner (5.5%) and helper bees (10.4%) (Fig. 4). The results were statistically higher than those of the reference group *ConQ* × *ConD* (beginner: GLMM, $p < 0.001$; CI 1.20; 2.21; and helper: GLMM, $p < 0.001$; CI 1.47; 2.53). The odds of group *SelQ* × *SelD* uncapping a parasitised cell were 5.5 times higher than those of the reference group (GLMM, OR = 5.5; CI 3.31; 9.09).

The second highest results were achieved when inseminating a queen from the VSH-selected line (*SelQ*) with sperm from drones coming from the nonselected line (*ConD*). Group *SelQ* × *ConD* showed the second highest

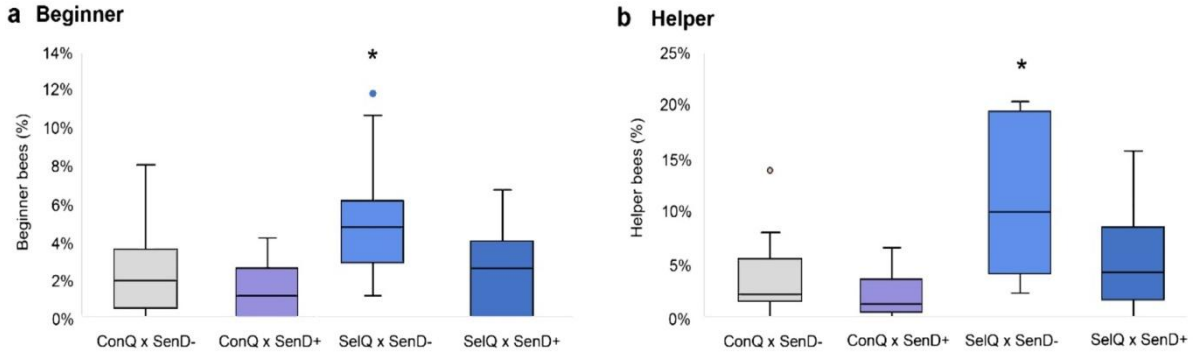

Figure 3. Boxplot of beginner and helper bees in groups based on the drones' olfactory sensitivity. Displayed are median, standard deviation and outliers for the beginner (**a**) and helper (**b**) categories for each group. The proportions of beginner (**a**) and helper (**b**) bees during the three courses of the video observation experiment are displayed for each group. One colony had 11.8% beginner bees (outlier—group *SelQ* × *SenD−*). One colony exhibited 13.8% helper bees (outlier—group *ConQ* × *SenD−*). The number of colonies tested per group was as follows: 11 (*ConQ* × *SenD−*), 8 (*ConQ* × *SenD+*), 9 (*SelQ* × *SenD−*) and 12 (*SelQ* × *SenD+*). *Proportion of beginner and helper bees at the level of 0.001 significantly higher than reference group *ConQ* × *SenD−* (grey colour).

	Queens	Offspring	Beginner		Helper	
Group	N	N	N	%	N	%
SelQ × *SelD*	11	1076	59	5.5	112	10.4
SelQ × *ConD*	10	1156	54	4.7	75	6.5
ConQ × *SelD*	9	1384	35	2.5	65	4.7
ConQ × *ConD*	9	1456	22	1.5	25	1.7

Table 2. Grouping considering genetic origin of queens and drones. Summary of the number (N) of inseminated queens per group and their offspring (worker bees). Displayed are furthermore the number of beginner and helper bees in each group (N) and the corresponding equivalent in percent per group (%).

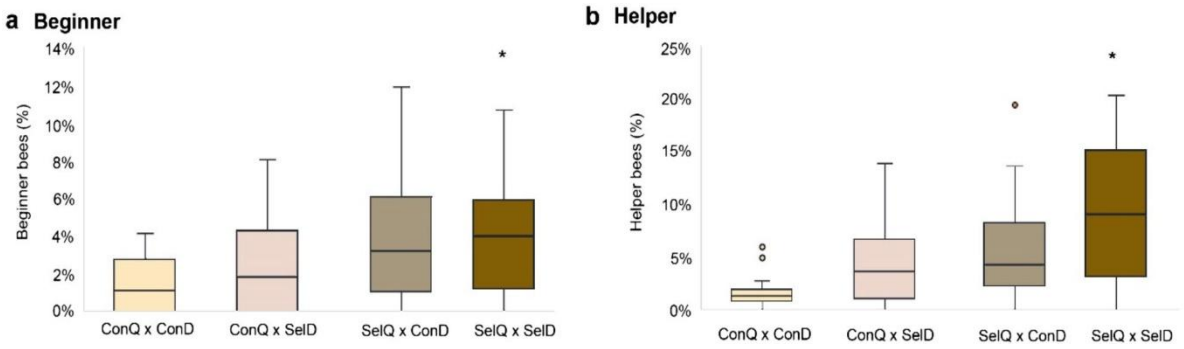

Figure 4. Boxplot of beginner and helper bees in groups based on the genetic origin of the queens and drones. Displayed are median, standard deviation and outliers for the beginner (**a**) and helper (**b**) categories for each group. The proportion of beginner (**a**) and helper (**b**) bees during the three courses of the video observation experiment are displayed for each group. One colony exhibited 19.4% helper bees (outlier—group *SelQ* × *ConD*). The number of colonies tested per group was as follows: 9 (*ConQ* × *ConD),* 9 (*ConQ* × *SelD*), 10 (*SelQ* × *ConD*) and 11 (*SelQ* × *SelD*). *Proportion of beginner and helper bees at the level of 0.001 significantly higher than reference group *ConQ* × *ConD* (beige colour).

activity (beginner: 4.7%, helper: 6.5%). This group performed significantly better than the reference group *ConQ* × *ConD* in both activity categories (beginner: GLMM, p=0.005; CI 0.44; 1.15; and helper: GLMM, p<0.001; CI 0.81; 1.93). The results are listed in detail in Suppl. Tables 5 and 6. The odds of this group uncapping a parasitised cell were 2.7 times higher (GLMM, OR = 2.7; CI 1.55; 4.66) than those of the reference group.

Group *ConQ* × *SelD* exhibited significantly higher performance than the reference group in the helper activity (GLMM, p = 0.003; CI 0.53; 1.68). While the worker bees' performance in the beginner category was higher than that of the reference group, the results were not significant (GLMM, p=0.18; CI – 0.24; 1.01).

The experimental course had no significant effect on the performance of the worker bees.

Control cells. To check the specificity of the VSH-behaviour, each test course contained five control cells. These cells were opened and resealed without being infested with a mite to consider the possibility that the workers only responded to the manipulation of the cell cap. In the first round of observation, none of the control cells was opened by the worker bees during the video observation. These cells were *Varroa*-free. During the second round, the brood from one cell was removed. The other four cells were *Varroa*-free. In the last round, one cell contained a single nonfertile mite; the other four were not parasitised.

Discussion

In the present study, 40 queens were each inseminated with sperm from one drone. A total of 5072 worker bees from the F1 generation were individually examined for their VSH. The aim of this multistage experiment was to assess the link between VSH and the drone's olfactory sensitivity, observed through conditioning the drones to an extract of *Varroa*-parasitised brood.

To our knowledge, this is the first conditioning experiment with drones using an extract from *Varroa*-parasitised brood. Compared to Chakroborty et al.[42] who used live parasitised pupae as a conditioning stimulus, the extracts used in this experiment were much less concentrated. For *extract-low*, our goal was to reach the threshold of perception for the *Varroa*-parasitised-brood extract and select the most sensitive drones. *Extract-high* had a concentration almost twice as high as *extract-low* and served the purpose of selecting for drones unable to perceive the *Varroa*-parasitised-brood odour. Although the chosen experimental setup does not provide proof that drones could perceive the difference between healthy and parasitised brood, it shows their ability to perceive the complex odour bouquet of *Varroa*-parasitised brood at a very low concentration. Masterman et al.[41] observed a difference in the discrimination abilities of hygienic and nonhygienic worker bees for brood odours. However, this does not seem to apply to drones. In contrast to worker bees, drone origin had no effect on their ability to perceive the CS+ during our experiment. Furthermore, the results from the PER conditioning experiment did not deliver any advantage to the F1 generation.

