The use of *A. mellifera* drone's olfactory sensitivity towards pathological odours as a selection trait in the breeding against *V. destructor*

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Research Article

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Abstract

The most effective strategy against brood diseases, such as infestation by the mite *Varroa destructor*, is the early detection and removal of sick brood. Recent findings suggest that genes associated with worker bees' olfactory perception play a central role in *Varroa*-sensitive hygiene (VSH).

In the following approach, *Apis mellifera* drones' odour sensitivity was examined through a standardised Proboscis extension response (PER) test. Individuals with a positive/negative conditioning outcome to two parasitised-pupae extracts (extract-low and extract-high) were used for breeding. Twenty-one queens from a VSH selection line (SelQ) and nineteen queens from an unselected line (ConQ) were single-drone-inseminated with drones that showed either a positive (SenD+) or a negative (SenD-) PER test. Individual VSH behaviour of a total of 5072 offspring of these combinations (SelQ x SenD+, SelQ + x SenD-, ConQ x SenD+, ConQ x SenD-) was subsequently observed in a specially designed unit with infrared light. The results from the observation were also separately examined, considering the hygienic status of the participating queens and drones.

The results of the PER test of the drones were not significantly reflected in the VSH results of the respective offspring. On the other hand, the participating queens/drones' hygienic status was crucial for the manifestation of VSH.

Introduction

The ectoparasitic mite *Varroa destructor* plays a dominant role in colony losses in 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. Studies have shown an alarming tendency of increasing mite resistance against miticides [4–6]. While current treatment methods provide only temporary benefits, breeding colonies resistant against *V. destructor* is considered the only long-term solution [7, 8].

One of the natural defence mechanisms of honeybees that has proven effective against *V. destructor* is hygienic behaviour. This trait is observed in many social insects [9–11]. In the honeybee, hygienic behaviour consists of detecting, uncapping, and removing damaged brood [12–14]. The term "Suppressed of Mite Reproduction" (SMR) was created by Harbo and Harris [15] to describe the lack of viable progeny observed in resistant colonies during their experiments. Subsequently, SMR was described as a result of removing reproductive mites and not of an inhibited reproduction of *V. destructor* in resistant colonies [16, 17]. Nowadays, *Varroa*-sensitive hygiene (VSH) is considered a more accurate description of the defence mechanism against *V. destructor* [18, 19].

VSH is assumed to be based on the differential expression of genes responsible for the olfactory system and perception [20–24]. Colonies bred for VSH target cells containing multiple mature females and higher numbers of offspring in highly infested patches [25]. Highly virulent strains of the Deformed Wing Virus

(DWV) in foundress mites are also described as possible triggers of VSH [3, 26, 27]. Mondet *et al.* [28] presented evidence that all worker bees can detect *Varroa*-parasitisation-specific compounds, but only bees bred for VSH can distinguish those from healthy brood odour. This discrimination most probably takes place on the central lobes and the mushroom bodies [28, 29].

In some cases, parasitised brood cells are opened and recapped multiple times. This behaviour disrupts the mite's reproduction and allows the pupae to develop into an adult bee, thus minimising the colony loses [30, 31]. Research has shown that selective breeding can improve the colonies' performance regarding their hygienic behaviour against *V. destructor* [7, 16, 32, 33].

Masterman *et al.* [34] conducted laboratory tests on the olfaction of hygienic and non-hygienic bee colonies and observed discrepancies in the perception of pathological odours emitted by sick pupae. The hygienic selection lines performed much better during the learning process than the non-hygienic ones. However, there were no significant differences between the groups trained with flower odours. The authors suspected a genetically induced increased specific odour sensitivity to pathogens in the selection lines, allowing bees to remove sick individuals from the population faster. As a method, they 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 [35]. Honeybees usually exhibit this behaviour while foraging. PER is easily replicated under laboratory conditions. Based on the Pavlovian classical conditioning, the PER-Test was first introduced by K. Takeda in 1961 and has been used as a foundation for many olfactory experiments ever since [35–37]. In the honeybee, it is widely applied for observing the learning ability of individuals [38, 39], the odour sensitivity connected to VSH [28, 40] and the adverse effects of pesticides on the honeybee's behaviour [41, 42]. Bees learn to associate a conditioned stimulus (CS+) – usually an odour – with an unconditioned stimulus such as a sugar solution [43]. The odour presentation leads to the spread of the mouthpart (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.

While current breeding strategies concentrate on worker bees and their ability to recognise mite-infested cells, our focus lies in identifying the drone's role as a genetic carrier for the manifestation of VSH. Drones are haploid, and their offspring wholly inherit their genetic material. Because drones exhibiting a higher number of sensory cells in their antennae [44], we speculated that the use of individually tested drones (consequently flying gametes) could be a very efficient approach to significantly improve the genetic progress in the *Varroa*-resistance. By measuring the odour sensitivity of drones to an extract of *Varroa*-parasitised pupae, we bypassed the randomness of the meiosis to observe whether the PER-test results are reflected in the VSH of the F1-generation.

Results

Drone Conditioning

The GLMM binomial model showed no statistically significant difference between the results of selection and control drones. These findings applied to both extract-low and –high (Table 1 and Table 2).

Table 1
– Results from GLMM with a binomial distribution and a logit function for predicting the conditioning outcome for the lower-concentrated pupae extract depending on the drones' origin.

