

The parasitic mite *Varroa destructor* affects non-associative learning in honey bee foragers, *Apis mellifera* L.

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Abstract The parasitic mite *Varroa destructor* influences flight behavior, orientation and returning success of forager honeybees (*Apis mellifera*) infested as adults. As impaired orientation toward the nest entrance might be due to deficiency in recognition and responsiveness to stimuli in the environment, we examined effects of *V. destructor* on sensory responsiveness, non-associative and associative learning of honey bee foragers by using proboscis extension reaction paradigm (PER). Although infested and uninfested workers were initially equally responsive to different concentrations of sugar water, we found differences in non-associative learning. In habituation, PER to repeated sugar stimulation of the antennae occurred faster in infested

foragers compared to uninfested foragers. In sensitization, infested foragers showed a lower response to an odor stimulus following sugar stimulation than non-infested foragers. Differences in non-associative paradigms were more pronounced in bees with lower responsiveness to sucrose. In conditioning learning experiments, a significant reduction in proboscis extension response was found 1 min but not 12 min after a single conditioning trial indicating that *V. destructor* predominantly affects the non-associative components of learning and its underlying neural and molecular processes.

Keywords *Apis mellifera* · *Varroa destructor* · Habituation · Sensitization · Learning

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Introduction

Life histories of hosts are often modified by parasites as an outcome of various responses from which both benefit: parasites from enhanced dispersal and hosts by several responses that eliminate or compensate negative effects of parasites (Poulin 1995; Jog and Watve 2005). Altered behaviors in favor of hosts may relate to avoidance or removal of parasites (Moore 2002; Poulin 1995), habitat preference (Moore and Freehling 2002; Müller and Schmid-Hempel 1993), mating (Hamilton and Zuk 1982), changing diet (Moore 2002; Poulin 1995) and increasing activity which in the most extreme cases increases risk of predation (suicidal behavior, Smith-Trail 1980; McAllister et al. 1990).

Behavioral modifications reducing the infestation rate, such as grooming and hygienic behavior, are well documented in honey bees and most of them are

described in the relation to the parasitic mite *Varroa destructor* (Spivak and Reuter 1997; Gilliam et al. 1983; Boecking and Drescher 1992; Peng et al. 1987; Büchler et al. 1992; Fries et al. 1996). Recently it has been shown that there is a marked difference in mite load between foragers leaving and returning to the hive (Fuchs and Kutschker 2000). Foragers infested by *V. destructor* showed prolonged absence from the colony and lower returning success to the colony (Kralj 2004). In particular, an orientation assay testing the accuracy to orient toward the nest entrance suggested the possibility, that the lower returning rate might be a result of reduced sensory and/or neural processing capabilities involved in navigation during foraging flights.

A simple experimental paradigm to test for alterations in sensory processing, motor output and learning is the proboscis extension response paradigm (Kuwabara 1957). Stimulation of contact chemosensory sensilla on antennae or the mouthparts with sugar solution elicits extension of the proboscis. Sugar threshold concentrations for the reaction vary with the satiation state, behavioral task, age and genetic background and can be modulated by brood pheromone (Page et al. 1998; Pankiw and Page 1999, 2000; Pankiw 2004; Scheiner et al. 2001a, b, 2003). Continuously stimulating the antennae with sucrose solution leads to a suppression of the proboscis extension (= habituation, Braun and Bicker 1992; Bicker and Hahnlein 1994). In addition, sugar stimulation of the antennae increases the spontaneous proboscis extension to floral odors (= sensitization) shortly after stimulation (Hammer et al. 1994; Menzel et al. 1999). Habituation and sensitization represent simple forms of non-associative learning which are based on short-lasting modifications of synaptic transmission (Kandel 1991; Menzel 1999). Pairing the sugar stimulation with an odor stimulus results in a long-lasting conditioning of the odor stimulus (associative conditioning), which is able to elicit proboscis extension when presented alone (Takeda 1961; Bitterman et al. 1983). Habituation and odor conditioning of the proboscis extension reaction has been shown to depend on the sucrose threshold of the individual bees (Scheiner et al. 1999, 2001b; Scheiner 2004); bees with lower sugar thresholds show a slower habituation, and a stronger and longer lasting conditioning.