The drones' olfactory sensitivity to *extract-low* was not represented in the VSH of the drones' offspring. Moreover, the group with the highest results contained the sperm of drones that were insensitive to the *Varroa*-parasitised brood odour (*SelQ* × *SenD−*).

When mated with queens from the VSH-selected line, the *Varroa*-parasitised-brood-odour sensitive drones produced colonies with more active beginner and helper bees than did the reference group *ConQ* × *SenD−*. However, those results were significant only for the helper activity (GLMM, $p < 0.001$; CI 0.39; 0.95). Provided that the single drone's perception ability is crucial for the manifestation of VSH in the next generation, we would have expected groups *SelQ* × *SenD+* and *ConQ* × *SenD+* to exhibit the highest activity in the observation. Contrary to our hypothesis, the *SelQ* × *SenD−* group produced the most active offspring in the three repetitions of the experiment.

Furthermore, the *ConQ* × *SenD*+ group scored lower than the reference group, although the differences were not significant. Thus, our assumption that the negative conditioning outcome from the experiments with *extract-high* would be a reliable exclusion criterion, was incorrect.

There may be various reasons behind the inability of the conditioning experiment to ensure higher VSH activity in the next generation. The individual drones' sensitivity to sucrose at the time of the experiment might have been different. Pankiw et al.[57] described handling stress as one of the factors responsible for differences in sucrose sensitivity. From our observations, drones proved to be much more sensitive to conditioning length and weather conditions than worker bees. We observed a greater unwillingness of drones to respond to the CS+ and the sugar solution on cold or rainy days, although the temperature in the laboratory was regulated. Our observations corroborate earlier research conducted on drones[58,59]. Benatar et al.[58] deemed the usual protocols used on worker bees unfit for drones. During our preliminary tests, we also observed high drone mortality if drones were treated according to existing bee protocols. Vareschi[59] described differences between worker bee and drone conditioning, stating that drones are more "nervous" than worker bees. We, too, observed such a tendency. Throughout the experiment, we ensured the same nursing conditions for all test subjects through the drones' collective upbringing in one hive. We strived to ensure that the laboratory conditions were as uniform as possible. The number of trials was modified from eight to six to keep the drones as fit as possible for insemination. Nevertheless, the stress tolerance threshold of each individual differs[60] and is a factor that is difficult to measure.

Another reason for the unsuccessful phenotyping of the drones through conditioning might be the strong sex dimorphism in the olfactory system of eusocial insects such as honeybees[55]. While queens and drones specialize in behavioural tasks such as mating, workers have a more diverse task range. Such specialization is also typical for other species, such as moths[61,62], bark beetles[63], cockroaches[64], and ants[65,66]. The differences between both sexes encompass all stages of the olfactory pathway. The antennae of drones and workers exhibit sex-specific molecular specialization[67,68]. Drone antennae have a higher number of sensory cells (~ 339,000) than worker bees (~ 65,000)[69]. Of these, only one type—the so-called placoid sensilla—is present in large numbers in the drone's antennae, while the other types are either diminished in

numbers or completely missing[55]. Most of the receptors on the drone's antennae are connected to the perception of the queen pheromone 9-ODA. Workers, on the other hand, exhibit receptors connected to pheromone communication, cuticular hydrocarbon perception and distinction of floral odours[68].

Different epigenetic mechanisms, such as DNA methylation and histone posttranslational modifications, regulate the expression of receptor genes[70]. Kucharski et al.[71] examined the expression of one odourant binding protein (OBP) gene—*obp11*—on the antennae of workers. OBP11 is also found in the *sensilla basiconica* of female ants[72]. It is involved in the accurate perception of cuticular hydrocarbons and pheromones, enabling workers to interact with each other and fulfil their social duties. While *obp11* is expressed in worker bee *sensilla basiconica*, it is silenced through methylation on drone antennae[71].

According to Arnold et al.[73], a well-pronounced sexual dimorphism in the glomeruli of the antennal lobe can be observed between worker bees and drones. While worker bees display only two structural types of glomeruli, drones exhibit a third glomerulus type, which is hypertrophied and responsible for the detection of queen pheromones[74]. Plant odours, on the other hand, are processed in the ordinary glomeruli of the antennal lobe[74]. While we proved that drones could perceive the extract used in our experiment, this ability is probably as unimportant to the drone's mating success as the distinction between two floral odours. It is therefore possible that the drone's ability to sense the odour of brood parasitised by *V. destructor* per se is of no advantage for the improvement of VSH. Moreover, the genes that are silenced in drones and cannot be measured by conditioning most likely play a larger role in the enhancement of VSH. If that is the case, odour conditioning would be unsuitable for detecting the best drones for breeding purposes.

The conditioning experiment might have also selected drones solely based on their better or worse learning abilities[58]. To rule out this possibility, we selected sensitive drones not only based on the results of the unrewarded tests but also on their whole performance during the experiment. Only drones that perceived the odour and distinguished it correctly from the CS- every time during the last trials and the unrewarded tests were chosen for insemination. While we acknowledge that the performed conditioning has some limitations for the achievement of our goal, we are optimistic regarding the potential of PER conditioning as a means for phenotyping

drone olfactory sensitivity. Phenotyping in relation to an odour that is very easy to perceive for drones opens up the possibility of indirectly recognizing their general odour sensitivity. If used for breeding, this trait could lead to an increase in odour sensitivity in the drone's female offspring towards *Varroa*-parasitised brood. Through an optimization of the exclusion criteria and the choice of another odour in a low concentration—for example 9-ODA—it might be possible to better select for odour sensitivity in the drone and pass on this trait to the next generation.

Our experiments also provide new information on the inheritance of VSH. When the group results were analysed with the genetic origin in mind, the number of beginner and helper actions increased when drones and/or queens of the VSH-selected line were used. The origin of the queen proved to play an even larger role than that of the drone. This observation was in accord with the substantial effect of the queen's origin (VSH-selected/nonselected line) on the beginner activity when the results were analysed based on the PER conditioning experiment. The *Sel* queens produced offspring with a higher VSH activity when inseminated with sperm from *Con* drones than did *Con* queens inseminated with sperm from *Sel* drones. The odds of commencing a beginner activity compared to the reference group were as follows: 1.5-times higher for *ConQ* × *SelD* (OR; CI 0.79; 2.73), 2.7-times higher for *SelQ* × *ConD* (OR; CI 1.55; 4.66), and 5.5-times higher for *SelQ* × *SelD* (OR; CI 3.31; 9.09). The same tendency was observed for the helper activity: 3-times higher than the reference group for *ConQ* × *SelD* (OR; CI: 1.71; 5.38), 3.9-times higher for *SelQ* × *ConD* (OR; CI: 2.24; 6.86) and 7.4-times higher for *SelQ* × *SelD* (OR; CI 4.33; 12.53). These results lead us to believe that maternal effects play a significant role in the manifestation of VSH. Maternal effects shape behaviour and help offspring better adapt to changes in the environment. Maternal effects have been observed in many species[75–78], including honeybees. Dloniak, French and Holekamp[78] described rank-related maternal effects on offspring phenotype in spotted hyenas (*Crocuta crocuta*). Dominant females exhibited higher androgen concentrations in late pregnancy, which shaped the behaviour and social structure of the new generation. Storm and Lima[79] described an "adaptive transgenerational maternal effect on offspring antipredator behaviour" in crickets. The offspring of mothers exposed to *Hogna helluo* spiders survived longer than the offspring of naive mothers. The forewarned crickets exhibited a behavioural change that manifested in a mobility reduction. Such behavioural changes have also been described in bees. Unger and Guzmán-Novoa[80]

experimented with crossbreeding of highly hygienic Russian bee strains and less hygienic Ontario bee strains. The hybrid bees with a "hygienic mother" and "control father" exhibited higher results for individual bees uncapping cells as well as removing the brood. On the other hand, "control queens" and "hygienic drones" produced an F1 generation with weaker hygienic behaviour. Spivak and Reuter[81] assessed colonies with queens from a VSH-selected line naturally mated with unselected drones. Compared to unselected colonies, the hygienic colonies displayed a reduced mite load. Our findings further strengthen these observations.