Extract-Low (15 pupae)						
Breeding line drone	Reg.-Coeff.	S.E.	Sig.	95% CI	OR	95% CI(OR)
Drones selection line (SelD)	-0.021	0.3056	0.945	-0.625; 0.582	0.979	0.536; 1.790
Drones control line (ConD)	0 [a]	-	-	-	-	-
[a] – reference group						

Table 2
Results from GLMM with a binomial distribution and a logit function for predicting the conditioning outcome for the higher-concentrated pupae extract depending on the drones' origin.

Extract-High (25 pupae)						
Breeding line drone	Reg.-Coeff.	S.E.	Sig.	95% CI	OR	95% CI(OR)
Drones selection line (SelD)	0.089	0.3102	0.774	-0.523; 0.702	1.093	0.593; 2.017
Drones control line (ConD)	0 [a]	-	-	-	-	-
[a] – reference group						

For both extract-high and -low none of the effects – temperature and drone's mother – played a significant role in the conditioning.

The number of drones conditioned successfully to extract-low was 39% and 46% to extract-high. During the experiment, the drones excluded for not responding to the stimulus amounted to 22% for extract-low and 16% for extract-high.

Video observation

VSH of groups considering PER-test outcome. The number of bees in each group and the total count of active bees are listed in Table 3. Group SelQ x SenD- exhibited the highest number of active bees in the two categories, followed by group SelQ x SenD+. Group ConQ x SenD- scored higher than group ConQ x

SenD+. The variation between each colony's average activity in its corresponding group is displayed in Figs. 1 and 2.

Table 3
– Number of beginner and helper bees per group. Each activity is presented as an absolute number and in per cent per group.

Breeding line queen/outcome PER-test drone	N	Queens (N)	Beginner		Helper	
			Count	%	Count	%
SelQ x SenD+	1382	12	47	3.4	92	6.7
SelQ x SenD-	850	9	66	7.8	95	11.2
ConQ x SenD+	1273	8	18	1.4	32	2.5
ConQ x SenD-	1567	11	39	2.5	58	3.7

During the video observation group SelQ x SenD- exhibited the highest beginner (GLMM, $p < 0{,}001$; CI: 0.842; 1.640) and helper (GLMM, $p < 0.001$; CI: 0.846; 1.537) results. The odds of SelQ x SenD- uncapping a parasitized cell were 3,5-times higher compared to the reference group (GLMM, OR = 3.459; CI: 2.322; 5.153).

Group SelQ x SenD + displayed a slightly higher but non-significant uncapping activity than the reference group (GLMM, $p = 0.225$; CI: -0.159; 0.675). The odds of initiating the uncapping of a parasitised cell were 1,3-times higher than the reference group (GLMM, OR = 1.294; CI: 0.853; 1.963).

Group ConQ x SenD + did not perform higher than the reference group in any of the activities (see Tables 4 and 5, supplementary information). The origin of the drones chosen for the insemination played a significant role in the helper activity – selection line drones produced a significantly higher scoring F1-generation (GLMM, $p < 0.001$; CI: 0.394; 0.947). The queen's effect was statistically significant for the beginner activity - $p < 0.001$ (CI: 0,623; 1.467).

Table 4
Number of beginner- and helper bees per phenotype group. Each activity is presented as an absolute number and in per cent per group.

Breeding line queen/breeding line drone	N	Queens (N)	Beginner		Helper	
			Count	%	Count	%
SelQ x SelD	1076	11	59	5.5	112	10.4
SelQ x ConD	1156	10	54	4.7	75	6.5
ConQ x SelD	1384	9	35	2.5	65	4.7
ConQ x ConD	1456	9	22	1.5	25	1.7

Table 5

Mating design for one-drone insemination. Hygienic Queens (*SelQ*) and non-hygienic queens (*ConQ*) were inseminated with sperm from drones, which participated in the PER-conditioning. Drones with positive outcome were marked with *SenD+*. Those with a negative outcome – with *SenD-*. The number of bees in each group participating in the experiment is listed next to the mating combination.

Breeding line queen / PER-test drone	**Drone with positive learning outcome (*SenD+*)**		**Drone with negative learning outcome (*SenD-)***	
		N		N
Hygienic Queen (SelQ)	*SelQ* x *SenD+*	1382	*SelQ* x *SenD-*	850
Non-hygienic Queen (ConQ)	*ConQ* x *SenD+*	1273	*ConQ* x *SenD -*	1567

The bees scored significantly higher in their beginner actions in course two (GLMM, p = 0.017; CI: 0.094; 0.969) and three (GLMM, p < 0.001; CI: 0.583; 1.358) compared to the reference course. Course three also exhibited the highest results for the helper activity (GLMM, p = 0.02; CI: 0.048; 0.571).

When the groups were combined to incorporate only the PER-test outcome - SenD + and SenD- - group SenD- was set as a reference group. The offspring of the drones with a positive outcome during the conditioning (SenD+) displayed significantly lower beginner results compared to the reference (GLMM, p < 0.001, CI: -0.925; -0.283). These results were further portrayed in the average distribution of the active bees in the two groups (Fig. 3, 4). The father drones' origin (selection/control line) had no significant effect on the results (GLMM, p = 0.19, CI: -0.107; 0.539). The queen's effect was significant (GLMM, p < 0.001, CI: 1.580; 2.379).

In the third course of observation, a significantly higher number of beginner bees was exhibited than during the reference course one (GLMM, p < 0.001, CI: 0.460; 1.255).

The helper activity showed no statistically significant difference between the two groups. The drones' origin had a significant positive effect on the helper activity – drones from the selection line fathered a higher number of bees who conducted a helper activity (GLMM, p = 0.001, CI: 0.226; 0.704). The queens' effect was non-significant.