In this study we tested sugar responsiveness, non-associative and associative learning of the proboscis extension reaction in uninfested and infested foragers with *V. destructor*. We show that the mite parasitism does not affect sugar responsiveness and associative odor learning, but affects sensitization and habituation of the proboscis extension response.

Materials and methods

Bees and *Varroa* infestation

Experiments were conducted in Würzburg Beegroup using workers from uninfested or infested *Apis mellifera carnica* colonies. Conditioning experiments were conducted in September 2003; habituation in April 2004 and sensitization assays in May 2004.

For the habituation and sensitization experiments foragers were caught when leaving the hive entrance of an uninfested colony. Half of these workers ($n = 20\text{--}40$) were artificially infested with mites and half of them served as a control ($n = 20\text{--}40$). Mites were transferred onto the bees' bodies with a fine brush. After infestation bees were caged for 18–24 h (overnight), a parasitism period sufficient to affect flight behavior of infested bees (Kralj 2004). Control bees were caged separately. Both groups were provided with the same quantity of water and sugar candy made from sugar and honey. On the following day, prior to the experiments, bees were checked for the presence of mites. Workers that lost the mites during caging (about 25%) were excluded from the experiments. Bees were chilled individually on ice until immobilization, mounted into metal tubes and tested 3 h later.

For the olfactory conditioning experiments we used uninfested and infested worker bees from a highly infested colony and uninfested workers from an uninfested control colony. Workers from both colonies were collected from the combs and immediately mounted into the metal tubes and tested 3 h later. Bees that lost the mites during the experiment were excluded from the analysis.

Sucrose response threshold assay

Prior to the different experiments responsiveness to sucrose was determined for each infested or uninfested worker. In the habituation and sensitization experiment bees were offered to drink water ad libitum 1 h before the gustatory test. Bees were examined for PER response by a standard procedure of touching the antenna with a droplet of water and further ascending sucrose solutions of 1, 1.6, 2.5, 4, 6.3, 10, 16, 25, 40% (weight/volume) chosen according to Scheiner et al. (2001a) to fit a logarithmic scale. The inter stimulus interval was 5 min to prevent sensitization effects between successive stimulations. The number of proboscis extensions to water and increased sucrose solution was determined as gustatory response scores. The maximum score was 10. Bees that could not extend their proboscis or did not respond even to the highest

sugar concentration were excluded from the experiment.

Habituation assay

Bees that did not respond to 40% sucrose stimulation were discarded from the experiment (uninfested bees $n = 20$, infested bees $n = 15$). The right antenna was continuously touched every 3 s with 40% sucrose solution to habituate the PER response. The criterion for habituation was the total number of PER responses per bee until three successive stimulations occurred without response. Bees that did not show dishabituation after stimulation of the contra lateral antenna with 40% sucrose immediately after habituation were discarded as unfit from the experiment. Habituation expressed as habituation scores was determined for 95 uninfested and 85 infested foragers.

Sensitization assay

Both antennae were stimulated with 40% sucrose solution simultaneously. Workers that did not extend the proboscis to the sugar solution were discarded from the trial (uninfested bees $n = 18$, infested bees $n = 23$). An odor (Neroli, flower odor extract from *Citrus aurantium*, Primavera, Life, 1:1,000 diluted in mineral oil) was applied to bees with the syringe 10 s or 1 min, respectively, after the sucrose stimulation. Bees that did not respond to the odor were tested for the proboscis extension subsequently by applying 40% sucrose solution on antennae again. Those that did not show a PER to this stimulation or had extended proboscis prior to the experiment were discarded from the experiment. Sensitization was scored as the proportion of bees showing a proboscis extension to the odor stimulation. Tests included 138 foragers (80 uninfested, 58 infested) which were tested 10 s, and 144 foragers (74 uninfested and 70 infested) which were tested 1 min after sugar stimulation.

