

# The glass is not yet half empty: agitation but not *Varroa* treatment causes cognitive bias in honey bees

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**Abstract** Honey bees (*Apis mellifera*) are prone to judge an ambiguous stimulus negatively if they had been agitated through shaking which simulates a predator attack. Such a cognitive bias has been suggested to reflect an internal emotional state analogous to humans who judge more pessimistically when they do not feel well. In order to test cognitive bias experimentally, an animal is conditioned to respond to two different stimuli, where one is punished while the other is rewarded. Subsequently a third, ambiguous stimulus is presented and it is measured whether the subject responds as if it expects a reward or a punishment. Generally, it is assumed that negative experiences lower future expectations, rendering the animals more pessimistic. Here we tested whether a most likely negatively experienced formic acid treatment against the parasitic mite *Varroa destructor* also affects future expectations of honey bees. We applied an olfactory learning paradigm (i.e., conditioned proboscis extension response) using two odorants and blends of these odorants as the ambiguous stimuli. Unlike agitating honey bees, exposure to formic acid did not significantly change the response to the ambiguous stimuli in comparison with untreated bees. Overall evidence suggests that the commonest treatment against one of the most harmful bee pests has no detrimental effects on cognitive bias in honey bees.

**Keywords** Proboscis extension reflex · Olfactory conditioning · Formic acid · Appetitive learning · Aversive learning · Invertebrate emotion

## Introduction

Cognitive bias is defined as the altered information processing resulting from background emotional state of an individual (Kloke et al. 2014). In humans, it is a well-known phenomenon that negative internal affective states result in enhanced attention to threatening stimuli and rendering it more likely to judge ambiguous stimuli more negatively (Eysenck et al. 1991; Matt et al. 1992; MacLeod and Byrne 1996; Williams et al. 1996; Armstrong and Olatunji 2012). Recently, similar cognitive biases have been observed in a number of different animal species indicating that the judgment of ambiguous stimuli depends upon the internal affective state (for an overview and methodological considerations see Gygas 2014). Thus, tests for cognitive bias have been suggested as a promising new indicator of animal emotion. Although it is debatable whether or not the concept of emotion applies to invertebrates (Sherwin 2001; Mason 2011; Mendl et al. 2011; Horvath et al. 2013), it has already been shown that putatively negative valenced lifetime events indeed affect information processing in western honey bees (*Apis mellifera*): Bateson et al. (2011) used two neutral, olfactory stimuli in differential conditioning experiments in worker honey bees. Half of the bees were shaken simulating a predator attack. Such agitated bees were shown to be prone to react to an ambiguous stimulus (a blend of the two odorants) as if they expected a punishment. Thus, agitating bees causes a negative cognitive bias that was suggested to indicate that honey bees exhibit emotions (Bateson et al. 2011).

Vigorous shaking of honey bees can reasonably be considered to simulate a predator attack, triggering a response based on dramatically increased motivation for defensive behavior, while the motivation to gather food is

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reduced simultaneously. Beyond attacks by large predators, western honey bees are threatened by numerous pathogens and parasites (Genersch et al. 2010). While the most prominent bee pest, *Varroa destructor*, might not infect all individuals, the treatment against it includes procedures that are applied to the whole hive and which might similarly affect motivational states of the bees.

The ectoparasitic mite *V. destructor* was accidentally introduced to *Apis mellifera* by host switching from the eastern honey bee (*Apis cerana*) around 50 years ago (Anderson and Trueman 2000). As of now, *V. destructor* has spread almost worldwide and is known to cause significant winter losses of bee colonies (Genersch et al. 2010). Since untreated colonies die after 2–3 years (Rosenkranz et al. 2010), pharmaceutical products have to be administered to the bees. One of the most common treatments is the application of formic acid (Rosenkranz et al. 2010 and references therein).

Formic acid is the simplest organic acid, and due to its small molecular size, it readily evaporates at room temperature. Once in vapor form, it can be used as a fumigant within the hive to kill *V. destructor*. Dissolving formic acid to 60 % and evaporating approximately 200 ml of this solution for 1–2 weeks in two subsequent applications interspersed by 3–4 weeks is a common and effective *V. destructor* mite treatment (Calderone 2000; Underwood and Currie 2003; Rosenkranz et al. 2010). Although the mechanisms of action of formic acid on *Varroa destructor* mites are not entirely understood, it is known that it results in inhibition of respiratory functions and acidification of body fluids (Bolli et al. 1993). Naturally, such a treatment does not only affect the mites but also the honey bees. It is common beekeepers' knowledge that the bees avoid contact with evaporators and audibly increase buzzing. Although a standard formic acid treatment does not kill the bees, in case of an overdose colonies leave through the hive entrance, apparently fleeing from the toxic vapor. In addition, queens may reduce or stop egg laying as a consequence of formic acid treatments (Satta et al. 2005) and it has been shown that *pkac*, a detoxification gene, was significantly upregulated in worker bees after being exposed to formic acid (Boncristiani et al. 2012). Hence, it can be assumed that a formic acid treatment is experienced negatively and thus possibly affects future expectations of honey bees.