VSH of groups considering the parental origin. During the three repetitions of the experiment, group SelQ x SelD exhibited the highest number of beginner and helper bees. Group SelQ x ConD performed the second highest activity. Group ConQ x ConD scored the lowest in the three categories. Differences in the average activity were observed amongst the hives in each group (Fig. 5, 6). The detailed results are listed in Table 4.

When comparing the groups' beginner and helper activities to the reference group ConQ x ConD, an increase from control to selection line was observed. The pairing of queens from the selection line with drones from the selection line delivered the highest statistically significant results (**beginner**: GLMM, p < 0.001; CI: 1.195; 2.207; and **helper**: GLMM, p < 0.001; CI: 1.465; 2.528). The odds of group SelQ x SelD

uncapping a parasitised cell were 5-times higher than the reference group (GLMM, OR = 5.482; CI: 3.305; 9.093).

The second highest results were achieved when inseminating a queen from the selection line with sperm from control drones (SelQ x ConD). This group performed significantly better than the reference group in both categories (**beginner**: GLMM, p = 0.005; CI: 0.437; 1.1538; and **helper**: GLMM, p < 0.001; CI: 0.805; 1.925). The results are listed in detail in supplementary tables 5 and 6. The odds of uncapping a parasitised cell were 2.7 times higher (GLMM, OR = 2.685; CI: 1.548; 4.657) than the reference group.

Group ConQ x SelD exhibited significantly higher performance than the reference group in the helper activity (GLMM, p = 0.003; CI: 0.534; 1.682). While the bees' performance in the beginner category was higher than that of the reference group, the results were not significant.

The experimental course had no significant effect.

Control Cells

During the first repetition, none of the control cells was opened. These cells were *Varroa*-free. During the second repetition, the brood from one cell was removed. The other four cells were *Varroa*-free. In the last repetition, one cell contained a single non-fertile mite; the other four were unparasitised.

Discussion

In the present study, 40 queens were each inseminated with sperm from one drone. A total of 5072 bees from the F1-generation were individually examined for their VSH. We aimed to display the connection between VSH and the drone's olfactory sensitivity by regrouping the data multiple times.

Masterman *et al.* [34] observed a difference in the discrimination abilities of hygienic and non-hygienic worker bees for brood odours. However, this does not seem to apply to drones. While the drones were able to perceive the extract of *Varroa*-parasitised pupae and be successfully conditioned to it, neither one of the groups (selection/control line) was more prone to learning the CS + than the other one. Moreover, the results from the PER-test did not deliver any advantage to the F1-generation. To our knowledge, this is the first conditioning experiment with drones using an extract from *Varroa*-parasitised pupae.

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 which did not learn the conditioning stimulus (SelQ x SenD-). When the results from the video observation were displayed for two groups on the base of the PER-test – SenD + and SenD- - the PER-test showed no significance for the manifestation of VSH.

When mated with queens from the selection line, the successfully conditioned drones produced colonies with more active beginner and helper bees compared to the reference group ConQ x SenD-. Yet, those results were significant only for the helper activity (GLMM, p < 0,001; CI: 0,394; 0,947). 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 x SenD + and ConQ x SenD + to exhibit the highest results in the observation. Contrary to our thesis, group SelQ x SenD produced the most active offspring in the three repetitions of the experiment. Further, group ConQ x SenD + scored lower than the reference group, although the differences were not significant. These observations led us to believe that the negative outcome from the conditioning with both extracts -high and -low did not prove to be a reliable exclusion instrument.

From our observations, drones proved to be much more sensitive to the conditioning temperature and length than bees. Vareschi [45] described differences between bees' and drones' conditioning, stating that drones are more "nervous" than bees. We, too, observed such a tendency. The PER-success depended on the physiological state of the tested individuals - factors such as the drone's disposition during the conditioning, hunger state, and environmental influences. Once the drones had ingested enough sugar, they stopped responding to the stimuli.

We ensured the same nursing conditions for all test subjects through the drones' collective upbringing in one hive. The laboratory conditions were as uniform as possible. Nevertheless, all bee species are known to be highly conscious of their natural surroundings [46], and the change in weather conditions [47] could trigger a physiological response. Li *et al.* [47] believed that high temperatures and humidity could influence oxidative stress in bees. The role of humidity as a factor is not yet clear; however, it might explain the unwillingness of some drones to respond to the CS+, for example, on cold or rainy days. Because of the PER-test drawbacks mentioned earlier, some drones with favourable genes for enhancing VSH might have potentially been excluded from the experiment or labelled with a "negative outcome", leading to the group outcomes we observed.

Brockmann *et al.* [48] conducted an electroantennogram (EAG) on bees and drones to study their reaction to the queen mandibular pheromone components. Drones exhibited an increased sensitivity towards 9-ODA, which acts as a sex pheromone and attracts drones during mating flights. Such specialisation is also typical for other species like moths [49, 50], bark beetles [51], cockroaches [52], ants [53, 54]. Brockman *et al.* [48] argued that while the sensitivity for 9-ODA in the honeybee drone was enhanced, the sensitivity to the other components was reduced. Whereas drones were specialised in detecting only one component, worker antennae showed a "generalised" antennal tuning. Bees exhibited no differences in the neurons sensitive for either individual pheromone components and those detecting other pheromonal or non-pheromonal components. These findings are compatible with the observations of Esslen and Kaissling [55] on the number of sensory cells in the antennae of bees (64 889) and drones (338 859). The worker bee exhibits a large number of secondary neurons relative to the number of sensory cells. The authors argued that these anatomical features could be the basis for a greater differentiation ability for scents. On the other hand, drones are assumed to be highly specialised in distinguishing only certain odours connected with their biological function. According to Arnold *et al.* [44], a well-pronounced sexual dimorphism in the glomeruli of the antennal lobe can be observed between bees and drones. While the worker bees displayed only two structural types of glomeruli, drones exhibited a third one. These large

glomerular complexes (macroglomeruli) of the drone were responsible for the detection of queen pheromones.

