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Original article

Elemental and non-elemental olfactory learning using PER conditioning in the bumblebee, *Bombus terrestris*

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Abstract – Learning olfactory stimuli and their implications is essential in bumblebees for orientation and recognition of nest sites and food sources. To evaluate associative learning abilities in bees under controlled environmental conditions, the proboscis extension response (PER) assay is a well-established method used in honeybees and has recently been successfully adapted to bumblebees. In this study, we examined the cognitive abilities of workers of the eusocial bumblebee, *Bombus terrestris*, by training individuals in different olfactory learning tasks using classical PER conditioning. We compared learning performance for four different floral odors. Individuals were able to solve absolute (A+) and differential (A+ vs. B−) conditioning tasks, and no differences were found between single odors and odor combinations, respectively. Furthermore, bumblebees performed well on a positive pattern discrimination task (A−, B− vs. AB+), but failed to solve the negative pattern discrimination (A+, B+ vs. AB−). Our results indicate that workers of *B. terrestris* possess elemental olfactory learning abilities, but, in contrast to previous findings in honeybees, fail in more complex tasks, such as negative pattern discrimination. We discuss possible ultimate causes that have led to the difference in learning capabilities between bumblebees and honeybees.

elemental learning / *Bombus terrestris* / proboscis extension conditioning / bumblebee / configural associations

1. INTRODUCTION

Associative learning forms the basis for efficient foraging behavior in bees. It allows bees to relocate specific food sources, efficiently extract pollen and nectar from various flower types, and orientate accurately in time and space (for review see: Menzel and Müller 1996; Dukas 2008). During appetitive conditioning, bees learn to associate a given olfactory, gustatory, visual, or tactile stimulus with a reward (e.g., sucrose solution) by forming an elemental association. In nature, stimuli often appear as multimodal blends rather than single elements. Under these conditions, associative learning involves relational dependencies, i.e., the meaning of a given stimulus varies as a function of its occurrence in combination with other stimuli. The processing and learning of complex stimuli is proposed to be accomplished in two different ways as follows (Rudy and Sutherland 1992): in an *elemental approach*, a complex stimulus is represented by its single elements, in a way that the association between the conditioned stimulus (CS) and the unconditioned stimulus (US) is formed by each element of the CS, i.e., the associative strength of a complex CS equals the sum of the strengths of its elements (elemental learning: Rescorla and Wagner 1972; Couvillon and Bitterman 1988). On the other hand, in a *configural approach*, the

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representation of a complex CS is achieved as an association, which is distinct from that of the sum of the individual elements. Thus, according to the "unique cue hypothesis," the learned multi-component stimulus bears specific information which makes it distinguishable from its single elements (configural learning: Rescorla 1972; Wagner and Rescorla 1972; Pearce 1987; Menzel et al. 1999).

Several studies have investigated the elemental (simple linking of two stimuli or a stimulus and a response) and non-elemental (beyond simple linking; Giurfa 2003) olfactory learning abilities in honeybees (Bitterman et al. 1983; Laloi et al. 2000; Deisig et al. 2002, 2003; Komischke et al. 2003), but only little is known about the capabilities of other (eusocial) bee species, e.g., the primitive eusocial bumblebees. Learning in bumblebees has usually been studied under free-flying conditions (Gumbert 2000; Fauria et al. 2002; Spaethe and Chittka 2003; Raine and Chittka 2008) that allow observations in more natural situations, but has the disadvantage of limited control over interindividual interactions and environmental effects. A method that provides the opportunity of Pavlovian (classical) conditioning under strictly controlled environmental conditions represents the proboscis extension response (PER) assay (Kuwabara 1957; for review see Giurfa and Sandoz 2012). The PER assay is well established in honeybee olfactory learning (Bitterman et al. 1983) and has recently been adapted for bumblebees (Laloi et al. 1999; Riveros and Gronenberg 2009b; Toda et al. 2009). Interestingly, recent findings by Laloi and Pham-Delègue (2004) provide evidence that bumblebees exhibit a strong learning asymmetry when they are asked to discriminate two different floral odors in a differential conditioning assay (Laloi and Pham-Delègue 2004). This effect has been reported in honeybees only when bees have to distinguish between one floral and one pheromonal odor (Sandoz et al. 2001) or between odors of different concentrations (Bhagavan and Smith 1997). Furthermore, honeybees and bumblebees also differ in solving complex learning tasks, with honeybees reaching a higher overall olfactory learning performance (Bitterman et al. 1983; Jakobsen et al. 1995; Laloi et al. 1999)

and acting fast, but more inaccurate in decision making (Morawetz and Spaethe 2012). While honeybees perform remarkably well in solving complex non-elemental learning tasks (Deisig et al. 2001; Giurfa 2007), only little is known about these skills in bumblebees.