Olfactory conditioning tests

In the conditioning experiment each bee was given one conditioning trial using Neroli as an odor conditioning stimulus. Each bee was first exposed to continuous air stream for 45 s to adapt antennae to the mechanical stimulation. The conditioning odor was pulsed into the continuous air stream for 2 s. Following the odor stimulation the bee was rewarded for 3 s with 40% sucrose solution. The response to odorant was tested 1 and 12 min after the conditioning trial. Responses 1 min after conditioning are mainly due to

sensitization effects, and responses 12 min after conditioning are mainly due to associative processes (Menzel 1999). Each bee was tested only for responses at one time point. Bees that did not respond to the odor stimulus were subsequently stimulated with 40% sucrose solution to test whether the proboscis extension remained intact. Bees that did not respond to sucrose stimulation were excluded from the experiment. The response was tested in 49 infested and 101 uninfested workers after 1 min, and in 50 infested and another 101 uninfested workers 12 min after sugar stimulation.

Results

Gustatory responsiveness

The response of bees to sucrose increased with the increasing concentration regardless of infestation (Fig. 1). The median gustatory response scores of infested workers did not differ from those of uninfested workers in all three separate experiments (habituation, sensitization and conditioning PER response 1 and 12 min after learning trial, Mann–Whitney U tests, $P > 0.05$, Table 1). However, gustatory response probabilities strongly varied between experiments and were much higher in the experiments performed in fall (conditioning) compared to those in spring (habituation and sensitization) and they even differed between experiments done in April and May (Mann–Whitney U tests, $P < 0.005$).

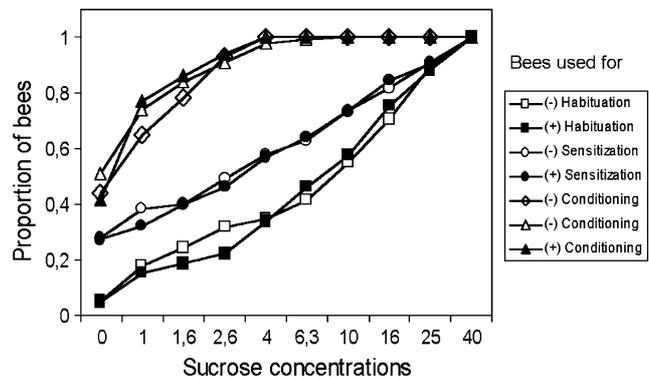


Fig. 1 Gustatory responsiveness expressed by the proportion of positive responses of uninfested (–) and infested honey bees (+) to water and sucrose solutions in increasing concentrations for bees used in the experiments of habituation, sensitization and conditioning. Bees sampled from the same colony at the same time are marked with a same symbol. Numbers of infested bees: 85 (habituation) and 128 (sensitization). Numbers of uninfested bees: 95 (habituation) and 154 (sensitization)

Table 1 Gustatory response scores of uninfested (–) and infested (+) workers in conditioning experiments using infested and not infested colony (c), habituation and sensitization experiments

	Infestation	Gustatory scores			
		Median	Min.	Max.	N
Habituation	+	4	1	10	85
	–	4	1	10	95
Sensitization 10 s	+	7	1	10	58
	–	6	1	10	80
Sensitization 1 min	+	6	1	10	70
	–	7	1	10	74
Conditioning 1 min	+	9	6	10	49
	– (infested c.)	10	5	10	50
	– (uninfested c.)	9	6	10	51
Conditioning 10 min	+	9	6	10	50
	– (infested c.)	9	4	10	50
	– (uninfested c.)	9	6	10	51

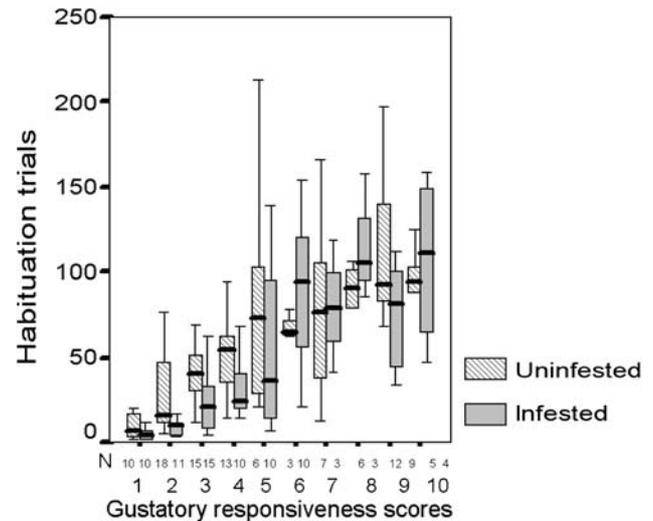
Habituation test

The degree of habituation, expressed by the number of stimuli needed until proboscis extension ceases, was positively correlated to gustatory responsiveness scores in both the infested (Spearman correlation, $r = 0.676$, $P < 0.01$, $n = 85$) and uninfested workers (Spearman correlation, $r = 0.746$, $P < 0.01$, $n = 95$).