Plant odours were processed in the ordinary glomeruli of the antennal lobe [56]. Having this in mind, it is likely that the extract of *Varroa*-parasitised brood is processed in the drone's ordinary glomeruli. When it comes to VSH, Mondet *et al.* [28] strengthened the view that all worker bees can detect the *Varroa*-parasitisation-compounds, but only VSH bees can distinguish the difference between healthy and diseased brood. The VSH is a sex-specific function. While we proved that drones can perceive the parasitised pupae extract, this ability is probably as unimportant to the drone's mating success as the distinction between two floral odours.

Eusocial insects are known for their caste systems and behaviours performed by each cast (foraging, nursing, mating). Different epigenetic mechanisms like DNA methylation and histone posttranslational modifications regulate these behaviours in ants [57], bumblebees [58], honeybees [59]. Flores *et al.* [59] suggested that certain environmentally induced non-heritable methylations of DNA could lead to better survival outcomes and in time become permanent, heritable methylations. Kucharski *et al.* [60] examined the expression of one Odorant binding protein (OBP) gene – *obp11* – on the antennae of bees. OBP11 is also found in the *sensilla basiconica* of female ants [61]. 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 female bees' *sensilla basiconica*, it is silenced through methylation on drones' antennae [60]. This observation is not surprising since the drones' only role is mating with a virgin queen. Epigenetic changes connected to labour division between the sexes could explain why the drones' PER-conditioning results did not prove useful for enhancing the VSH of the F1-generation.

Our experiments also add new information on the inheritance of VSH. When the group results were analysed with the hygienic status in mind, the number of beginner and helper actions increased when drones or/and queens of the selection line were used. The origin of the queen proved to play an even bigger role than that of the drone. This observation was in accord with the substantial effect of the queen origin (selection/control line) on the beginner activity when the results were analysed based on the PER-test. The Sel queens produced offspring with a higher VSH-activity when inseminated with sperm from Con drones compared to Con queens inseminated with sperm from Sel drones. The odds of commencing a beginner activity compared to the reference group were as followed: 1,5 times higher for ConQ x SelD (OR; CI:0,791; 2,732), 2,7-times higher for SelQ x ConD (OR; CI: 1,548; 4,657), 5,5-times higher for SelQ x SelD (OR; CI: 3,305; 9,093). The same tendency was observed in the helper activity: 3,0-times higher than the reference group for ConQ x SelD (OR; CI: 1,706; 5,378), 3,9-times higher for SelQ x ConD (OR; CI: 2,237; 6,858) and 7,4-times higher for SelQ x SelD (OR; CI: 4,327; 12,528). These results lead us to believe that maternal effects play a significant role in the manifestation of VSH.

Maternal effects affect behaviour and help offspring better adapt to changes in the environment. Maternal effects have been observed in many species [62–65], including honeybees. Dloniak, French and

Holekamp [65] described rank-related maternal effects on offspring's phenotype in spotted hyenas (*Crocuta crocuta*). Dominant females exhibited higher androgen concentrations in late pregnancy, which shaped the new generation's behaviour and social structure. Storm and Lima [66] 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 been described by bees as well. Unger and Guzmán-Novoa [67] 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 the removal of brood. On the other hand, "control queens" and "hygienic drones" produced an F1-generation with weaker hygienic behaviour. Spivak and Reuter [68] assessed colonies with queens from a VSH-selection 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 demonstrated drones' ability to sense odours connected to the distress signalling of brood infested with *V. destructor.* The PER-test proved a non-suitable selection tool for the enhancement of VSH. While an additive genetic effect was observed when drones from the selection line were paired with queens from the selection line, there was a tendency that maternal effects also played an important role. Since both genders inherit the same genes from their mother, it would be a big step towards creating a breeding strategy against *V. destructor* if a worker bees' odour sensitivity could be measured on her fathering gametes (drones). The odour sensitivity to parasitised brood of worker bees is the key factor in *Varroa*-resistance. Therefore, further research is necessary to find odours and suitable test methods to phenotype the drones' unspecific odour sensitivity. If the heritability of such test results is sufficient, VSH can be improved more efficiently by the breeding use of such individually tested drones.

Materials And Methods

Extract Preparation

An extract from *Varroa*-parasitised brood was created to mimic the complex composition of the distress signals emitted by 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. Four mites were inserted pro cell 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 the time had elapsed, 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 a basis for the extract. The pupae were washed in 4ml isopropanol for 10 min. The supernatant was decanted in special 2ml 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). It was presumed that drones who cannot perceive and learn the highly concentrated extract (extract-high) were inferior in their olfactory sensitivity compared to drones who perceived the lower concentrated pupae extract (extract-low).

PER-test

The antennae of bees play an essential role in perceiving their environment and communicating within the hive [69, 70]. Amid this process, both olfactory and tactile stimuli are perceived and processed.

Signals from the damaged brood are presented on the cell caps [71]. During the execution of hygienic behaviour, the infested cell is visited by nursing bees. Its cell cap and those of neighbouring cells are touched multiple times by the antennae. The bee performs typical movements with its head, enabling it to localise the damaged brood very accurately [72].