In this study, we first aimed to investigate whether asymmetrical discrimination is a general trait in bumblebees when facing different floral odors (perhaps due to different innate biological meanings of the respective odors; Laloi and Pham-Delègue 2004), as it was found in solitary stingless bees (Mc Cabe and Farina 2010). Second, we intended to evaluate the abilities of bumblebees to solve complex non-elemental olfactory learning tasks, e.g., when they have to discriminate stimuli comprising of more than one odor component.

2. MATERIALS AND METHODS

2.1. Animals and setup

Colonies of *Bombus terrestris* (Koppert B. V., Netherlands) were kept in a two-chambered nest box (each chamber was 240×210×110 mm in size) at 25 °C and 70 % relative humidity under a 12/12 h photoperiod. Commercially available Apiinvert (a mixture of sucrose, fructose, and glucose) and pollen was provided ad libitum. Individual workers of unknown age and various sizes (inter-tegula span between 2.7 and 4.6 mm) were captured and chilled on ice for 45 min. They were then harnessed in tubes (7 mm in diameter, 35 mm in length) and fixed with a "yoke" made from a paper clip (adapted from Riveros and Gronenberg 2009b), leaving the head free to enable stimulus perception and proboscis extension. After fixation, individuals were allowed to feed ad libitum on a 0.5 M sucrose solution and were kept for 18 h at 20 °C and 70 % relative humidity. Before the experiment started, individuals were tested for a proper PER by carefully touching the antennae with a toothpick soaked with 0.5 M sucrose solution. Only individuals showing a PER were used for further experiments and treated following a standard protocol for olfactory conditioning in bees (Bitterman et al. 1983; Laloi et al. 1999; Deisig et al. 2001; Riveros and Gronenberg 2009b). Bumblebees were placed one

at a time in front of an exhaust system that ensured the extraction of any residual odor and rested for 20 s to become familiarized. The respective odorants were diluted 1:100 in paraffin oil. This concentration was chosen to avoid habituation or repelling effects and has been used in other studies (Wright et al. 2009; Reinhard et al. 2010; Brill et al. 2013). Five microliter of the diluted odor was applied to a 2×20 mm piece of filter paper and placed in a 20 ml plastic syringe. Odor was applied for 6 s, and, after touching the antennae with the wetted toothpick, 0.5 M sucrose solution was presented simultaneously for 3 s after a delay of 3 s, and the bee was allowed to lick. Afterwards, the bee could rest for another 15 s and was then replaced by the next individual. The used intertrial interval (ITI) was 8 min (see also Bitterman et al. 1983; Deisig et al. 2001; using 8 min ITI; Riveros and Gronenberg 2009b; using 10 min ITI). An extension of the proboscis during the odor presentation and before sucrose delivery was scored as a positive response.

For the CS, we used four different common floral odors (Knudsen et al. 1993) which were also used in earlier studies (1-nonanol (>99.5 %), linalool (>95 %; both Fluka, St. Louis, USA), citral (96 %), and phenylacetaldehyde (>90 %; both Sigma-Aldrich, Taufkirchen, Germany)) (Deisig et al. 2001; Laloi and Pham-Delègue 2004; Hussaini et al. 2007; Dacher and Smith 2008).