In order to investigate whether the standard treatment against *Varroa destructor* mites causes a cognitive bias (CB) in bees, we conducted a series of experiments deploying the proboscis extension response (PER), a classical conditioning paradigm as a basis for further testing. We modified a published version (Bateson et al. 2011) of a cognitive bias test based on other literature records (Harding et al. 2004; Gyga 2014; Kloeke et al.

2014; Bethell 2015) and our own experience. In the process of optimization of the learning protocol, we tested honey bees on reversal of odor reward and odor punishment combination, and different numbers and sequential variations of conditioning trials. Thus, another aim of this study was to present findings from evaluation and the final application of the modified version of the procedure to test CB in honey bees in addition to investigating whether formic acid affects future expectations of honey bees.

## Materials and methods

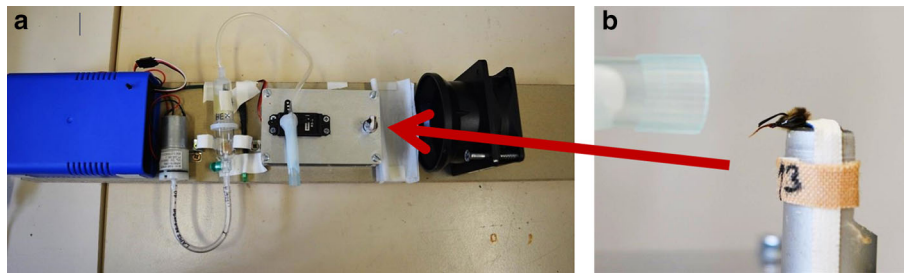
### Honey bees

All honey bees (*Apis mellifera*) were collected from a single colony that was kept in our apiary in Osnabrueck, Germany, and was headed by a naturally mated queen. Thus, all sampled bees had the same mother and little genotypic variability, which is desirable as high genotypic variability might increase behavioral variance (Giray and Robinson 1994). All honey bees were worker bees collected at the hive entrance when they returned to the hive. Only bees that did not forage for pollen were used in the experiments. The bees were collected in the afternoon the day before they were conditioned.

### Classical conditioning of bees and general setup of cognitive bias tests

Excluding preliminary experiments, for this study 972 bees were collected in vials of 22 mm diameter and chilled on ice in order to facilitate handling (Scheiner et al. 2013). Immobilized bees were fastened in aluminum tubes using adhesive tape that was shielded with a thin plastic strip to prevent sticking on the bees (see Bitterman et al. 1983). As we could not judge satiety of the bees, all individuals were fed a 30 % sucrose solution until they stopped consuming the food. The sucrose solution was provided using a syringe (Braun, Inkjet<sup>®</sup>, 2 ml with Braun Sterican<sup>®</sup> Gr. 20, Ø 0.40 × 20 mm). After feeding, bees were left untouched until the next day in order to increase motivation for food reward.

1-Hexanol and 2-octanone (≥98 % purity, Sigma-Aldrich, Germany) were used as odorants for conditioning. These substances were successfully used previously in olfactory classical conditioning experiments (e.g., Guerrieri et al. 2005; Wright et al. 2008; Bateson et al. 2011). We developed an apparatus for training and testing the bees (Fig. 1). Three microliters of one of the two odorants was applied to a piece of filter paper that was placed in a modified drop chamber of an infusion set. An