This research demonstrates drones' ability to perceive low concentrations of brood-emitted odours. PER conditioning with the selection criteria used in this experimental setting proved unsuitable for the enhancement of VSH. While an additive genetic effect was observed when drones from the VSH-selected line were paired with queens from the VSH-selected line, there was a tendency for maternal effects to also play an important role. Since both sexes inherit the same genes from their mother, it would be a big step towards creating a breeding strategy against *V. destructor* if a worker bee's odour sensitivity could be measured on the haploid father's side. Workers' odour sensitivity towards parasitised brood is the key factor in *Varroa*-resistance. Therefore, further research is necessary to identify odours and suitable test methods to phenotype drones' odour sensitivity. If the heritability of such test results is sufficient, VSH can be improved more efficiently by the use of such individually tested drones in breeding.

Materials and methods

Extract preparation. An extract from *Varroa*-parasitised brood was created to mimic the complex composition of the distress signals emitted by the parasitised brood. A total of 190 mites were collected from a *Varroa*-infested colony at our institute. A brood frame with newly capped brood from a *Varroa*-free colony was chosen. The cell caps were cut open and lifted on one side using a razor blade. Only brood cells containing prepupae (9–10 days old) were infected. In each brood cell four mites were inserted using a moistened brush. The caps were subsequently resealed. The location of the parasitised cells was marked on translucent projector foil. The brood frame was placed back into the hive for two hours for the small incisions on the cell caps to be sealed by the nursing bees. After that, the frame was kept in an incubator for four days.

After that time, the parasitised pupae were extracted from the brood cells without damage. During the preparation process, the pupae were stored in an incubator at 35 °C on damp filter paper. Isopropanol was used as the base for the extract. The pupae were washed in 4 ml isopropanol for 10 min. The supernatant was decanted in special 2 ml glass vials with PVC lids and stored at − 20 °C. Two extracts with different concentrations were produced for this experiment—one extract obtained from 15 pupae (*extract-low*) and one from 25 pupae (*extract-high*).

Testing for odour sensitivity. Having the process of localizing and uncapping parasitised brood cells in mind, we decided to present the odours in a manner that would allow direct contact with the stimulus and ensure that non-volatile chemicals such as oleic acid, the brood ester pheromone and tritriacontane are perceived[36,82–84]. We chose filter paper as a medium that was presented with the help of tweezers.

During the olfactory conditioning experiment, the solvent isopropanol—used during the preparation of the two extracts—was chosen as a CS−. As isopropanol was present in both the CS+ and the CS−, only drones that perceived the solved brood components sensed the difference between the two stimuli. If this were not the case, we expected that insensitive drones would show similar proboscis extension rates to both the CS+ and CS−.

Two PER conditioning experiments were carried out for the selection of drones that were to be used for artificial insemination:

1. Selecting *Varroa*-parasitised-brood-odour sensitive drones: 5 µl *extract-low* (see above) as the positive stimulus CS+ and 5 µl isopropanol as the negative stimulus CS−.
2. Selecting *Varroa*-parasitised-brood-odour insensitive drones: 5 µl *extract-high* (see above) as the positive stimulus CS+ and 5 µl isopropanol as the negative stimulus CS−.

For the conditioning experiments, eight colonies were chosen, and 100 newly hatched drones per origin were marked on the dorsal thorax with a chip. The drones were placed in a nursing hive with an unmated queen. Four of the chosen colonies came from a line selected for VSH, and the other four were of a nonselected line. A queen excluder was used to prevent drones from leaving the hive. After the drones reached reproductive age (14 days), the conditioning experiments were started.

The drones were collected from the hive shortly before the start of each conditioning and strapped in small metal tubes with paraffin tape. The immobilized drones were kept in a rack with numbered slots. A 50% sugar solution was used for the experiments. Only the drones that readily stretched their proboscis during the presentation of the sugar solution were used in the experiment.

The drones were presented with plain filter paper three times before the beginning of odour conditioning. This was done to prevent proboscis extension solely due to mechanical irritation from the filter paper. Each conditioning group consisted of eight drones. We aimed to equally represent every origin in these groups. Two conditioning experiments were conducted daily—one with each of the extracts. The chronological order of the tests (conditioning for sensitive drones, conditioning for insensitive drones) was changed each day to eliminate any bias due to the time of day.

During extensive preliminary experiments, we observed a decrease in drone reactions and difficulty collecting sperm after long-lasting conditioning experiments. Therefore, we modified the trial sequence of the conditioning described by Matsumoto et al.[46] to shorten the experimental time.

The modified conditioning consisted of six trials with a specified order of stimuli presentation: CS+, CS−, CS−, CS+, CS+, CS−. The CS+ was enhanced by the administration of an unconditioned stimulus (US) in the form of a sugar solution. This was done with the help of a toothpick. The CS− was not reinforced. Each CS lasted 6 s. During the CS+ trials, the US was applied during the last 3 s of CS+ presentation. The intertrial interval was 5 min. No unpaired conditioning or exchange of the odours (isopropanol as CS+ and brood extract as CS−) was performed, as it was considered unnecessary for the achievement of our goals. The conditioning was used solely as a means of testing for odour perception and not to analyse learning behaviour.

The conditioning success was subsequently examined and recorded by a presentation of the two stimuli without the reward.

The following drones were considered for artificial insemination:

1. *Varroa*-parasitised-brood-odour sensitive drones displayed excellent odour perception of *extract-low* (15 pupae extract) by responding to the CS+ but not the CS− during the last two trials and during the unrewarded tests. (Trials: CS+, CS−, CS−, CS+, **CS+, CS−;** Unrewarded test: **CS+, CS−**).

2. *Varroa*-parasitised-brood-odour insensitive drones responded to the US throughout the experiment with *extract-high* (25 pupae) but showed no positive responses to the CS+ during the last two trials, and the unrewarded tests indicated a negative conditioning outcome and the inability to perceive the extract of *Varroa*-parasitised pupae.

A total of 223 drones were tested with *extract-low*, while 202 drones were assessed using *extract-high*. Drones that stretched their proboscis at the first presentation of the CS+ were excluded as well as those that stopped responding to the stimulus during the experiment. The number of excluded drones amounted to 22% for *extract-low* and 16% for *extract-high*.