2.2. Absolute conditioning

To prove that the four tested odors can be learned to a comparable extent, bumblebees were first trained in an absolute conditioning paradigm. For each odor (citral, 1-nonanol, linalool, and phenylacetaldehyde), two groups of randomly chosen bumblebees were tested. One group was subjected to a forward paired presentation of CS and US, and a control group to an unpaired presentation of the same stimuli. The paired group was trained with ten conditioning trials as described above. Bees of the unpaired group underwent the identical protocol, but with a total of 20 trials, consisting of 10 CS-only trials and 10 US-only trials, presented in a pseudo-randomized order. During CS-only trials, solely the odor was applied for 6 s, whereas during US-only trials, only the sucrose solution was presented for 3 s. With this procedure, an equal number of stimuli presentations in paired

and unpaired groups were assured. To exclude that the bumblebees learned the air flow as the CS, an additional control group was trained to pure paraffin, but did not learn at all (see electronic supplemental material, Fig. S1). The final number of individuals used for the experiments was $N=25\text{--}37$ in the paired group and $N=12\text{--}14$ in the unpaired group.

2.3. Differential conditioning

The bumblebees' ability to discriminate between different floral odors was tested in a differential conditioning paradigm. Using the same protocol as described above, each individual was given ten reinforced conditioning trials with one odor as the rewarded stimulus (S+) and ten unreinforced trials with a second odor (S−), presented in a pseudo-randomized order. In addition, a second group of bees was trained in a balanced manner, i.e., the meaning (which odor was S+ and which S−, respectively) of the same odor stimuli was reciprocally changed. Experiments were done with the odor pairs citral vs. 1-nonanol and linalool vs. phenylacetaldehyde, respectively. The total number of individuals subjected to the experimental procedure was $N=25\text{--}26$ per group.

2.4. Positive and negative patterning discrimination

To study whether bumblebees possess the ability to solve complex learning tasks, two groups of bumblebees were tested in either a positive (A−, B−, AB+) or negative (A+, B+, AB−) pattern discrimination paradigm. In this experiment, the odors citral and 1-nonanol were tested. In the first group (positive patterning group), the presentation of the pure odorants (A−, B−) remained unrewarded, whereas the presentation of the odor mixture (AB+) was reinforced by a sucrose reward. The second group (negative patterning group) was subjected to the same odor presentations, but here, the individual odors were rewarded (A+, B+), and the mixture remained unrewarded (AB−). Each bee underwent 32 trials, consisting of 8 presentations of citral alone, 8 presentations of 1-nonanol alone, and 16 presentations of the mixture. With this procedure, the bees were faced an equal number of CS+ and CS− presentations (for honeybees, see Deisig et al. 2001). The sequence of CS+ and CS− trials was pseudo-

randomized with no more than two repeats of the same stimulus presentation in a row.

2.5. Learning abilities and body size

To test if body size correlates with learning performance, the size of each individual was estimated by measuring intertegulae span (Kapustjanskij et al. 2007). Intertegulae span was correlated with three measures of learning performance: the total number of correct responses for each individual and the occurrence of the first response to the CS+ (in absolute and differential conditioning; Riveros and Gronenberg (2009a)), as well as the discrimination index (DI; in differential conditioning and the patterning discrimination tasks). The DI was calculated as the sum of an individual's responses to the CS+ minus the sum of its responses to the CS− (see Pelz et al. 1996).

2.6. Data analysis

Statistical analysis was performed on the sum of an individual's responses during all conditioning trials. Each individual was ranked according to its number of correct responses (0–9 in experiments 1 and 2; 0–15 in experiment 3). In the first experiment, the ranks of the individuals were compared between the paired and the unpaired group using a Mann–Whitney U test and among different odors using a Kruskal–Wallis test. For the second experiment, the ranks were compared between the responses to the rewarded and the unrewarded stimulus, using a Wilcoxon test. A Wilcoxon test was also applied in the third experiment, where the ranks of the responses to the rewarded and the unrewarded stimulus were compared. Additionally, a χ^2-test was used to compare the number of responders to the CS+ and the CS− in the final conditioning trial (trial number 16) in the third experiment. Correlation of learning performance (measured as total number of correct responses, the occurrence of the first correct response, and as the DI) and body size was tested by a Spearman correlation test.