**Fig. 1** Apparatus used **a** consists of an electronic pump that blows odorized air to a bee mounted in a fixed distance. On initiation of each trial, the pump is started and a servo motor brings the air stream through an inverted pipette tip close to the bee (**b**). The stimulus is

presented for 5 s. The experimenter touches the antennae of the bee with a droplet of either a rewarding sucrose solution or a punishing NaCl solution

electric air pump was attached via a plastic tube to one side of the chamber, and a reversed pipette tip was connected to the tube on the other side of the chamber. The pipette tip was mounted on the arm of a servomotor allowing pointing the odorized air stream to the bee and away from the bee without interference by the experimenter. The aluminum tube containing the bee was placed 1 cm from the final position of the pipette tip prior to initiating the air stream and the movement of the servomotor. This apparatus allowed presenting the odor stimuli in a standardized and repeatable way. Each odor was presented to a bee for a total of 5 s. An acoustic signal was given by the apparatus after 3 s in order to remind the experimenter to present the unconditioned stimulus (US). The US was a 30 % NaCl solution as a positive punishment (according to R. Scheiner, pers. comm.; Wright et al. 2008) and a 30 % sucrose solution as a positive reinforcement. The US was provided to the bee by touching the antennae with a droplet of the solution. If the bee fully extended the proboscis (proboscis extension reflex; PER), it was rated as a positive reaction and the bee was allowed to consume a small amount of the solution (see Bitterman et al. 1983). A missing PER was noted as a negative reaction. If the bee had not shown a clear positive reaction, e.g., an incomplete PER, the reaction was considered as an intermediate reaction but excluded from further analysis.

Initially, we tested whether or not the different odorants were equally suitable for reinforcement or punishment. According to the results of this experiment 1 (see below) in the two subsequent experiments, 2-octanone was positively reinforced (CS+) and 1-hexanol was conditioned to punishment (CS−). The whole olfactory conditioning procedure consisted of 16 trials in a pseudorandom order (ABBABAABAABBABAB, A = CS+, B = CS−). The number of 16 trials was based on results from preliminary tests which revealed a higher proportion of successfully trained bees compared with 12 trials as used by Bateson et al. (2011). Additionally, our preliminary tests revealed

that a higher proportion of bees learned to differentiate between CS+ and CS− if the first trial was a CS+, while there was no significant effect of the last training being CS+ or CS−. A bee was considered a successful learner if she survived the complete testing regime and reacted as expected in the last four trials toward the odors. Additionally, the bee had to show at least once a positive reaction toward the salt solution prior to the correct choices, in order to ensure knowledge about the character of the punishment. Only bees which met these criteria were used in the cognitive bias trials in which a mixture of the positive and the negative conditioned odors in the ratio of 2:1, 1:1, or 1:2 was presented. There was no reinforcement or punishment in the cognitive bias trial, but a syringe with water was presented for consistency of the testing procedure. Each bee was tested only once and with only one mixture in order to avoid confounding effects of previous mixtures on the reaction toward the ambiguous stimuli. Although this restriction reduces the number of individuals that could be tested for each mixture, the overall accuracy was thought to be increased by testing bees that were naïve with regard to ambiguous stimuli. After being tested in the cognitive bias trial, the bees' reactions toward pure 1-hexanol and 2-octanone were tested again to confirm the initial conditioning. As in the cognitive bias trial, a syringe filled with water was used instead of reinforcement or punishment.

We investigated the impact of three factors on cognitive judgment bias. First, we interchanged the two odorants with regard to the kind of reinforcement. Secondly, we agitated bees through shaking, and as a third factor, we tested bees subjected to formic acid treatment. Bees not agitated through shaking and without formic acid treatment served as a control group. There were no bees that received a formic acid treatment and were also shaken. Bees were released after the experiment, but they were marked with a red color pen (UNI Posca PC5M) in order to exclude recapturing the same individuals at the hive entrance.

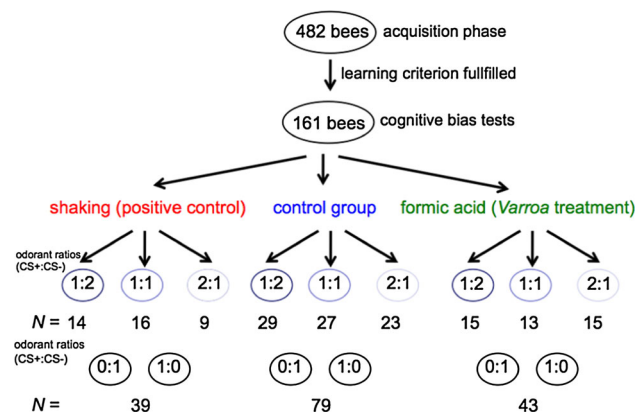
## Experiment 1: interchanging odors and unconditioned stimuli

To investigate whether learning and the subsequent cognitive bias test is influenced by the combination of odor and conditioned stimulus, we interchanged 2-octanone as being rewarded and 1-hexanol being punished for the opposite combination (2-octanone as CS<sup>-</sup> and 1-hexanol as CS<sup>+</sup>) while keeping everything else equal. The number of bees trained with 1-hexanol as CS<sup>+</sup> was 259, and the number of bees tested with 2-octanone as CS<sup>+</sup> was 268. As shown in the results of experiment 1 and discussed below, only bees treated with 2-octanone as CS<sup>+</sup> were found to be suitable for all subsequent experiments.