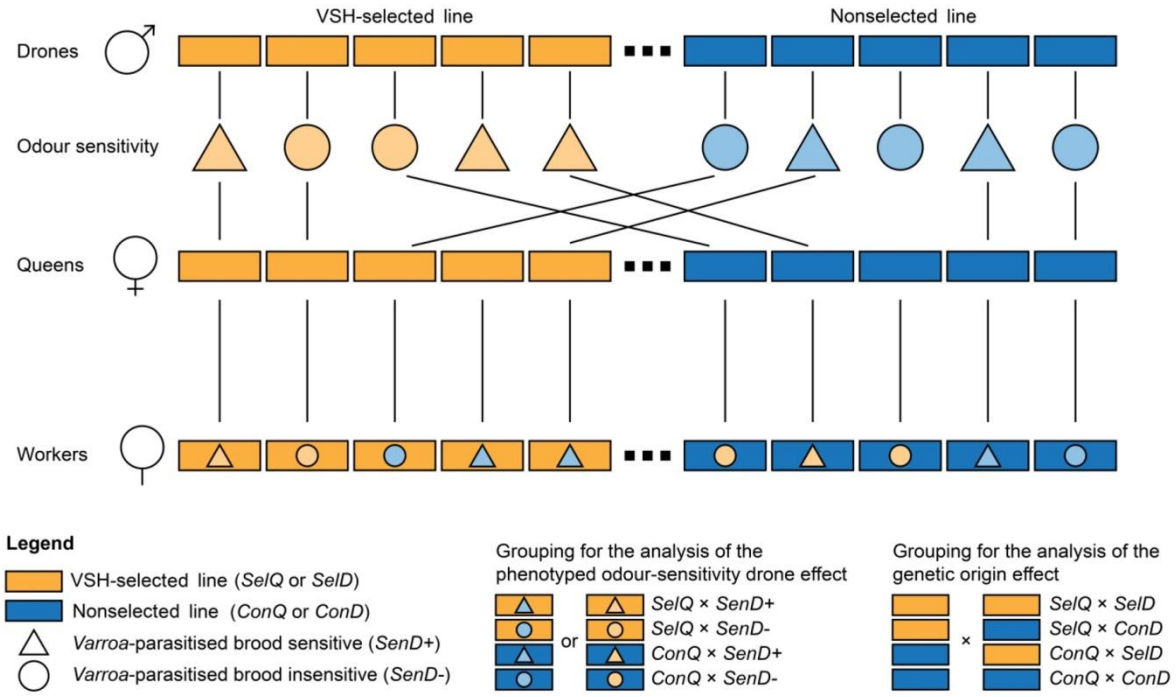

Figure 5. Mating scheme used during one-drone insemination. Drones from each of the two lines—VSHselected line (*SelD*, yellow colour) and nonselected line (*ConD,* blue colour)—were tested for their odour sensitivity towards the extract of *Varroa*-parasitised brood. Drones, which perceived the *Varroa*-parasitised brood-odour were referred to as "*Varroa*-parasitised-brood-odour sensitive" (*SenD*+) and marked with a triangle. Drones, that did not perceive the odour were referred to as "*Varroa*-parasitised-brood-odour insensitive" (*SenD*−) and marked with a circle. The tested drones were subsequently used for the insemination of queens from both VSH-selected (*SelQ*, yellow colour) and nonselected line (*ConQ*, blue colour). The offspring workers were placed into four groups considering the queen's genetic origin and the drone's olfactory sensitivity towards the *Varroa*-parasitised-brood extract. The workers were subsequently assessed for their VSH in a video observation test.

Artificial insemination. The drones were brought back to the hive after each conditioning for recovery before the sperm were extracted. Sperm extraction took place immediately before insemination[85].

The queens originated from lines selected for their hygienic behaviour towards *V. destructor* (VSH-selected line) and from institute-owned lines (nonselected line). The one-drone insemination was conducted using the mating scheme displayed in Fig. 5.

Of a total of 50 queens, 40 took part in the experiment. The rest did not produce enough eggs in time for the video observations or died. The inseminated queens were housed in mini nucleus hives (Segeberger®) with young bees. All mini nucleus hives were located on the institute terrain in close proximity to one another. The mini nucleus hives were fitted with two food frames each (honey and pollen) and two brood frames. The worker bees for the mini nucleus hives came from colonies kept in the institute, especially for the purpose of queen rearing. Each mini nucleus hive received approximately the same number of worker bees. The worker bees were supplied with feed dough to ensure adequate food storage. The flight hole was narrowed to prevent possible robbing behaviour. Once all the inseminated queens had started laying eggs, each mini nucleus hive received an empty brood frame at the same time to ensure that all the bees for the infrared video observation were of the same age.

After the young bees hatched, they were collected daily within a week and marked individually with a numbered plate on the dorsal thorax. Afterwards, they were placed in the video surveillance unit described by Bienefeld et al.[37]. A *Varroa*-free brood frame with freshly capped brood was taken from an institute-owned hive, and 60 brood cells were infected with one mite each. Five control cells were opened and resealed without being artificially infested. The brood frame was placed in the observation unit, and the recording was started.

For six days, bee activity was monitored using an infrared camera. The video recording analysis was carried out manually with the help of a software program—Beehaviour—specially created for this purpose (Batz et al., submitted).

Statistical analysis. *Analysis of PER conditioning experiment.* The drones were split into two groups for the statistical analysis, considering their origin (VSH-selected line/nonselected line). Acquisition curves were plotted in addition to the analysis.

The outcome (0—unsuccessful, 1—successful) of the unrewarded tests was examined using a binomial generalized linear mixed model (GLMM) with a logit function in SPSS V. 25. The alpha-level was set at 0.05. The drones coming from the control line were set as a reference group by the model. The stimulus effect (reinforced, non-reinforced) was also considered. The temperature during the experiment and the mother of each drone were both set as random factors.

Video-observation analysis. While observing the VSH recordings of the drones' offspring, two activities were used to evaluate the VSH of the new generation. The beginner activity was defined by the first worker opening an infested cell and the helper activity—the workers that enlarged the hole after the beginner had created it. If the cell caps were opened and resealed multiple times, the new beginner and helper bees were written down. One course of video observation was completed in year one. In the second year, two courses of video observations were performed. A total of 5072 bees were recorded during the experiment: 1694 in course one, 1696 in course two and 1682 in course three. More detailed information on the composition of each group and the number of worker bees is described in Suppl. Tables 7 and 8.

VSH of groups considering the conditioning outcome. The video recording results were analysed through a binomial GLMM with a logit function in SPSS V.25.

Group *ConQ* × *SenD*− was used as a reference. The courses of observation—one, two or three—and the drone's origin (VSH-selected line, nonselected line) were considered fixed effects. By including the drone's origin in the regression, the model provided more accurate insight into the PER conditioning and its explanatory power for the results. Course one and nonselected lines were chosen as reference values. The individual effect of each queen mother on the VSH of her offspring was set as a random factor in the regression model.

VSH of groups considering the parental origin. In a second step, the video observation results were analysed with consideration of the parental origin of queens and drones and ignoring the PER conditioning results. The statistical analysis was conducted using a binomial GLMM with a logit function. Group *ConQ* × *ConD* was set as the reference group. The course of observation was again considered a fixed effect. Course one was set as a reference.

Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

K.B. conceived the study. I.I. performed the experiments, analysed the results, and wrote the manuscript. K.B. supervised the study and assisted with the interpretation of the results and writing of the manuscript.

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4. General Discussion

4.1 Research objectives and suitability of the experimental design

The *V. destructor* parasitisation has remained one of the main causes for colony losses all around the world since the introduction of the mite in *A. mellifera* populations (Guichard et al., 2020). Beekeepers are forced to treat their colonies with acaricides every year to prevent colony collapse (Rosenkranz et al., 2010). Over the years, the *Varroa*-mite has developed resistances against some of the most commonly used products containing amitraz and flumethrin (Rodríguez-Dehaibes et al., 2005; Millán-Leiva et al., 2021). Commercially available acaricides can also have adverse effects on the colony health (Tihelka, 2018). The increasing frequency of severe weather events associated with climate change is a further factor responsible for making current treatments less effective and honeybee colonies even more vulnerable to *V. destructor* (Vercelli et al., 2021).