3. RESULTS

Almost all bees survived the 18 h resting period, and about 30 % of them showed a proper PER when sucrose solution was delivered to the antennae and could be used in the following experiments.
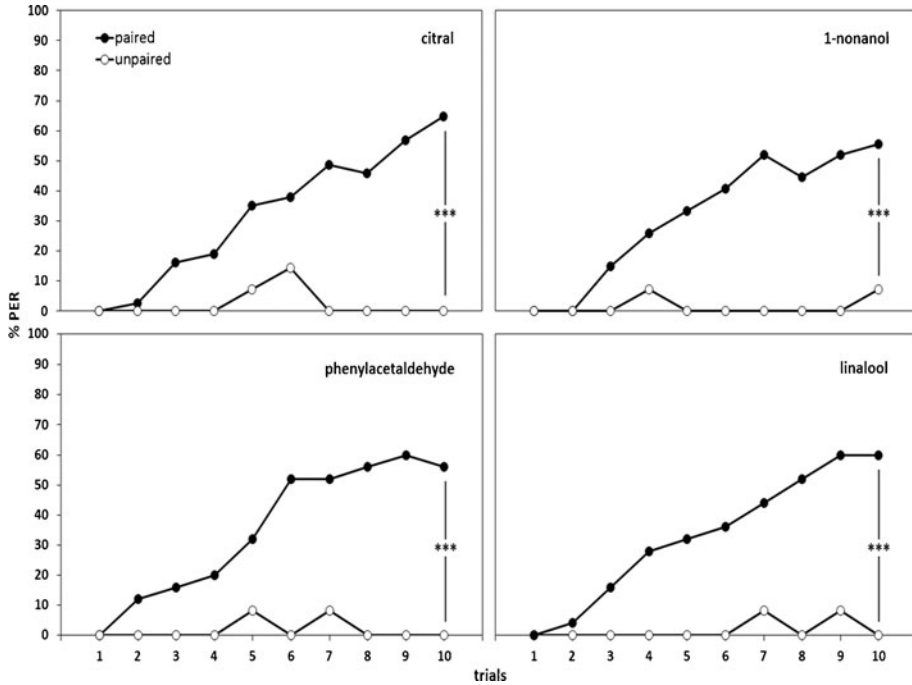
3.1. Absolute conditioning

Bumblebee workers responded significantly more often to the CS odor in the paired than in the unpaired group (citral: $Z=-3.856$, $P<0.001$; 1-nonanol: $Z=-3.947$, $P<0.001$; phenylacetaldehyde: $Z=-3.418$, $P<0.001$; and linalool: $Z=-3.627$, $P<0.001$) and reached a mean response level of ca. 60 % after ten trials (Figure 1). Cross comparison among odors revealed no significant differences in learning performance ($\chi^2=0.13$, $P>0.05$, $N=114$).

3.2. Differential conditioning

Individuals trained with one rewarded (S+) and one unrewarded odor (S−) could significantly discriminate between both stimuli (Figures 2 and 3). Bees responded to the S+ at a similar level observed in absolute conditioning, whereas almost no response to the S− occurred (Figures 2 and 3). Interestingly, bees in all experimental groups learned equally well independent of what odor was presented as the rewarded or unrewarded one (citral(+) vs. 1-nonanol(−): $Z=-3.736$, $P<0.001$, DI=5.4; 1-nonanol(+) vs. citral (−): $Z=-3.850$, $P<0.001$, DI=5.6; linalool(+) vs. phenylacetaldehyde(−): $Z=-3.631$, $P<0.001$, DI=5.4; and phenylacetaldehyde(+) vs. linalool(−): $Z=-3.310$, $P=0.001$, DI=5.4). Learning performance did not differ among the four experimental groups ($\chi^2=1.71$, $P>0.05$, $N=101$). We found no evidence for asymmetrical learning in any of the odor combinations.