## Experiment 2: cognitive bias in response to agitation and formic acid treatment

Agitating of the bees was achieved in a similar way as in Bateson et al. (2011). Three to five minutes after the 16 learning trials, half of the still restrained bees were put into an Eppendorf Thermomixer at room temperature and shaken at a frequency of 1200 rpm for 60 s. Of the 268 bees that were treated with 2-octanone as CS<sup>+</sup> in experiment 1, only those bees fulfilling the learning criterion (see above) were either shaken or used as controls. The cognitive bias test was performed immediately after shaking.

In addition to shaking, a total number of 259 bees were tested with regard to possible effects of an in-hive formic acid treatment on CB. The treatment was conducted as part of the yearly bee keeping routine which includes two formic acid exposures for 2 weeks interspersed by 4 weeks. A 60 % formic acid solution (the concentration legally approved in Germany as a treatment against *V. destructor*) was constantly evaporating from Nassenheider evaporators during 14 days. There was one vertical evaporator per box of the hive. This treatment resulted presumably in a maximum concentration of about 500 ppm of formic acid in the hive (Charrière et al. 1992; Bolli et al. 1993; Ostermann and Currie 2004). Tests were performed in autumn during the second formic acid treatment with 119 bees. The control group consisted of 140 bees that were tested 1 week prior to the formic acid treatment. Control groups of shaking and formic acid treatment were pooled for further model analysis as they did not differ significantly with regard to PER following different odor combinations. Of the total number of 527 bees, 45 did not survive. Noteworthy, the mortality rate was lower than the natural mortality rate of forager bees (Dukas 2008). The numbers and group sizes are summarized in Fig. 2.



**Fig. 2** Summary of experimental setup showing sample sizes of bees in the acquisition phase (16 learning trials) and the cognitive bias test. The three treatment groups of experiment 2 each comprised two pure odors which were presented to all bees, while each bee experienced only one out of three different ambiguous odor ratios

## Experiment 3: in-cage formic acid treatment

In order to assess whether a higher concentration of formic acid might cause a cognitive bias, we additionally carried out a formic acid treatment of bees in cages. Control group bees ( $N = 94$ ) and treated bees ( $N = 92$ ) were kept overnight in closed plastic boxes (1.62 L) after they had been collected, restrained, and fed as described above. These bees were taken from the same colony but were not included in any of the other experiments. The treatment group box contained a filter paper to which four microliters of formic acid (98–100 %, pure Food grade, AppliChem, Germany) was applied. In the box containing the bees of the control group, a filter paper was sprinkled with four microliters of water. Since the formic acid completely evaporated, the bees were exposed to about 1300 ppm of formic acid. Both groups of bees remained in the boxes for more than 14 h whereupon the bees were trained as described above. There were also 16 trials in the classical conditioning procedure in a slightly modified pseudorandom order (AABABAABBABBABAB, A = CS<sup>+</sup>, B = CS<sup>-</sup>). The cognitive bias trial, however, was carried out with a 1:1 ratio of the two odors only.

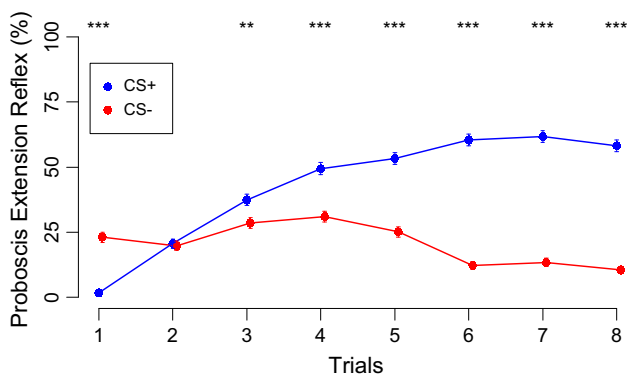
## Statistical analysis

The effect of odor type on conditioning and subsequent cognitive bias trials was analyzed using tests of equal or given proportions (Chi-square based). The same tests were used to analyze general learning performance during the acquisition phase by comparing the proportion of bees showing PER for each pair of ascendingly ordered CS<sup>+</sup> and CS<sup>-</sup> trials. The effects of formic acid and shaking, as well as the composition of the conditioned stimuli, were