Having this specialised behaviour 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 like oleic acid, the brood ester pheromone and tritriacontane are perceived [27,73–75]. We chose filter paper as a medium which was presented with the help of tweezers.

Two PER-tests were carried out for the selection of the drones which were to be used for the artificial insemination:

1. PER-test for a good odour perception: 5 µl extract-low (see above) as the positive stimulus CS + and 5 µl isopropanol as the negative stimulus CS-
2. PER-test for the absence of extract perception: 5 µl extract-high (see above) as the positive stimulus CS + and 5 µl isopropanol as the negative stimulus CS-

For the PER-tests, eight colonies were chosen, and 100 newly hatched drones per origin were marked with a chip on the dorsal thorax. The drones were placed in a nursing hive with an unmated queen. Four of the chosen colonies were of hygienic origin, and the other four of non-hygienic origin. After the drones reached reproductive age (14d), the PER tests were started.

The drones were collected from the hive shortly before the start of each conditioning and strapped in small metal tubes with tape. The immobilised drones were kept in a rack with numbered sloths. A 50% sugar solution was used for the tests. Only the drones that readily stretched their proboscis during the reward's presentation were used in the experiment. The drones were presented with plain filter paper multiple times before the beginning of the odour conditioning. This was done to prevent the proboscis's extension solely due to the filter paper's pure mechanical irritation. Each conditioning group consisted of eight drones. We aimed at the equal representation of every origin in these groups. Two PER-tests were

conducted daily – one with each of the extracts. The chronological order of the tests (good odour perceptions, absence test) was changed each day to ensure no bias due to daytime would occur.

The PER-test consisted of six trials with a specified order of stimuli presentation: CS+, CS-, CS-, CS+, CS+, CS-. The positive stimulus was enhanced by the administration of sugar solution with the help of a toothpick. Each CS's duration was 6 sec, with the reward for the positive stimulus being presented parallel to the CS + within the last 3 sec [76]. The learning success was subsequently examined and recorded by a presentation of the two stimuli without the reward (Fig. 7).

The following drones were considered for artificial insemination:

1. Drones with an excellent odour perception of extract-low (15 pupae extract) which showed the correct responses in the last two trials and during the outcome-examination.
2. Control drones were responsive throughout the experiment with extract-high (25 pupae) but showed no positive responses during the last two trials, indicating that the learning success was negative.

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.

Artificial insemination and infrared-video observation

The drones were brought back to the hive after each conditioning for recovery before the sperm was extracted. Sperm extraction took place right before the insemination [77]. A total of 40 queens took part in the experiment. The queens originated from lines selected for their hygienic behaviour (selection line) and from the institute-owned lines (control line). They were housed in mini mating hives with young bees. Once all the inseminated queens had started laying eggs, each mini-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 (Fig. 8). Afterwards, they were placed in the video surveillance unit described by Bienefeld *et al.* [72]. A *Varroa*-free brood frame with freshly capped brood was taken from an institute-own 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, the bees' activity was monitored using an infrared camera. The video recording analysis was carried out with the help of a software program - Beehaviour – specially created for this purpose (Batz *et al.*, submitted). Two activities were of interest – the beginner activity was defined by the first bee opening an infested cell and the helper activity - the bees which enlarged the hole after the beginner had created it. If the cells caps were opened and resealed multiple times, all the actions mentioned above were recorded each time. 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.

Mating design

Four groups were created during the one-drone insemination depending on the queen's affiliation to a VSH-selection line (SelQ) or the control-line (ConQ) [78]. For the results of the PER-test, the outcome of the single drones (SenD) was marked with "**+**" or "**-**", where "**+**" means a positive learning outcome and "**-**" - a negative learning outcome (Table 5).

The drones in each group came from both the selection and the control line. The participation of each line was as followed: ConQ x SenD- (64% selection line, 36% control line), ConQ x SenD+ (37,5% selection line, 62,5% control line), SelQ x SenD- (67% selection line, 33% control line), SelQ x SenD+ (42% selection line, 58% control line).

For the statistical analysis, the groups mentioned above were further combined to create two groups depending on the drone's PER-test results (Table 6).

Table 6

Group assembly depending on the PER-results of the drones. Group *SenD +* combines all bees with hygienic/non-hygienic mothers and a PER positive father. Group *SenD-* combines all hygienic and non-hygienic mothers inseminated with sperm of PER negative drones. The number of F1-bees in each group is listed in the table.

Breeding line queen / PER-test outcome drone	**Drone with positive learning outcome (*SenD+*)**		**Drone with negative learning outcome (*SenD-)***	
New Group	SenD+	N	SenD-	N
Queen (SelQ, ConQ)	*SelQ* x *SenD+*, *ConQ* x *SenD+*	2655	*SelQ* x *SenD-* *ConQ* x *SenD -*	2417

To determine the effect of the VSH-selection on the results, we lastly reassembled the four original groups based on the VSH of the parents (Table 7).

Table 7

– Group formation considering the hygienic status of the queen and drone. Queens and drones from the VSH-selection lines were marked with *Sel.* Queens and drones from the non-selection lines were marked with *Con* as control. The number of bees in each group participating in the experiment is listed next to the hygienic combination.