Habituation was affected by the mite infestation. Infested bees showed a faster habituation of the proboscis extension reaction to continuous stimulation with sucrose than uninfested bees (Fig. 2, t test on residuals of univariate ANOVA for levels of gustatory responsiveness, $t = 2.425$, $df = 178$, $P = 0.016$). The average number of habituations trials was 47 for infested bees and 57 for uninfested bees. The difference between infested and uninfested bees was more pronounced and significant only in bees with lower gustatory responsiveness. Infested bees with the gustatory scores between 1 and 5 habituated significantly faster compared to uninfested bees, while this was not the case for the bees with gustatory responsiveness levels between 6 and 10 (t test on residuals of univariate ANOVA for levels of gustatory responsiveness, $t = 3.375$, $df = 116$, $P = 0.001$ and $t = 0.129$, $df = 60$, $P = 0.898$, respectively).

Sensitization

Sensitization was affected by gustatory responsiveness; individuals with lower sugar response thresholds had a higher response probability to an odor stimulation in both the infested and uninfested groups (Spearman correlation: $r = 0.485$, $P < 0.01$, $n = 128$; $r = 0.449$, $P < 0.01$, $n = 154$, respectively).

**Fig. 2** Habituation trials (= number of trials to habituate PER) in infested and uninfested foragers with respect to gustatory responsiveness scores. The chart indicates medians, inter quartile ranges, 10 and 90% percentiles. Numbers of tested bees are indicated below the bars

Sensitization was influenced by the mite parasitism. Bees infested with *V. destructor* showed a lower response probability to an odour stimulus than non-parasitized individuals with the same level of gustatory responsiveness. This was significant when the odour stimulation followed the sucrose stimulation after 10 s, and was lower at each level of sucrose responsiveness (Fig. 3a, $P < 0.002$, Matched pair sign test). Overall, out of the total of 80 uninfested bees 43 (53.7%) responded to the odor stimulation while from 58 infested bees only 21 (36.2%) responded ($\chi^2 = 4.16$, $df = 1$, $P < 0.041$). Dividing the bees in groups of high and low gustatory scores showed that the difference in sensitization is based mainly on the bees of low gustatory responsiveness (levels 1–5: $\chi^2 = 4.54$, $df = 1$, $P = 0.033$; levels 6–10: $\chi^2 = 2.16$, $df = 1$, $P = 0.142$).

In both experimental groups, the response probability to an odour declined within the first minute after sugar stimulation. Thirty-two out of the 74 uninfested bees (43.2%) and 20 out of the 70 infested bees (28.6%) responded to the odour stimulus. The difference in the response probability between infested and uninfested bees was almost significant (Fig. 3b, $\chi^2 = 3.36$, $df = 1$, $P > 0.067$).

Olfactory conditioning test

A difference in PER response between infested and uninfested bees was found 1 min after conditioning. The proportion of PER response in the three groups was inhomogeneous ($G = 15.75$, $df = 2$, $P < 0.0005$),