analyzed using generalized linear mixed models (see Gygas 2014). In our models, we used composition of the odors (0:1, 1:2, 1:1, 2:1, 1:0 for experiment 2; 0:1, 1:1, 1:0 for experiment 3) and treatment (shaking, formic acid, control) as fixed effects. The IDs of the individual bees were included in the model as a random effect. We additionally compared models with different factor combinations using the Bayesian information criterion (BIC). This helps to assess whether the evidence favors certain factors to have no effect. Post hoc analysis was done using proportion tests. Data analysis and graphics were compiled using the statistical software “R” version 3.2.2 (R Core Team 2015).

## Results

For demonstrating the general suitability of our learning paradigm, we analyzed the acquisition rate of 482 bees that received 2-octanone as CS+ and 1-hexanol as CS−. The discrimination between CS+ and CS− was obvious after 3 positive and 3 negative trials with a significantly higher proportion of bees showing PER in response to CS+ compared with the proportion of PER shown in response to CS− (proportion test,  $\chi^2 = 8.17$ ,  $df = 1$ ,  $P = 0.0043$ ; Fig. 3). The percentage of animals showing a correct choice in both CS+ and CS− after three trials each was at 15 % and increased to 47 % correct choice in the last CS+ and CS− trials. The overall success rate according to our learning criterion of correct choices in the last four trials was at 33 % (161 of 482 see below) which is well above



**Fig. 3** Acquisition phase. Bees were trained to expect a reward when an odor stimulus consisting of 2-octanone was presented as CS+. Expectation of reward was measured as proboscis extension reflex (PER). The same bees were trained to withhold PER when 1-hexanol was presented as CS−. For ease of illustration, the percentage of bees showing PER  $\pm$  SEM following CS− and CS+ is plotted by the number of the respective CS+ or CS− trial. The first trial was a reward, and thus, the first CS− trial reflects conditioning to the test situation rather than to the specific odor. In trials 3–8, significantly more bees showed PER in CS+ trials. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ , proportion test,  $n = 482$

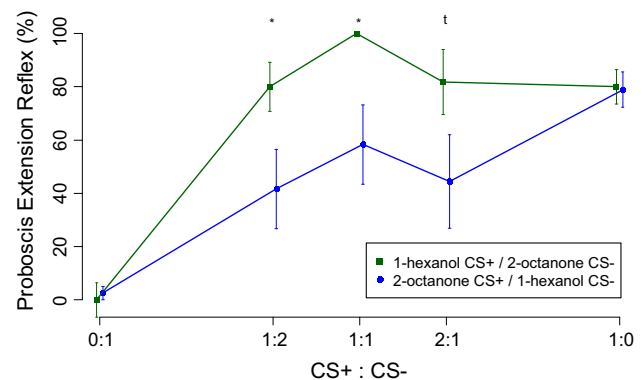
chance level compared with random performance in the last 4 trials (binomial test with probability of success =  $1/16$ ,  $P = 2.2e-16$ ).

## Experiment 1: interchanging odors and unconditioned stimuli

We tested two different combinations with each odor being used as either CS+ or CS−. There was no difference between the percentages of successfully conditioned bees when the different odors were used as pure CS+ or CS− (proportion test,  $\chi^2 = 0.07$ ,  $df = 1$ ,  $P = 0.79$ ). However, in the three ambiguous odor combinations PER was more likely to occur if 1-hexanol was used as CS+ compared with 2-octanone used as CS+ (proportion tests, CS+:CS− (1:2):  $\chi^2 = 4.89$ ,  $df = 1$ ,  $P = 0.027$ ; CS+:CS− (1:1):  $\chi^2 = 4.44$ ,  $df = 1$ ,  $P = 0.035$ ; CS+:CS− (2:1):  $\chi^2 = 3.04$ ,  $df = 1$ ,  $P = 0.081$ ; Fig. 4). These results challenged our assumption of the ambiguous nature of the combined odors as more than 80 % of the bees conditioned to expect a punishment, while smelling 2-octanone showed a PER if any diluted odor of 1-hexanol was present. Consequently, in subsequent experiments 2-octanone was always used as the CS+ and 1-hexanol as CS−.