To date, selective breeding for tolerance is considered the only long-term and sustainable solution to the *Varroa*-problem (Harbo and Harris, 1999; Pérez-Sato et al., 2009; Rosenkranz et al., 2010; Locke, 2016a; Wagoner et al., 2018; Guichard et al., 2020). Current selection programs are time-consuming, lack rapidity and have not yet achieved global success (Guichard et al., 2020). Widespread implementation of a novel breeding strategy depends not only on genotype-environment interactions (Meixner et al., 2015), but also on the beekeepers' acceptance of the resistant stocks (Guichard et al., 2020). Other desired honeybee traits (e.g., high honey yield, decreased aggressiveness) must also be taken into account if a new breeding strategy is to be widely adopted in kept colonies. In Germany in particular, the implementation of a breeding strategy may prove difficult due to the small-scale nature of beekeeping – 96% of the beekeepers have fewer than 25 colonies (Deutscher Imkerbund. e.V., 2022). Against this background, the development of an efficient, inexpensive, and easily applicable resistance breeding method is urgently needed.

So far, one of the major problems of resistance breeding comes from the fact that selection programmes are based on the level of the colony, which consists of differently related worker bees (Blanchetot, 1991). As in other creatures, the female offspring of the honeybee are derived from a representative selection of the genetic material of the mother and the father. It follows that full siblings usually share an average of 50% of their genetic material. Due to the haploidy of the male sex in the honeybee, drones

pass on their entire genome to their offspring, without the usual Mendelean sample variance. Consequently, the offspring of a drone and a diploid mother share on average 75% of their genes (Blanchetot, 1991). Queen bees naturally mate with multiple drones, so that the bee colony consists of a mixture of different patrilines, with members within patrilines being 75% related, while members of different patrilines are only 25% related (Ratnieks, 1988; Blanchetot, 1991). Thus, resistance factors, which manifest themselves as individual behaviour (for example recognition and removal of infested brood) and also occur at a very low frequency – around 5% of the workers in the colony can detect and clear *Varroa*-parasitised brood cells (Bienefeld et al., 2015) – are extremely difficult to enforce through selection at colony level. For this reason, this project focussed on the individual's ability to detect and remove *Varroa*-parasitised brood. This ability correlates very strongly with *Varroa*-resistance, as the parasite is hindered in completing its reproduction cycle (Harris et al., 2010).

The haploidy of the drones promotes genetic differentiation and the emergence of "specialists" within the honeybee colonies, which is of great advantage for the honeybee colony and the division of labour within. This is especially true for hygienic behaviour towards *Varroa*-parasitised brood, which is carried out by only a few workers and requires a very high sensitivity of the olfactory organs due to the low stimulus threshold (Gramacho and Spivak, 2003). Thus, the haploidy of drones also opens up possibilities for a particularly efficient breeding program in which drones with sensitive olfactory organs would be utilized for the enhancement of *Varroa*-sensitive hygiene (VSH) in the next generation of workers. By artificially inseminating queens with only one drone, the genetic variability among the offspring is low, which allows testing the success of a fully new breeding design within a short time and with less effort.

For this new design, the PER conditioning was implemented in a breeding strategy for the first time. Two experiments were conducted in the course of this thesis. Proboscis extension response conditioning was chosen as the standard method for phenotyping drones' odour sensitivity because of its non-invasive nature and its simultaneous results.

In the first experiment, the PER conditioning was carried out on workers to determine whether differences in odour sensitivity between the VSH-selected line and a nonselected control line exist (Chapter 3.1). If so, could these differences be observed in the perception of brood odours in connection with the *Varroa*-parasitisation? The

results of the first experiment aligned with previous research showing a connection between olfactory sensitivity in honeybee workers and VSH (Gramacho and Spivak, 2003; Mondet et al., 2015). The method – PER conditioning – proved successful in measuring different olfactory sensitivity levels as well as differences in perception speed between workers subjected to resistance breeding and such coming from control lines. The first objective of this thesis was therefore considered accomplished. The differences in olfaction between the two lines were only observed in the perception of highly diluted odours emitted by the brood (in the form of an extract of *Varroa*-parasitised brood) but not in the perception of the flower odours which indicated a heightened specific olfactory sensitivity to brood cues as a result of breeding efforts.

In the second experiment, drones from both lines (VSH-selected line and a nonselected control line) were tested to determine whether they can perceive the odours emitted by *Varroa*-parasitised brood and if so, whether this odour sensitivity could enhance the colonies' VSH and be used to develop a breeding strategy. The ability of the drones to detect brood odours had not been previously tested in any published scientific work. Not only was the conditioning successful, but it also provided valuable information on the inheritance of VSH-mechanisms thus giving insights to the second objective of this work. As drones pass on their entire genome to their offspring, without the usual Mendelean sampling variance, higher odour sensitivity in drones to odours connected to VSH could potentially lead to faster breeding success (Chapter 3.2). It was assumed that drones able to perceive the low-concentrated extract of *Varroa*-parasitised brood would enhance their offspring's VSH compared to drones that would not exhibit a positive conditioning during the experiment. This hypothesis was rejected as the offspring's VSH could not be influenced by the ability of father drones to perceive a low concentration of the *Varroa*-parasitised brood odour. Compared to worker bees (Chapter 3.1.), no differences in odour sensitivity were observed between drones from the VSH-selected and control lines. Nevertheless, the results of the experiment indicated that maternal effects played a significant role in the manifestation of VSH, and additive genetic effects were present when drones from the VSH-selected line were paired with queens from the VSH-selected line.

While the first two study objectives could be answered through this dissertation, the question whether the PER conditioning can be used as a selection tool for drones, remains unanswered. The incorporation of PER conditioning as a selection tool in

breeding programmes against *V. destructor* had never been attempted before, which made it particularly interesting for research. So far, only a few studies have concentrated on the selection of drones for the enhancement of the F1 generation's performance, demonstrating the potential of the haploid genotype of drones for selection purposes. Ferguson et al. (2001) improved the ability of worker progeny to reverse an already learned preference during odour conditioning. By selecting drones for their fast reversal performance, the trait was enhanced in the F1 generation showing a significant hereditary component. In another experiment Benatar et al. (1995) described alterations in worker bees olfactory learning ability based on the choice of the father drone's phenotype. Father drones with a low learning performance led to a decrease in the ability of the worker bee progeny to differentiate between stimuli.

Although it is possible to select for better learning ability, VSH is a behavioural adaptation which leads to a fitness advantage (Neumann and Blacquière, 2017; Blacquière and Panziera, 2018) and cannot be improved by learning (Trump et al., 1967; Cremer et al., 2007). In addition, the perception of odours connected to *V. destructor* is not crucial for the survival or mating success of drones and the recognition of such odours does not carry the same importance as it does for workers (Avalos et al., 2016). Nevertheless, the drones' ability to perceive the odours emitted by *Varroa*-parasitised brood has been demonstrated and the possibility that drone's olfaction can be used as a selection tool for breeding purposes, should not be ruled out. A possible reason for the failure to improve the VSH of the F1 generation in the course of this work may lie in the experimental design. The difference in potency of the extracts may not have been great enough to distinguish between drones with a heightened olfactory sensitivity and drones unable to perceive the highly diluted extract. It is also possible that the stimulus itself had a varying degree of impact on the drone's motivation to carry out a behavioural reaction (Robinson et al., 2014). While one individual might readily react to the *Varroa*-parasitized brood extract, another individual might be able to perceive the odour but will not show any reaction to it. Also, an individual pre-exposed to the Cs in the rearing environment, could exhibit a delay in the establishment of a connection between Cs and Us during trials (Fernández et al., 2009). This could have direct consequences for the classification of the individual's discrimination abilities in an experimental design such as the one used in the course of this thesis (odour-sensitive vs. odour-insensitive drones, depending on the count of right/wrong

answers during conditioning trials). With this in mind, simply distinguishing between a rewarded and an unrewarded stimulus may not be precise enough as a selection criterion for drones. A possibility for modifying the experimental setting would be the use of differential conditioning with a rewarded (Cs+) and punished stimulus (Cs-, where the odour is connected to an aversive reinforcer) which might prove more accurate for the selection of suitable drones (Benatar et al., 1995). By introducing punishment in connection with the Cs-, the unwillingness to stretch the proboscis when the Cs- is presented, would give more accurate evidence of discrimination between both odours.