### 3.3. Positive and negative patterning discrimination

When bumblebees had to solve the positive pattern discrimination task, they were able to distinguish between the reinforced mixture and the non-reinforced single odorants ($Z=-4.563$, $P<0.001$; on trial 16: $\chi^2=4.08$, $P=0.043$; Figure 4). In contrast, in the negative patterning
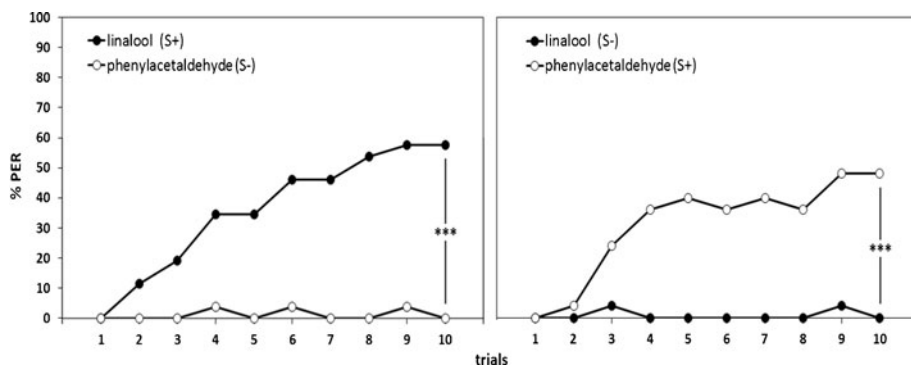
Figure 1. Proboscis extension response (*PER*) during absolute conditioning of four different odors. Bumblebees were trained either paired or unpaired (see Material and methods) with the odors citral (paired: *N*=37; unpaired: *N*=14), 1-nonanol (*N*=27, *N*=14), phenylacetaldehyde (*N*=25, *N*=12), and linalool (*N*=25, *N*=12), respectively. Learning performance did not differ among odors (see text for statistics). ***P<0.001.

discrimination task, bumblebees failed to discriminate between the rewarded single odorants and the unrewarded mixture (*Z*=−1.758, *P*>0.05 and on trial 16: χ^2=0.13, *P*>0.05; Figure 4). None of
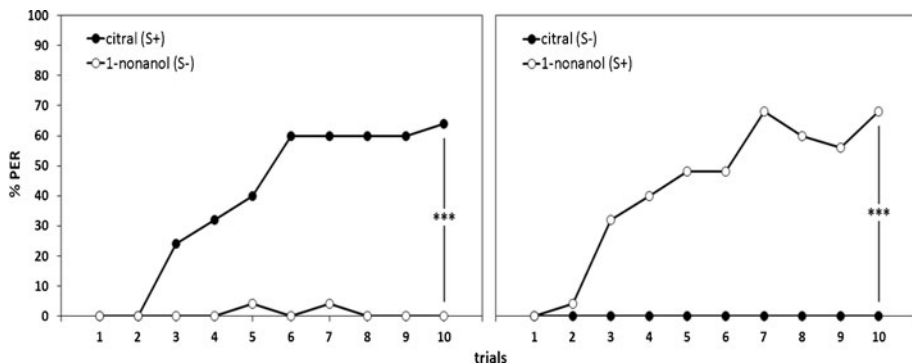
Figure 2. Proboscis extension response across trials of differential conditioning with the odors citral vs. 1-nonanol. On the left, workers (*N*=25) were trained with citral as the rewarded stimulus (S+) and 1-nonanol as the unrewarded stimulus (S−); in the right panel, both odors were interchanged (*N*=25). The learning performance did not differ between both situations (see text for statistics). ***P<0.001.

Figure 3. Proboscis extension response across trials of differential conditioning with the odors linalool vs. phenylacetaldehyde. On the *left*, linalool was the rewarded stimulus (S+) and phenylacetaldehyde the unrewarded stimulus (S−) (*N*=26); in the *right* panel, both odors were interchanged (*N*=25). No significant differences were found between the two groups (see text for statistics). ***P<0.001.

the bees tested performed the task correctly (for all individuals DI was ≤2).

3.4. Learning abilities and body size

A size-effect on the learning performance was observed for two of the tested odors in the absolute conditioning experiment. When trained to 1-nonanol and phenylacetaldehyde, body size correlated positively with the number of responses (1-nonanol: $r_s=0.552$, $P=0.009$; phenylacetaldehyde: $r_s=0.709$, $P<0.001$) and negatively with the first response (1-nonanol: $r_s=−0.433$, $P=0.05$; phenylacetaldehyde: $r_s=−0.765$, $P<0.001$). In contrast, no size effects were found for citral (size vs. number of responses: $r_s=−0.230$, $P=0.240$; size vs. first response: $r_s=0.321$, $P=0.096$), linalool (size vs. number of responses: $r_s=−0.091$, $P=0.710$; size vs. first response: $r_s=−0.245$, $P=0.312$), and in all of the other experiments (differential conditioning and pattern discrimination; all $P>0.05$).