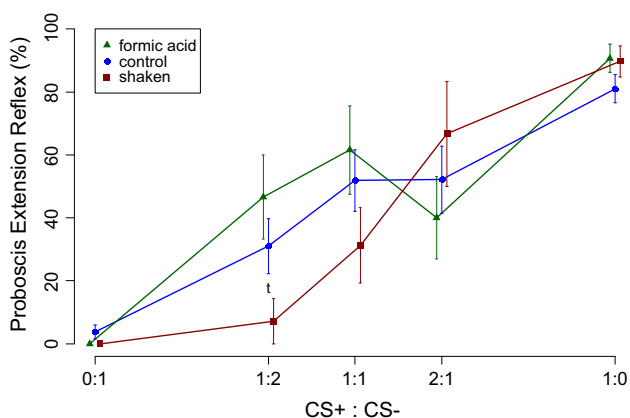
## Experiment 2: shaking and in-hive formic acid treatment

Of the 482 bees which were subjected to conditioning, 383 received at least once a NaCl solution as a consequence of



**Fig. 4** Expectation of reward depending on combination of the odor to CS+ and CS− at different odorant concentrations was measured as percentage of bees showing PER  $\pm$  SEM. The comparison of the two different combinations of CS+/CS− revealed significant differences with 1-hexanol as CS+ rendering proboscis extension reflex significantly more likely even at low concentrations. Expectation of reward was measured as proboscis extension reflex (PER)  $\pm$  SEM. \* $P < 0.05$ ,  $t$ :  $P < 0.1$ , proportion test,  $n$ (2-octanone as CS+, 1:2, 1:1, and 2:1) = 13,  $n$ (1-hexanol as CS+, 1:2) = 20,  $n$ (1-hexanol as CS+, 1:1) = 9,  $n$ (1-hexanol as CS+, 2:1) = 11,  $n$ (1-hexanol as CS+, 0:1, 1:0) = 21,  $n$ (2-octanone as CS+, 0:1, 1:0) = 36

showing a PER in response to CS− which was a prerequisite for further CB testing. Of these 383 bees, 161 fulfilled the learning criterion of correct choices in the last four trials. These bees were confronted with ambiguous stimuli combinations as well as with pure CS+ and CS− odors for verification of successful conditioning. In this verification, 26 bees did not respond as expected in one of the two trials with both pure odors. Figure 5 shows the percentage of bees showing a PER reaction in relation to the amount of CS+ present in the air flow. GLM model analysis revealed significant effects of concentration ( $P = 3.84e-11$ ) and of shaking the bees prior to testing ( $P = 0.032$ ). Effects of formic acid treatment did not reach significance ( $P = 0.537$ ). In addition, we performed a model comparison taking into account different factor combinations in the cognitive bias test. The model that turned out to fit our data best included the ratio of CS+ to CS−, ID as a random factor, and shaking of the bees (BIC = 304.4). An alternative model including CS+ to CS− ratio, ID as random factor and formic acid treatment instead of shaking turned out to be less well fitting (BIC = 335.3). Post hoc analysis revealed a trend that bees that were shaken prior to the cognitive bias task were less likely to extend their proboscis compared with controls at a 1:2 ratio of the CS+ (proportion test,  $n_{\text{control}} = 29$ ,  $n_{\text{shaken}} = 14$ ,  $\chi^2 = 3.02$ ,  $df = 1$ ,  $P = 0.082$ ). The proportion of PER did not differ significantly between shaken bees and controls at 1:1 nor at 2:1 ratio. PER behavior did also not differ between the groups when pure CS+ or pure CS− was presented.



**Fig. 5** Cognitive bias test. The percentage of bees showing PER  $\pm$  SEM was measured in response to different blends of CS+ and CS−. Model analysis revealed significant effects of concentration ( $P < 0.001$ ) and of shaking the bees prior to testing ( $P < 0.032$ ). There was no significant effect of formic acid treatment.  $t$ :  $P < 0.1$ , proportion test,  $n$  (formic acid, 0:1, 1:0) = 43,  $n$  (formic acid, 1:2, 2:1) = 15,  $n$  (formic acid, 1:1) = 13,  $n$  (control, 0:1, 1:0) = 79,  $n$  (control, 1:2) = 29,  $n$  (control, 1:1) = 27,  $n$  (control, 2:1) = 23,  $n$  (shaken, 0:1, 1:0) = 39,  $n$  (shaken, 1:2) = 14,  $n$  (shaken, 1:1) = 16,  $n$  (shaken, 2:1) = 9