Breeding line queen/breeding line drone	**Hygienic Drone (*SelQ*)**		**Non-hygienic Drone (*ConD*)**	
		N		N
Hygienic Queen (SelQ)	*SelQ* x *SelD*	1076	*SelQ* x *ConD*	1156
Non-hygienic Queen (ConQ)	*ConQ* x *SelD*	1384	*ConQ* x *ConD*	1456
Main Figures				

Statistical analysis

PER-test analysis. During the conditioning, the drones from all origins were treated the same way. The question of whether there was a statistically significant difference between the selection and the control drones was of interest to our research. Therefore, the drones were split into two groups for the statistical analysis, considering their origin (selection line/control line).

The drone conditioning results were examined using a Binomial 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 temperature during the experiment and the mother of the drone were both set as random effects.

Drones tested on days with very low overall drone activity, mostly on days with bad weather conditions, did not produce sperm. Therefore, they did not procreate and will not be considered a vital factor in the video observation.

Video-observation analysis

VSH of groups considering PER-test outcome. The video recording results were analysed through a Binomial GLMM with a logit function in SPSS V.25.

Group ConQ x SenD- was used as a reference. The course of observation - one, two or three - and the drone's origin (selection line, control line) were considered fixed effects. By including the drone's origin in the regression, the model provided more accurate insight into the PER-test's explanatory power for the results. Course one and control line were chosen as reference values. The individual effect of each queen mother was set as a random factor for the regression.

To better understand the possible effect of the PER-test on the manifestation of VSH, we further examined the VSH of the offspring as two groups (SenD- and SenD+), considering only the odour sensitivity of the fathers to the extract. The analysis was conducted using the same statistical model as when analysing the groups considering the mother's origin and the fathers' sensibility (Binomial GLMM with logit-function) but with the difference of group "SenD-" having been set as a reference.

VSH of groups considering the parental origin. The analysis was conducted using a Binomial GLMM with a logit function. Group ConQ x ConD was set as the reference group. The course of observation was again considered a fixed effect. Course one was set as a reference.

Declarations

Acknowledgements

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Author Contributions

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

Additional information

Competing interests

The authors declare no competing interests.

Data availability

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

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Figures

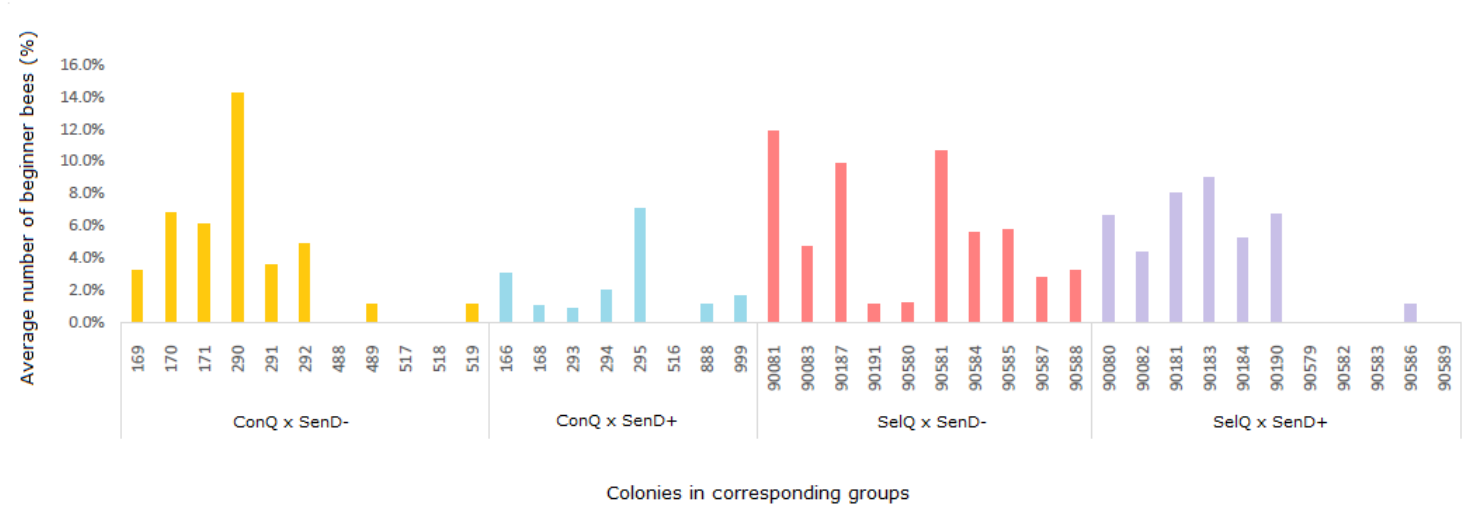

Figure 1

Average beginner number of tested bees per colony in per cent. Each colony is displayed in its corresponding group. Colonies without beginner bees are shown without bars.