4. DISCUSSION

We could show that workers of *B. terrestris* are able to solve the absolute conditioning task, i.e., they are able to associate a specific odor with a sugar reward. The learning performance reached a level of more than 50 % correct responses after

seven trials and ca. 60 % after ten trials. Previous studies have found a variable learning performance in bumblebees, ranging from ca. 20–30 % in *B. terrestris* (Laloi et al. 1999) to 50–70 % in *Bombus occidentalis* (Riveros and Gronenberg 2009b). These differences might be due to methodological (e.g., starvation period, amount and concentration of sugar solution, and mounting procedure; Toda et al. 2009) or species-specific differences (see below).

In our study, we found no differences in the memory acquisition curves and the learning performance among different odor groups (Figure 1). Thus, stimulus reception, processing, and memory acquisition in the absolute conditioning essay seem to be similar for all tested odors. Our findings also suggest that, in contrast to previous studies, *B. terrestris* shows only a slightly poorer learning performance compared to honeybees both in speed (slope of learning curve) and accuracy (plateau of learning curve) (honeybee: Bitterman et al. 1983; *B. terrestris*: Laloi et al. 1999; *B. occidentalis*: Riveros and Gronenberg 2009b).

Interestingly, bumblebees were able to learn both odor combinations in the differential conditioning essay equally well, irrespective of which odor was the rewarding or unrewarding stimulus (Figures 2 and 3). After seven trials, the average response level reached 50–60 % and thus was
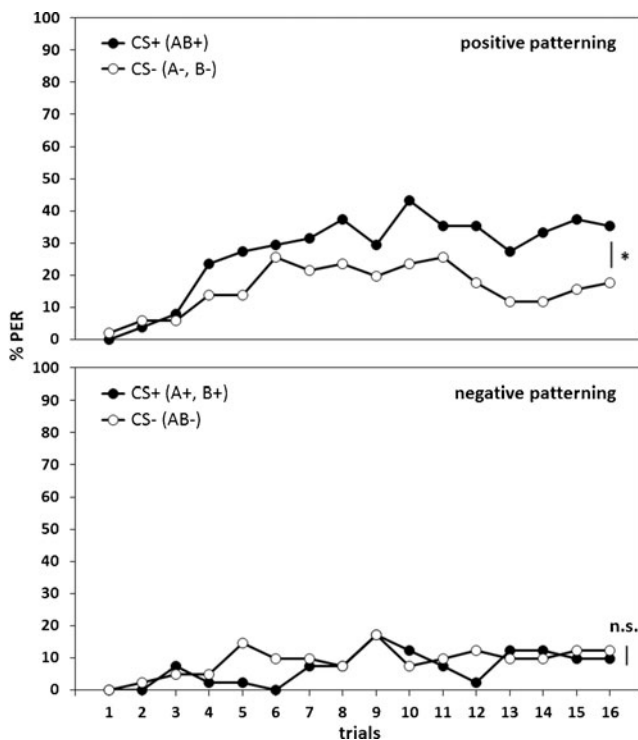
Figure 4. Proboscis extension response during trials of positive ($N=51$) and negative ($N=41$) patterning discrimination. In the *upper panel*, the individual odors (citral, 1-nonanol; CS−) remained unrewarded, while the compound (CS+) of both odors was reinforced. Individuals learned to discriminate between CS+ and CS−. In contrast, in the *lower panel* the individual odors were rewarded (CS+), while the compound (CS−) remained unrewarded. Here, the bees were not able to discriminate between CS+ and CS− (see text for statistics). * $P<0.05$; *ns*, not significant.

similar to the level found in the absolute conditioning essay. Our results contrast with Laloi and Pham-Delègue (2004), who reported an asymmetrical discrimination effect in bumblebees when using linalool and phenylacetaldehyde. They found that bumblebees could easily discriminate between both stimuli when linalool was the rewarded stimulus and phenylacetaldehyde was the unrewarded one, but failed when the odors were exchanged. This discrepancy might be explained by various factors, for example, experimental procedure, colony specificity, or starvation level of the tested bumblebees. In the study by Laloi and Pham-Delègue (2004), bees were starved for a shorter time than in our case and most other studies (5 h vs. 12–18 h; Riveros and Gronenberg 2009b; Anfora et al. 2011) and were

probably fixed in a way usually used for honeybees (although a detailed description is missing in their paper). Both factors strongly affect PER performance (Bitterman et al. 1983; Toda et al. 2009; Matsumoto et al. 2012) and may, thus, explain the overall low response level of ca. 30 %.