### Experiment 3: in-cage formic acid treatment

Bees that were kept in cages and subjected to a high dose of formic acid (about 1300 ppm) immediately before conditioning did not differ from controls in proportion of bees showing PER when confronted with an ambiguous 1:1 mixture of CS+ and CS− (proportion test,  $n_{\text{control}} = 31$ ,  $n_{\text{formic acid}} = 28$ ,  $\chi^2 = 0.16$ ,  $df = 1$ ,  $P = 0.69$ ). In this test, 46 % ( $\pm 9.6$  SEM) of the formic acid treated bees showed PER compared with 52 % ( $\pm 9.1$  SEM) of the control bees. Model comparison revealed the best fit for a model including the ratio of CS+ to CS− and ID as a random factor (BIC = 117.2). An alternative model additionally including the formic acid treatment as a fixed effect was less well fitting (BIC = 124.1).

### Discussion

One-third of the bees were successfully conditioned to two different odors after 16 trials. Literature data suggest a considerable higher number of bees (up to 80 %) to associate successfully an odor with a reward if only one stimulus is used (e.g., Bitterman et al. 1983). However, differential learning procedures including a reward as well as a punishment in the same procedure were previously described to be complex and more challenging to train (Bateson et al. 2011). Given that we applied more demanding learning criteria, our success is in line with literature data on comparable conditioning experiments in honey bees (Smith et al. 1991; Wright et al. 2008). In addition, it is known that time of year can affect learning in honey bees (Scheiner et al. 2003). By means of applying a strict learning criterion selecting only bees that clearly learned, we thought to rule out any such seasonal effects.

Initially, we planned to counterbalance both odors to be used as CS+ and CS−. We knew that both odors are learned equally well and are readily distinguished by honey bees (Guerrieri et al. 2005; Wright et al. 2008; Bateson et al. 2011). Noteworthy, our honey bees indeed learned equally well during the acquisition phase regardless of the odor used as CS+ or CS− (Fig. 2, pure CS+ and CS− ratios) as it is the case with 1-nonanol and 2-hexanone used in an experiment investigating appetitive and aversive olfactory learning in bees (Bos et al. 2014).

However, much to our surprise bees that were trained with 1-hexanol as CS+ were not suitable for subsequent cognitive bias testing due to an unusually high PER rate in all ambiguous odor combinations. The overly positive reaction in the cognitive bias test of these bees could be due to the CS− not being considered aversive enough or the CS+ being so exceedingly rewarding that even a putative punishment was dared. If a false-negative reaction

(no reward due to missing PER following CS+) is more costly than a false positive (punishment due to PER following CS–), the reaction is likely to be shifted to riskier behavior (Leonard et al. 2011). Such a shift might be due to the fact that 1-hexanol is commonly found in natural flower scents such as baneberry, strawberry, hyacinth, rose, and clover compared with 2-octanone which is only described to be found in much more scarcely spread orchids of the genus *Ophrys* (Knudsen et al. 1993) which do not even provide nectar. Thus, bees might have taken priors, either phylogenetically or ontogenetically acquired, into account. Even though we cannot finally decide why 1-hexanol was so attractive, one should keep the different valence of the two odors in mind. This is a vital point to be considered for future studies testing cognitive bias in honey bees.

In line with results from Bateson et al. (2011), shaking the bees did not affect general learning performance indicated by similar PER probabilities following pure CS+ or pure CS–. General learning performance was also not affected by treatment with formic acid, neither in the hive nor at a higher formic acid concentration in a box during the night preceding the experiment.

In experiment 2, we could confirm the results of Bateson et al. (2011) with regard to a pessimistic cognitive bias following vigorously shaking of the bees. A low ratio of CS+ to CS– rendered the shaken bees more likely to be “pessimistic” and withholding PER. Our experimental design differed from that of Bateson et al. (2011) with regard to the number of training trials, using NaCl as an US, and by testing each bee in only one of the ambiguous odor combinations. These changes did not affect the main conclusion of the test, and thus, shaking for 60 s can be considered a reliable means to affect the cognitive bias in honey bees. It was argued that analogously to other studies using cognitive bias to measure emotions (for overviews see Mendl et al. 2009; Gygas 2014; Bethell 2015), honey bees should be considered to be capable of exhibiting negative emotional states (Bateson et al. 2011; Mendl et al. 2011). Our data provide strong evidence supporting the argument of negative emotion-like states in honey bees. However, we also suggest that such states are strongly dependent upon motivation for foraging versus defensive behavior and might only be of short duration. This has to be tested in further experiments, for example by observing whether or not shaking affects CB for an extended period of time. Admittedly, a direct comparison between the formic acid treatment and the shaking would be more comparable if the formic acid stimulus were also presented after the acquisition phase for a short time. However, this would not reflect common beekeeping procedures to treat bees against *Varroa* mites. We refrained from this experiment as our goal was to investigate a potential effect on valence of future expectations under conditions as close as

possible to what bees experience when beekeepers treat their colonies.