Even with a different experimental design, the success of PER conditioning is influenced by several factors – some are directly related to the competence of the experimenter (avoiding unintentional mixing of odour and sucrose solution, not causing additional stress to the test subjects etc.; Matsumoto et al., 2012), while others are related to the external conditions. Little is known how or if seasonal variations influence PER conditioning results (Ray and Ferneyhough, 1997). An entirely different method for measuring olfactory sensitivity - such as an electroantennography (EAG) - might prove more suitable for breeding purposes. A recording electrode is placed at the tip of one of the antennae of the tested individual, the reference electrode is connected to the base of the same antenna via a hole in the cuticula. Electrical signals are recorded when an odour is presented to the antenna of the tested individual (de Jong and Pham-Delègue, 1991). While this method is more sensitive and less susceptible to biases as it tracks neurophysiological mechanisms instead of behavioural reactions, it is also more invasive and time-consuming than PER conditioning (Patte et al., 1989). If it was used for screening drones' ability to perceive highly diluted odours, results might differ from those seen in PER conditioning. Individuals which are able to perceive low concentrations of the chosen odour but would not show a reaction during a PER conditioning could be detected. Even though this method sounds promising, there is no data on whether and to what extent the invasiveness of the EAG procedure affects drones' health, sperm quality or survival chances after the procedure. More research is needed to answer these questions. A comparison of the sensitivity of both PER conditioning and EAG would be useful for better understanding olfactory sensitivity.

Despite having some drawbacks, PER conditioning has a huge advantage over other methods of phenotyping olfactory sensitivity as no special equipment is required for

the experimental set-up. Proboscis extension response conditioning is non-invasive, cost-effective and provides fast results. The use outside of laboratories and universities can easily be achieved and can encourage beekeepers to get involved in a new breeding programme. As already seen in workers, it is possible to detect differences in olfactory sensitivity between VSH-selected and nonselected individuals (see Chapter 3.1) and thus validate the success of a breeding effort. The possibility of drones from different origins exhibiting differences in their olfactory sensitivity should not yet be excluded as only one odour was tested so far. By testing odours with various levels of biological relevance to drones, such as 9-ODA, citral, geraniol, it may be possible to display contrasts in olfactory sensitivity between *Varroa*-tolerant/resistant and susceptible colonies. As drones are haploid, the genetic potential for *Varroa*-resistance can be assessed directly on the gametes. This would allow genetic differences to be recognised significantly better and enable faster selection success. More research is needed to back this hypothesis.

4.2 Inheritance mechanisms of resistance breeding

Selecting the right individuals for resistance breeding is a difficult task as inheritance mechanisms are not yet fully understood (Le Conte et al., 2020). By examining existing research on resistance traits one thing is clear – resistance cannot be examined as a fixed trait or a fixed set of traits but is the product of interactions between different adaptive traits and the local environment (Le Conte et al., 2020). The heritability of hygienic behaviour is considered being ~ 0.6 (Harbo and Harris, 1999; Lapidge et al., 2002) meaning that only about half of the phenotypic variance in a population can be explained by genetics. The other half is related to factors with an unknown origin (Oxley et al., 2010).

Only a small number of workers – around 5% - actually engage in hygienic behaviour in the colony (Bienefeld et al., 2015; also see Chapter 3.2). Understanding the genetic mechanisms involved is therefore essential. In honeybee subpopulations in different parts of the world, similar selection pressures from natural levels of mite infestation have led to the parallel evolution of different mechanisms for reducing mite reproductive success (Locke, 2016b; Le Conte et al., 2020; Luis et al., 2022). Colonies in Gotland, Sweden are known for smaller brood nests, high recapping activity and a delay of egg-laying in mites, probably caused by pupal volatile odours (Locke et al.,

2012; Oddie et al., 2021). Colonies in Cuba exhibit a high recapping and mite removal behaviour, as well as low mite reproduction (Luis et al., 2022). The honeybee population in Avignon, France and Russian Primorsky colonies both display efficient detection and removal of mites (Unger and Guzman-Novoa, 2010; Locke et al., 2012; Le Conte et al., 2020). Colonies specially bred for *Varroa*-resistance also show a combination of these resistance traits. Although these resistance traits all result in similar resilience levels towards *V. destructor*, the traits often do not share the same molecular pathways and the same traits are sometimes expressed through different molecular pathways in different populations (Mondet et al., 2020). The genetic mechanisms of inheritance for these traits therefore also differ greatly – Gotland colonies are thought to have a dominant genetic component of their resistance traits (Locke, 2016a), Russian Primorsky bees show evidence of maternal effects for the inheritance of hygienic behaviour (Unger and Guzman-Novoa, 2010).

The colonies tested in the course of this thesis also displayed maternal effects for the inheritance of VSH. Maternal effects are considered coming from genes which are located in the queen's genome. Two hypothetical mechanisms come in question for the expression of these effects – epigenetic mechanisms and cytoplasmic inheritance of mitochondrial genes (Unger and Guzman-Novoa, 2010). Epigenetic effects are influenced by DNA methylation patterns. Unlike mammals, insects do not undergo a DNA methylation reprogramming during embryogenesis and honeybee workers inherit methylation patterns from both parents (Yagound et al., 2020). Depending on the parent, from whom the methylation patterns are inherited, the phenotype of the offspring might differ (Wu et al., 2020). Alleles coming from the mother can either silence the paternal alleles or enhance the expression of the maternal alleles (Guzman-Novoa et al., 2005; Unger and Guzman-Novoa, 2010). While this mechanism is considered advantageous in some cases such as the inheritance of genes connected to defensive behaviour in the honeybee, it does not provide a greater advantage to the inheritance of VSH compared to cytoplasmic inheritance (Guzman-Novoa et al., 2005). If epigenetic effects were responsible for the VSH in the experiments conducted in Chapter 3.2, the influence of the drones on the VSH would have been greater.

Cytoplasmic inheritance of mitochondrial genes, on the other side, provides a more plausible explanation for the inheritance of VSH as seen during the conducted experiments. As in mammals, mitochondria in honeybees are maternally inherited

(Behura, 2007). If genes connected to the expression of VSH are located in the mitochondria, VHS would be mainly inherited from the mother's side. The choice of father drones would in this case have limited influence on VSH. Even if queens mated with drones from colonies that did not exhibit VSH, it would be less likely that the expression of VSH would be reduced in the offspring. This hypothesis is in accordance with the findings described in Chapter 3.2. The differences in VSH between the offspring of VSH-selected queens and nonselected queens was significant. The choice of father drones only slightly influenced the F1 generation. Nevertheless, additive genetic effects were present when drones from the VSH-selected line were paired with queens from the VSH-selected line, suggesting that the role of the drone in selection breeding should not be underestimated. Stochastic simulations show that no genetic gain for honeybee breeding efforts can be achieved in the long run, if controlled mating is not performed and drones are left out of the selection process (Plate et al., 2019).