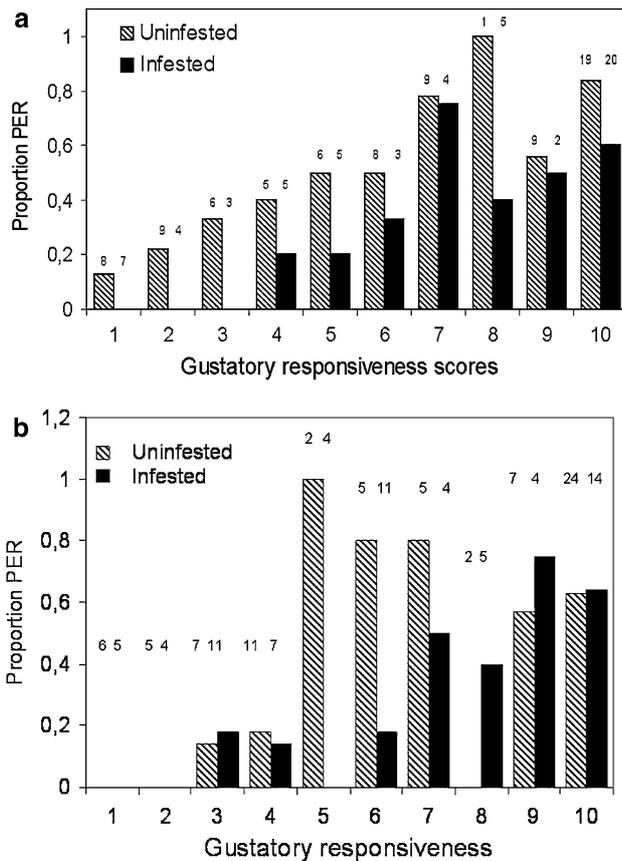
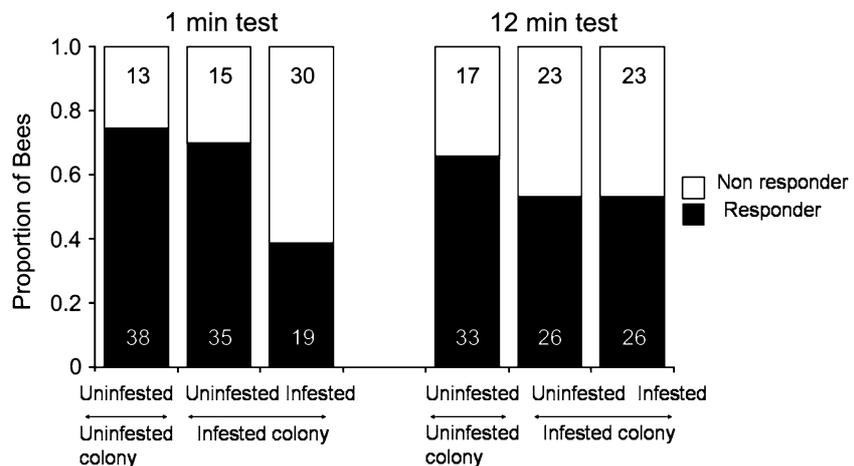


Fig. 3 Sensitization response (= proportion of PER response to an odor) 10 s (a) and 1 min (b) after sugar stimulation in uninfested and infested bees. Bees are grouped according to gustatory responsiveness scores. Numbers of tested bees are given above the corresponding bars

and the difference between infested and uninfested bees either from the same infested colony or the other uninfested colony was highly significant (same colony: $G = 9.90$, $df = 1$, $P < 0.005$; different colony: $G = 13.32$, $df = 1$, $P < 0.001$, alpha level adjusted for two planned comparisons). In contrast, no difference was found

Fig. 4 Response probabilities to an odor stimulus 1 or 12 min after one conditioning trial for uninfested and infested bees. Control included uninfested workers from the uninfested colony. Numbers of bees corresponding to CS stimuli are indicated on the bars



between the groups of bees when tested 12 min after conditioning (Fig. 4). Although not significant, both bee groups from the highly infested colony show a slight reduction in the 12 min response compared to the bee group from the non-infested hive.

Discussion

Gustatory responsiveness

The positive relationship between increasing sucrose concentrations and proboscis extension response concurrently demonstrated by other researchers (Page et al. 1998; Pankiw and Page 1999; Scheiner et al. 2004) was not affected by the mite parasitism. The majority of infested and uninfested bees responded to the higher sucrose concentrations, while fewer were sensitive enough to respond to lower concentration of sucrose. However, the sucrose response thresholds strongly differed between the time the experiments were done, which corroborates the findings by Scheiner et al. (2003), who for the first time demonstrated seasonal variation in sucrose response thresholds.

Influence of gustatory responsiveness on non-associative learning

The level of non-associative learning was positively affected by increased gustatory responsiveness. Bees with low sucrose responsiveness showed more rapid habituation and were less responsive to odor stimulation in the sensitization test in both infested and uninfested group. This is consistent with Scheiner (2004) and shows that bees with high gustatory responsiveness need a higher number of stimulations until they habituate.