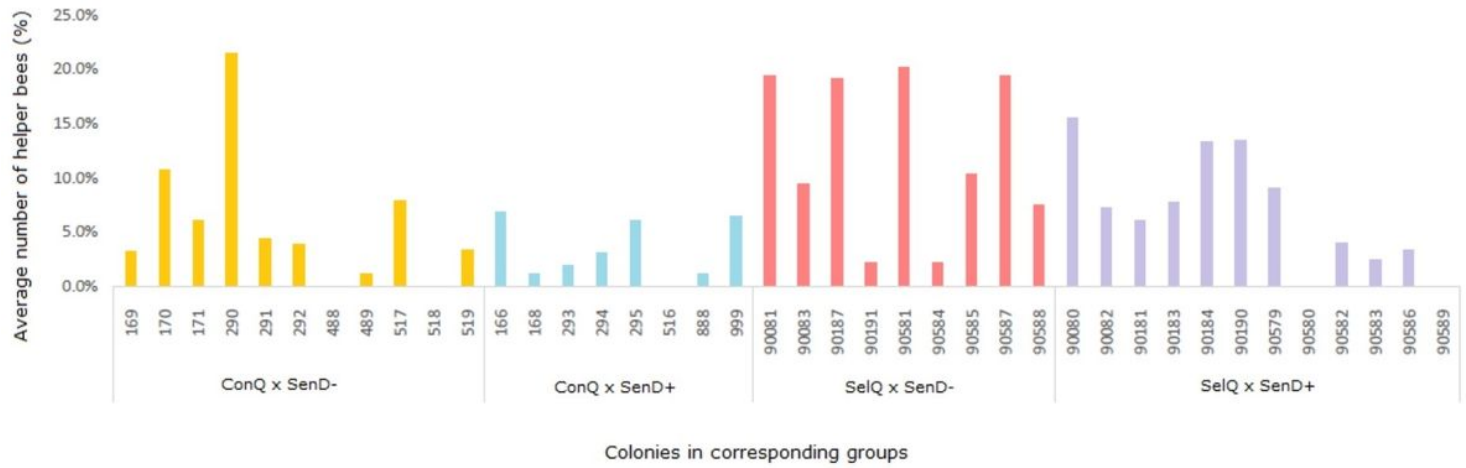

Figure 2

Average helper number of tested bees per colony in per cent. Each colony is displayed in its corresponding group. Colonies without helper bees are shown without bars.

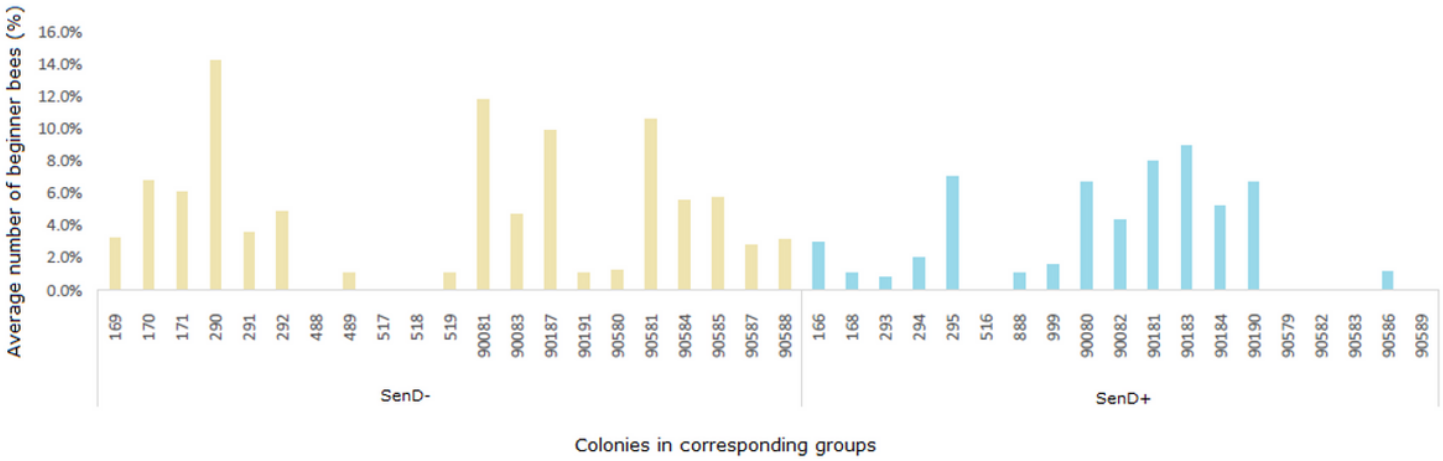

Figure 3

Average number of beginner bees displayed for SenD- and SenD+. The values are shown in percent for each hive.

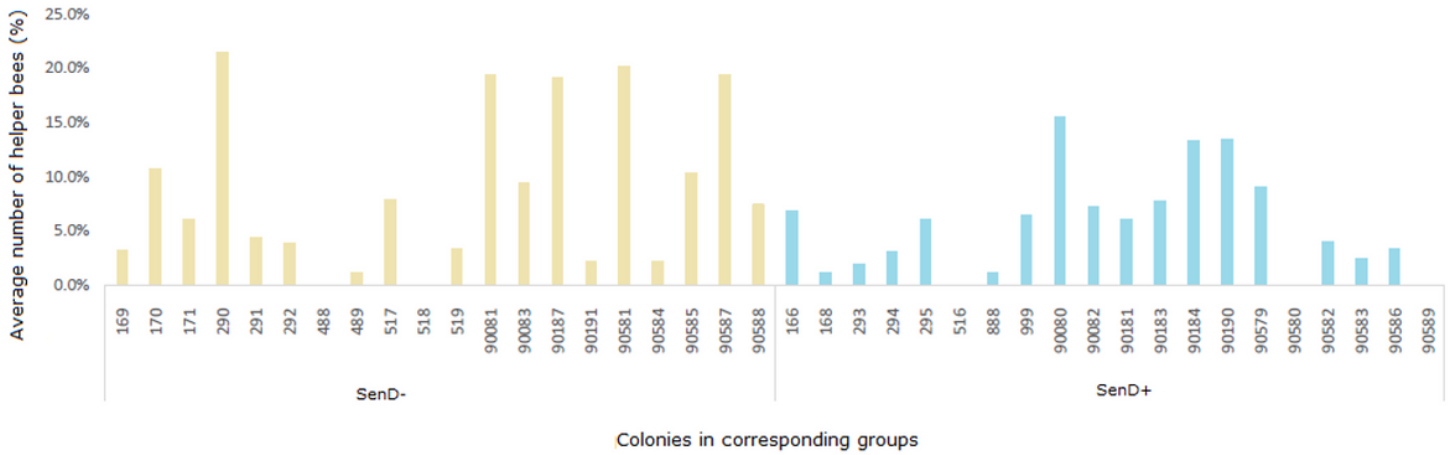

Figure 4

Average number of helper bees displayed for SenD- and SenD+. The values are shown in percent for each hive.