In order to establish a successful breeding strategy, the molecular mechanisms behind desired traits should be better understood. A big drawback of existing studies on the molecular mechanisms responsible for resistance traits is that the findings of these studies do not overlap. One of the reasons behind this is the use of diverse experimental methods (transcriptomics, genomics, proteomics) and protocols as well as different ages, casts and tissues for the analysis (Mondet et al., 2020). In the field of quantitative trait loci (QTL) analysis alone, research shows very different results. Behrens et al. (2011) mapped three QTLs on chromosomes 4, 7 and 9 which showed significant impact on mite suppression. Oxley et al. (2010) discovered six QTLs on chromosomes 2, 5 and 16 – three of which influenced the likelihood of engaging in hygienic behaviour, two being responsible for uncapping behaviour and one linked to removal of parasitised brood. These included genes responsible for odour detection and odour-mediated behavioural responses. Guichard et al. (2022) observed two QTLs on chromosomes 4 and 5 connected to recapping of infested brood cells. As QTL analysis does not indicate expression patterns of the candidate genes, it has to be combined with a transcriptome analysis. This is not the case in many of the studies. Furthermore, the use of different breeding lines of honeybees for the QTL analysis and transcriptome analysis might distort the results (Le Conte et al., 2011). Despite the difficulties in consolidating the results of different studies some of the functions associated with the candidate genes are consistent in different publications - visual signalling, olfactory perception, circadian rhythm, nutrient intake, neural sensitivity,

signal transmission (Oxley et al., 2010; Le Conte et al., 2011; Mondet et al., 2015, 2020; Guichard et al., 2022).

Once important QTLs have been identified, candidate genes need to be studied so as to identify variations in the genome responsible for VSH. Identifying mutations of single nucleotides in the honeybee genome – so-called single-nucleotide polymorphisms (SNP) – can make marker assisted selection (MAS) of desirable traits possible (Spötter et al., 2012, 2016; Tsuruda et al., 2012; Sainsbury et al., 2022). Sainsbury et al. (2022) attempted MAS in honeybee colonies in New Zealand based on an adenine/guanine single nucleotide polymorphism located on the 9th chromosome in the honeybee genome. During the experiments, a reduction in the mean *Varroa*-population level of 28.5% in colonies headed by a queen that carried two copies of the guanine allele was present. While the selection of only one SNP did reduce the *Varroa*-mite count, treatments were still necessary for the survival of the tested colonies. The simultaneous selection for multiple markers, on the other hand, can prove more robust and lead to the desired traits (Spötter et al., 2012; Sainsbury et al., 2022). The combination of MAS with behavioural observations of honeybee colonies' performance can further close the research gap on honeybee resistance knowledge.

4.3 Conclusion and outlook

The research questions were answered for the most part. Proboscis extension response conditioning has proven a reliable method of researching olfactory sensitivity in workers and drones connected to the parasitisation with *V. destructor*. The test can not only display differences in odour sensitivity between different selection lines of worker bees but can also give information on the speed of perception of different highly diluted odours. Furthermore, with the help of PER conditioning the ability of drones to detect odours emitted by the brood during a *V. destructor* parasitisation could be confirmed for the first time. Unfortunately, this ability was not beneficial for the enhancement of the VSH of drones' offspring. Nevertheless, the possibility of discovering an odour which is perceived differently by drones coming from honeybee colonies selected for better resistance against *V. destructor* in comparison with nonselected colonies, should not be ruled out. Such a difference in olfactory sensitivity might prove a suitable marker for resistance breeding and considerably shorten the time and effort for creating resistant honeybee stocks. The search for a specific odour

marker suitable for quantifying olfactory sensitivity of drones and, at the same time, allowing the enhancement of the olfactory receptors of the drones (and their progeny) in terms of *Varroa*-parasitisation recognition, is a difficult task which might exceed the boundaries of the chosen experimental method (PER conditioning). Nevertheless, the scientific approach is extremely promising. The research conducted in the course of this thesis forms an integral part of the fundamental understanding of honeybee olfaction in connection with the parasitisation of *V. destructor*.

Future research can involve testing substances that can be perceived by drones at low doses and whether differences in perception can be found between VSH-selected and nonselected colonies. The effects of such potential differences on the offspring's olfaction could bring valuable information for future breeding strategies. By also including queens in the olfactory experiments, an even better understanding of olfactory sensitivity and its inheritance can be achieved. Furthermore, desired traits can be bred with even less genetic variance and ensure even faster breeding success when both the maternal and paternal sides are carefully chosen.

Mapping the genetic profile of drones, queens and workers participating in olfactory experiments should be considered. By combining PER conditioning and SNP analysis or another 'omics approach, differences seen in behaviour and olfactory sensitivity of the tested individuals can directly be linked to different genetic profiles or expression of important proteins.

5. Summary

Use of *Apis mellifera* drone's olfactory sensitivity towards pathological odours as a selection trait in the breeding against *Varroa destructor*

The *Varroa destructor* parasitisation has remained one of the main causes for colony losses all around the world since the introduction of the mite in *Apis mellifera* populations. One of the natural defence mechanisms used by the honeybee in the fight against *V. destructor* is hygienic behaviour, particularly a specialized form of it – *Varroa*-sensitive hygiene (VSH) – which includes the uncapping and removing of parasite-infested, or dead brood. When infested, capped brood changes its cuticular profile sending an olfactory signal which only a few workers in the colony can sense. Subsequently, the brood cell is opened, and the mite removed.

Hygienic behaviour and olfactory sensitivity to brood-related odours are observed to be strongly influenced by genetics. Breeding efforts with the goal of enhancing the European honeybee's resilience have been ongoing for more than three decades, but to date no breeding strategy has reached a broad-scale host-parasite balance. Due to the strong environmental dependence of resistance traits, the reproductive and genetic peculiarities of the honeybee, as well the small-scale structure of the German beekeeping community, very labour-intensive traditional breeding programmes for this species are in need of transformation. Having this in mind, this project focused on the development of a new strategy that has the potential to make breeding in honeybees significantly more efficient by utilising drones' olfactory as a selection trait. As drones are haploid, the genetic potential for *Varroa*-resistance can be assessed on the gametes. This allows genetic differences to be recognised significantly better and enables faster selection success.

The proboscis extension response (PER) conditioning as a non-invasive method of observing olfactory sensitivity in both workers and drones enabled the gathering of insights into VSH and its inheritance and was chosen for the selection of the most suitable individuals. In a first step, workers bred for VSH and nonselected control line workers were tested via PER conditioning for differences in their speed and perception ability when presented with highly diluted stimuli. Two pairs of odours (Pair 1: citral as Cs+: linalool as Cs-; Pair 2: *Varroa*-parasitised brood extract as Cs+, isopropanol as

Cs-) were used as tactile stimuli for the differential conditioning. Citral – a floral odour – and the brood extract were especially chosen in order to observe whether breeding for resistance in one of the tested groups had an effect on odour sensitivity to all or only to special odours connected to VSH. The VSH-selected line exhibited a significantly higher speed of perception for the parasitised brood extract than the nonselected line. The two lines showed no differences when conditioned with the floral stimulus citral as Cs+. The results suggested an increased specific sensitivity to chemical stimuli emanating from the brood in VSH-selected workers, which could play a role in recognising and removing *V. destructor*.

In a second step, the odour sensitivity of drones to the *Varroa*-parasitised-brood extract was examined through PER conditioning. Sperm from drones, sensitive/insensitive to two *Varroa*-parasitised-brood odours concentrations was extracted, and queens from VSH-selected and nonselected lines were inseminated accordingly, following a mating scheme. The VSH behaviour of the offspring was observed, and the genetic origin of queens and drones as well as the drones' perception of the brood odour were considered. While drone PER conditioning did not significantly correlate with VSH results, the genetic origin of participating queens and drones played a crucial role in VSH manifestation. A tendency for maternal effects for the inheritance of VSH was observed, suggesting that the choice of father drones would have less influence on VSH than that of mothers as genes connected to VSH are inherited via mitochondrial DNA of the mother. Additive genetic effects were also observed when drones from the VSH-selected line were paired with queens from the VSH-selected line, suggesting that drone's genes nevertheless play an important role in resistance breeding. The role of the drone should therefore not be underestimated.

To summarise, the study highlighted the importance of genetics in the expression of VSH in honeybee populations. The use of PER conditioning for observing differences in honeybee olfaction proved very promising. Odours with biological relevance to drones need to be tested in order to determine how drones' olfaction would affect the choice of fathers and the sensitivity of their offspring to *Varroa*-parasitised brood. Furthermore, if PER conditioning is to be combined with a genetic or transcriptome analysis, differences seen in behaviour and olfactory sensitivity of the tested individuals can be directly linked to different genetic profiles or expression of important proteins.