Influence of infestation on non-associative and associative learning

The results show that actual infestation with *V. destructor* (i.e. Varroa feeding) leads to a reduction in non-associative learning performance. In particular, mite infested foragers showed a faster habituation and a lower degree of sensitization than uninfested foragers with similar gustatory responsiveness.

Effects of the mite on sensitization were marked when the odor was presented 10 s after sugar stimulation but less pronounced 1 min after sugar stimulation. The lower difference in the response probability after 1 min might be due to the general decline of sensitization within the first minute after sugar stimulation (Menzel 1999).

Sensitization and habituation experiments also indicate that effects of mite infestation are more pronounced in bees with lower gustatory response thresholds. As these bees are already less responsive to environmental stimuli like flower odors and pollen (Scheiner et al. 2004) and poorer learners than bees with high sucrose thresholds (Scheiner et al. 1999, 2001a), we assume a synergistic effect for both conditions on the behavioral and physiological level. This could mean that bees weakened by other factors might strongly react to the parasitism, while not afflicted bees might be able to compensate negative effects of the mite on sensory responsiveness.

In the conditioning experiments, a difference between infested and uninfested foragers was observed in the 1 min, but not in the 12 min retrieval test. As the early short-term memory 1 min after conditioning is still dominated by sensitization, the effect of infestation on conditioning might be restricted to the non-associative components. Interestingly, the effect of infestation on the response probabilities in the 1 min retrieval test was more pronounced than that found in the 1 min sensitization test. The main difference between both experiments was a different exposure to the mites. In the conditioning test we used bees from a heavily infested colony (i.e. about every 10th bee was infested with a mite), whereas in the sensitization test we used bees from uninfested colonies that were artificially infested with mites for about 20 h. Thus, although artificial infestation for a short time period is sufficient to demonstrate an effect of mite infestation on non-associative learning; the effects caused by natural infestation are probably stronger (Kralj 2004).

In contrast to non-associative learning, there were no differences between infested and uninfested bees in associative learning. The differences in PER response vanished 12 min after conditioning indicating that

V. destructor influences early and not later stages of learning processes, the latter at least not at levels detectable in this experiment. As bees in conditioning experiments conducted during fall were in general more responsive to sucrose than those in experiments testing non-associative learning conducted during spring, possible differences between infested and uninfested bees might not have been detected. The slight reduction in associative response between workers from the highly infested colony compared to the workers from the uninfested colony might be due to additional stress factors like secondary infections, upregulation of the immune response (Mallon et al. 2003; Ridell and Mallon 2006) or other causes such as decline in temperature during pupal development (Tautz et al. 2003) in highly infested colonies.

Infestation by the mite could operate through unspecific physiological interactions, such as energy deficiency (Contzen et al. 2003) and eliciting an immune response (Mallon et al. 2003). However, *V. destructor* might also induce specific physiological changes. Recently it has been reported, that pupae and emerging workers infested by *V. destructor* showed suppressed antimicrobial peptides expression (Gregory et al. 2005; Yang and Cox-Foster 2005).

Honeybee behavior in natural context

The demonstrated deficits in non-associative learning forms show that short-term mite infestation can affect behavioral responses to stimuli. The facts that the sucrose response thresholds did not differ between infested and uninfested bee groups, but the sensitizing and the habituating function of the sucrose stimulus did suggest that mite infestation might affect primarily modulatory processes rather than stimulus perception. Effects of mite infestation should thus be more general and not limited to the specific behavior tested. Although the results of our experiments do not allow any conclusions about which processes necessary for proper orientation and navigation are affected by mite infestation, they support the general idea that mite infestation alters the bee's behavior by interfering with neural processes.

Kralj and Fuchs (2006) have discussed the possibility that the reduced ability of foragers infested by *V. destructor* may result from natural selection. A benefit for the mite would be that the infested foragers have a reduced ability to find their own colony and thus have higher drifting rate (Sakofski 1990). The benefit for the bees would be reduced infestation due to loss of infested bees (Kralj and Fuchs 2006). Both, the parasite and host thus might benefit through deficiency in

sensory and/or neural processing. As *V. destructor* is relatively recent parasite of western honey bees and specific adaptations could not be expected, lowered neural abilities are likely to be a more general response of diseased forager bees.

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