Attentional modulation of elemental olfactory learning has been put forward as an alternative explanation for the behavioral change after a negative experience (Giurfa 2013) avoiding the term pessimism. However, notwithstanding that it might be desirable to use language that is not centered around emotion especially in insect behavior, the basic point of altered information processing due to a negative experience remains untouched by invoking attentional modulation as a different description of the phenomenon.

Beehives are attacked by large predators including honey badgers (*Mellivora capensis*) in Africa and striped skunks (*Mephitis mephitis*), black bears (*Ursus americanus*), Virginia opossums (*Didelphis virginiana*), or raccoons (*Procyon lotor*) in the USA (Storer and Vansell 1935). In Europe, attacks by large wild animal predators are rarely seen. However, brown bears (*Ursus arctos*) are repopulating Western Europe and there are a few documented attacks on beehives by brown bears. Any of such attacks will agitate the bees and a shift of priority from foraging or nursing to defense has been positively selected during evolution. We therefore believe that the observed behavior is biologically meaningful as vigorously shaking naturally would require immediate action. As the cognitive bias was measured within a few minutes after agitating the bees, it reflects short-term effects on emotion-like states. It is not known to date how long these effects might prevail. Future studies have to reveal whether or not other aversive treatments are potent for revealing long-term effects on emotion-like states.

We did not observe a pessimistic CB following an in-hive formic acid treatment nor following an in-cage formic acid treatment. The simplest explanation would be that bees are not expecting a higher likelihood of something detrimental happening being more likely after a formic acid exposure. This might be due to adaptation to being exposed to formic acid over a long time, or formic acid might indeed not change the motivational state and future expectations of honey bees at all. Other possible explanations which are not mutually exclusive are that bees are not able to remember the aversive experience long enough or that the bees' cognitive, motivational, and physiological conditions involved in mood are not affected for more than a few hours by an unpleasant treatment.

It is known that bees can recall memories up to 10 days after training (Giurfa and Sandoz 2012), and thus, we think it is unlikely that the memory of the formic acid treatment has vanished. As we collected foraging bees, these animals should have been used to changes in acid concentrations inside and outside the hive. Thus, we would expect that these bees were aware of the in-hive air quality, but this did

not affect their motivational state at the time we conducted the cognitive bias test. As opposed to shaking, a formic acid treatment is not imposing a direct threat and thus does not require taking immediate action. Thus, there might be no fitness consequences of any motivational change related to the in-hive air quality.

Admittedly, the treatment with formic acid was not acute as the shaking was. However, a short-term formic acid treatment would not be a realistic scenario in beekeeping practice. Here we can conclude that there are no long-lasting consequences of formic acid treatment on cognitive bias. Nevertheless, it has not been investigated yet if shaking also causes long-term changes in cognitive bias.

Cognitive biases have been demonstrated in a number of species, mostly in vertebrates. Although the basic experimental design has been described more than 10 years ago (Harding et al. 2004), there are still considerable difficulties in establishing stable, reproducible, and feasible experimental designs that are generally accepted and effectively used. For example, one of the most widely used animal models in current research is the house mouse (*Mus musculus*). However, there is up to now no stable protocol that would allow a reasonable throughput for testing cognitive biases in this species (Kloke et al. 2014). In our experiments, a relatively high number of bees could not be used for testing. However, unlike working with vertebrates, experiments with bees are much more feasible as hundreds of working bees can be easily screened for suitability before being finally tested.

## Conclusion

We presented a working version of a cognitive bias task for honey bees that can be easily applied in future studies. Noteworthy, the odor combination associated with CS+ and CS− is a vital point for successfully conducting CB tests. Our results show that agitated bees were more pessimistic, but formic acid treatment was not capable of shifting cognitive bias. This might be due to the duration of changes in motivational state, and thus, it has to be further evaluated whether any treatment might indeed bring about long-term changes in emotion-like behavior in honey bees.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that there is no conflict of interest with any of the funding agencies of the research reported herein.

**Ethical statement** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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