Nutzung der Geruchsempfindlichkeit von *Apis mellifera*-Drohnen gegenüber pathologischen Gerüchen als Selektionsmerkmal in der Zucht gegen *Varroa destructor*

Seit der Einschleppung der Milbe *Varroa destructor* in Populationen der europäischen Honigbiene *Apis mellifera* zählt die Milbe als eine der Hauptursachen für Völkerverluste weltweit. Wie alle Insekten verfügen auch Honigbienen über natürliche Abwehrmechanismen gegen Krankheiten. Der zentrale Mechanismus im Kampf gegen *V. destructor* ist das Hygieneverhalten, insbesondere die spezielle Form, die *Varroa*-sensitive Hygiene (VSH), die das Entdeckeln und Entfernen parasitierter oder toter Brut umfasst. Bei Befall verändert die verdeckelte Brut ihr Duftprofil und sendet ein Signal aus, das nur einige Arbeiterinnen im Volk wahrnehmen können. Daraufhin wird die Brutzelle durch die Arbeiterinnen geöffnet und die Milbe entfernt.

Das Hygieneverhalten und die Sensitivität der Arbeiterinnen gegenüber dem Duft kranker Brut sind stark genetisch beeinflusst. Zuchtversuche mit dem Ziel, Resistenzeigenschaften wie das Hygieneverhalten zu verbessern, werden seit mehr als zwei Jahrzehnten durchgeführt. Bis heute ist es jedoch nicht gelungen, mit einer Zuchtstrategie die Behandlung befallener Völker überflüssig zu machen. Resistenzmerkmale, die bei der Honigbiene beobachtet werden, sind von der Umwelt abhängig. Aufgrund dessen und unter Berücksichtigung der reproduktiven und genetischen Besonderheiten der Honigbiene sowie der kleinbäuerlichen Struktur der deutschen Imkerschaft ist eine effizientere Gestaltung der sehr arbeitsintensiven traditionellen Zuchtprogramme für *A. mellifera* dringend nötig. Aus diesem Grund konzentrierte sich dieses Projekt auf die Entwicklung einer neuen Zuchtmethode, mit dem Potenzial, die Resistenzzucht der Honigbiene wesentlich effizienter zu gestalten, indem die Geruchssensitivität der Drohnen als Selektionsmerkmal genutzt wird. Da Drohnen haploid sind, kann das genetische Potenzial für *Varroa*-Resistenz direkt an den Gameten bewertet werden. Dadurch lassen sich genetische Unterschiede deutlich besser erkennen und ermöglichen schnellere Selektionserfolge.

Die am besten für die Selektion geeigneten Individuen hinsichtlich ihrer Geruchssensitivität wurden mit Hilfe der Rüsselreflex-Konditionierung (Proboscis Extension Response, PER) ausgewählt. Diese nicht-invasive Methode ermöglichte es, die Geruchssensitivität sowohl bei Arbeiterinnen als auch bei Drohnen zu beobachten

und Erkenntnisse über VSH und deren Vererbung zu gewinnen. In einem ersten Schritt wurden VSH-selektierte Arbeiterinnen und nicht-selektierte Arbeiterinnen der Kontrolllinie mittels PER-Konditionierung auf Unterschiede in ihrer Geschwindigkeit und Wahrnehmungsfähigkeit stark verdünnter Duftreize getestet. Zwei Duftstoffpaare (Paar 1: Citral als Cs+: Linalool als Cs-; Paar 2: *Varroa*-parasitierter Brutextrakt als Cs+, Isopropanol als Cs-) wurden als taktile Reize für die differentielle Konditionierung verwendet. Citral - ein Blumenduftstoff - und der Brutextrakt wurden mit dem Ziel ausgewählt, zu untersuchen, ob die Resistenzzucht die Geruchsempfindlichkeit der Arbeiterinnen aus der VSH-selektierten Linie gegenüber allen oder nur gegenüber VSH-spezifischen Duftstoffen beeinflusst. Die VSH-selektierte Linie zeigte eine signifikant höhere Wahrnehmungsgeschwindigkeit für den Extrakt aus parasitierter Brut als die nicht selektierte Linie. Die beiden Linien zeigten keine Unterschiede, wenn sie mit dem Blütenduftstoff Citral als Cs+ konditioniert wurden. Die Versuchsergebnisse deuteten darauf hin, dass die VSH-selektierten Arbeiterinnen eine erhöhte spezifische Sensitivität gegenüber chemischen Reizen der Brut aufweisen, was bei der Erkennung und Entfernung von *V. destructor* eine Rolle spielen könnte.

In einem zweiten Schritt wurde die Geruchssensitivität der Drohnen gegenüber dem Extrakt aus *Varroa*-parasitierter Brut durch PER-Konditionierung untersucht. Sperma von Drohnen, die empfindlich/unempfindlich auf zwei Konzentrationen des *Varroa*-parasitierte-Brut-Extrakt reagierten, wurde extrahiert, und Königinnen aus VSH-selektierten und nicht-selektierten Linien wurden nach einem entsprechenden Paarungsschema damit besamt. Das VSH-Verhalten der Nachkommen wurde unter Berücksichtigung der genetischen Herkunft der Eltern sowie der Fähigkeit der Drohnen, den Extrakt der parasitierten Brut zu erkennen, beobachtet. Während die PER-Konditionierung der Drohnen nicht signifikant mit den VSH-Ergebnissen des Nachkommens korrelierte, spielte die genetische Herkunft der beteiligten Königinnen und Drohnen eine entscheidende Rolle für die VSH-Manifestation. Eine Tendenz zu maternalen Effekten bei der Vererbung von VSH wurde ebenfalls beobachtet, was darauf hindeutet, dass die Wahl des Vaters bei der Besamung einen geringeren Einfluss auf VSH hat als die der Mutter, da die mit VSH assoziierten Gene über die mitochondriale DNA der Mutter vererbt werden. Additive genetische Effekte wurden ebenfalls beobachtet, wenn Drohnen aus der VSH-selektierten Linie mit Königinnen

aus der VSH-selektierten Linie gepaart wurden, was darauf hinweist, dass die Gene der Drohnen dennoch eine wichtige Rolle bei der Resistenzzucht spielen. Die Rolle der Drohnen sollte daher nicht unterschätzt werden.

Zusammenfassend lässt sich sagen, dass durch dieses Projekt die Bedeutung der Genetik für die Ausprägung von VSH in Honigbienenvölkern verdeutlicht wurde.

Der Einsatz der PER-Konditionierung zur Beobachtung von Unterschieden im Geruchssinn von Honigbienen erwies sich als sehr vielversprechend. Gerüche mit biologischer Relevanz für Drohnen müssen getestet werden, um festzustellen, wie und ob der Geruchssinn der Drohnen die Empfindlichkeit ihrer Nachkommen gegenüber *Varroa*-parasitierter Brut beeinflussen könnte. Durch die Kombination der PER-Konditionierung mit einer Gen- oder Transkriptomanalyse könnten Unterschiede im Verhalten und in der Geruchssensitivität der getesteten Individuen direkt mit unterschiedlichen genetischen Profilen oder der Expression wichtiger Proteine in Verbindung gebracht werden.

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Publications

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Conflict of Interest

The author has no conflict of interest to declare.

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Declaration of Authorship

I hereby certify that this thesis has been composed by me and is based on my own work, unless stated otherwise. No other person's work has been used without due acknowledgement in this thesis. All references and verbatim extracts have been quoted, and all sources of information, including graphs and data sets, have been specifically acknowledged.

Berlin, 13.12.2024 Ivelina Ivanova