Furthermore, our findings provide evidence that bumblebees manage to discriminate between single odors and mixtures containing the same odors in an appetitive positive patterning discrimination task. In contrast, our bees were not able to differentiate between single odorants and mixtures containing the same odors in the negative patterning approach. These results indicate deficiencies in configural learning abilities, compared to honeybees. While Deisig et al. (2001) demonstrated that honeybees solved both positive and

negative discrimination tasks, whereas bumblebees failed to solve the more complex latter task in our experiment. A positive patterning discrimination (A−, B− vs. AB+) can still be accomplished by *elemental learning*, since the response threshold could be exceeded by the associative strength of the combined elements, but not by that of each single element (elemental summation principle; Deisig et al. 2001). In contrast, solving the negative pattern discrimination (A+, B+ vs. AB−) can only be achieved by a *configural approach*, since the sum of the associative strengths of the elements of a complex CS would always be greater than that of the single elements themselves, i.e., individuals would respond more often to the "stronger" mixture than to the "weaker" single elements (for review see Pearce and Bouton 2001). However, in our experiments, bumblebees failed to process configural associations, and it seems that they cope with complex olfactory learning tasks in an elemental manner. Interestingly, bumblebees indeed possess configural learning abilities when they must associate a reward with visual stimuli in a complex task (Fauria et al. 2002). Although the neural mechanisms of the different learning strategies are still unclear, differences between honeybees and bumblebees can be considered in an ecological context, as they differ in the evaluation of stimuli modalities. While honeybees possess a pronounced flower constancy during a single foraging trip and rely stronger on olfactory cues (Grant 1950; Jakobsen et al. 1995), bumblebees are less faithful to one floral type and focus more on spatial information (Free 1970; Jakobsen et al. 1995; Raine and Chittka 2007). Thus, bumblebees may rely more on common floral cues like colors or shapes rather than species-specific odors when foraging and hence perform better in complex configural learning tasks when facing conditioned stimuli of non-olfactory modalities, e.g., visual or tactile stimuli.

A remarkable characteristic of bumblebees is the distinct size polymorphism of workers within a colony. Workers in our study differed in their intertegulae span by a factor of 1.7 (ranging from 2.7 to 4.6 mm). Our data revealed a significant correlation between body size and learning performance only in two experimental groups when the reinforced odor in absolute conditioning was 1-nonanol and phenylacetaldehyde, respectively. In general, this effect might be explained by differences in perceptual sensitivity between larger and smaller individuals (Spaethe et al. 2007). However, no size effect was observed either in the other absolute conditioning experiments or in the differential conditioning assays. This is consistent with the study by Raine and Chittka (2008), who found no correlations between body size and learning speed in *B. terrestris* under free-flying conditions. In contrast, some previous studies reported positive correlations between size and learning performance in *B. impatiens* (Worden et al. 2005) and size and memory consolidation in *B. occidentalis* (Riveros and Gronenberg 2009b). Taken together, these results suggest a distinct interspecific variation, although a more systematic evaluation of the size effect on various measures of learning performance (learning speed, maximum performance level, etc.) in different *Bombus* species is needed in future studies.

To summarize, we could show that PER conditioning is a well-suited paradigm for the investigation of memory acquisition and olfactory learning in *B. terrestris* under fully controlled environmental conditions. Bumblebees perform in absolute and differential olfactory conditioning in a way comparable to honeybees, but fail when facing a complex negative pattern discrimination task. Future studies should fathom the proximate and ultimate causes underlying the species-specific learning capabilities, in particular with respect to differences in the species' ecology and life history.

Apprentissage olfactif de base ou complexe par conditionnement du REP (réflexe d'extension du proboscis) chez le bourdon, *Bombus terrestris*

Apprentissage élémentaire / bourdon / REP / associations configurales

Elementares und nicht-elementares olfaktorisches Lernen bei der PER-Konditionierung der Hummel *Bombus terrestris*

Elementares Lernen / *Bombus terrestris* / Rüsselreflexkonditionierung / Hummel / konfigurale Assoziationen

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