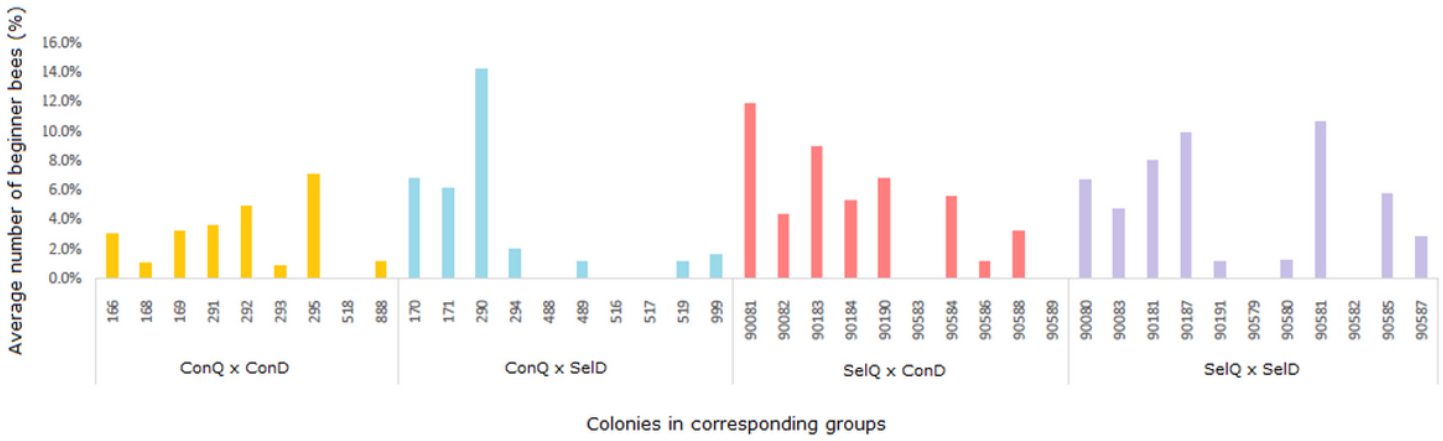

Figure 5

Average number of beginner bees displayed for each breeding combination. For each of the four groups, the corresponding colonies are exhibited. The values are shown in per cent.

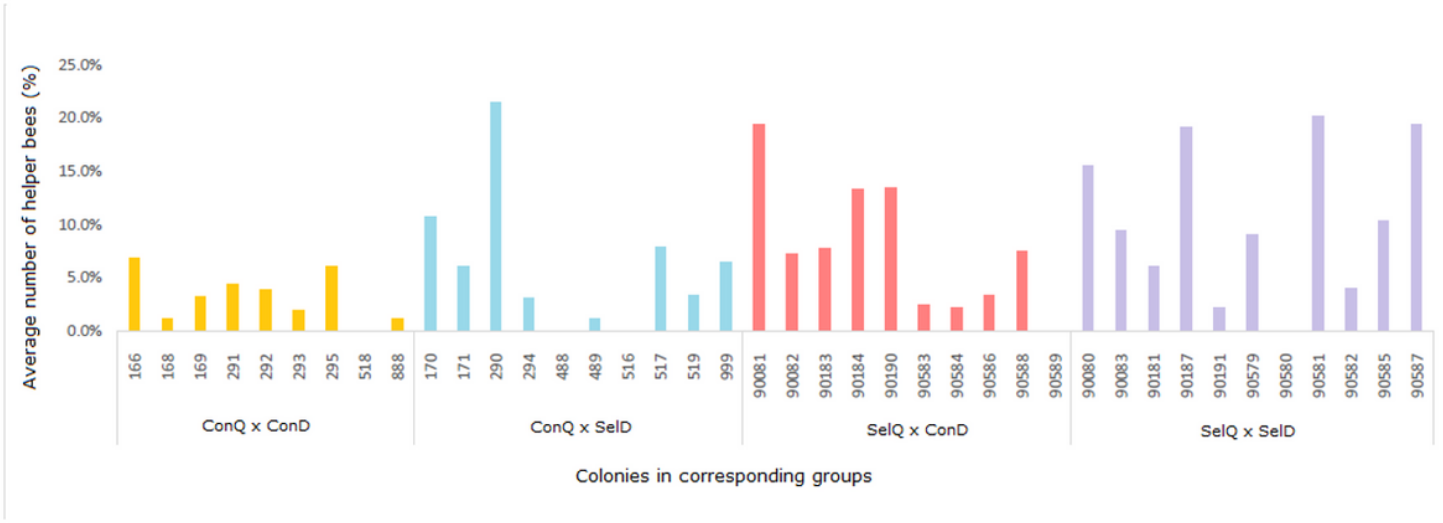

Figure 6

Average number of helper bees displayed for each breeding combination. For each of the four groups, the corresponding colonies are exhibited. The values are shown in per cent.

Figure 7

PER-test conducted on a drone. Tactile presentation of the CS+ using filter paper.

Figure 8

Marking the offspring with a plate on the dorsal thorax.

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