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RESEARCH ARTICLE

Learning of bimodal versus unimodal signals in restrained bumble bees

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ABSTRACT

Similar to animal communication displays, flowers emit complex signals that attract pollinators. Signal complexity could lead to higher cognitive load for pollinators, impairing performance, or might benefit them by facilitating learning, memory and decision making. Here, we evaluated learning and memory in foragers of the bumble bee *Bombus impatiens* trained to simple (unimodal) versus complex (bimodal) signals under restrained conditions. Use of a proboscis extension response protocol enabled us to control the timing and duration of stimuli presented during absolute and differential learning tasks. Overall, we observed broad variation in performance under the two conditions, with bees trained to compound bimodal signals learning and remembering as well as, better than or more poorly than bees trained to unimodal signals. Interestingly, the outcome of training was affected by the specific colour–odour combination. Among unimodal stimuli, the performance with odour stimuli was higher than with colour stimuli, suggesting that olfactory signals played a more significant role in the compound bimodal condition. This was supported by the fact that after 24 h, most bimodal-treatment bees responded to odour but not visual stimuli. We did not observe differences in latency of response, suggesting that signal composition affected decision accuracy, not speed. We conclude that restrained bumble bee workers exhibit broad variation of responses to bimodal stimuli and that components of the bimodal signal may not be used equivalently. The analysis of bee performance under restrained conditions enables accurate control of the multimodal stimuli provided to individuals and to study the interaction of individual components within a compound.

KEY WORDS: Associative learning, Conditioning, *Bombus impatiens*, Proboscis extension reflex, PER

INTRODUCTION

Flowers have been described as multisensory billboards adapted to attract visitors that act as pollinators in exchange for rewards such as nectar and pollen (Chittka and Raine, 2006; Raguso, 2004). Flowers display signals of various odours, colours, patterns, textures, shapes and sizes (Faegri and van der Pijl, 1971; Harder and Barrett, 1992; Leonard et al., 2012; Raguso, 2004). Thus, for pollinators, competing plants create an enormous sensory load that must be processed in order to make appropriate decisions and optimize energy use during foraging (Chittka and Raine, 2006; Knauer and Schiestl, 2015).

Among the components of signals provided by flowers, olfactory and visual elements are the most conspicuous. Learned associations of these components in particular have been well studied in a unimodal sensory context. Under unimodal conditions, bees readily learn olfactory (e.g. Giurfa and Sandoz, 2012; Guerrieri et al., 2005; Matsumoto et al., 2012) and visual cues (Giurfa et al., 1996; reviewed by Avarguès-Weber and Giurfa, 2014), exhibiting remarkable capacities for shape discrimination (Hempel de Ibarra and Giurfa, 2003), motion learning (Hori et al., 2007) and long-term retention (Menzel, 1999). Yet, because bees are most commonly exposed to multimodal environments, a full understanding of information use requires exposing pollinators to controlled compound signals. This in turn requires considering that individual floral cues may interact in shaping the receiving pollinator's response. Such interactions may depend upon factors such as stimulus salience, temporal sequence and innate biases, which may affect learned acquisition and retention. Moreover, one must consider potential differences in processing time across modalities and the required coincidence upstream in central brain areas. So far, behavioural observations of compound bimodal learning in free-flying pollinators have revealed that components of signals may interact synergistically to increase pollinator performance (Goyret et al., 2007; Kulahci et al., 2008; Kunze and Gumbert, 2001; Leonard et al., 2011a,b; Leonard et al., 2012; Raguso and Willis, 2002). In nature, this modulation of learning might impact the efficacy of floral visitation and reward collection (Giurfa et al., 1995). For instance, bumble bees learn bimodal stimuli faster and remember them longer (Kulahci et al., 2008), consequently improving the rate of floral visits and therefore the rate of reward collection.

Precisely characterizing the interactions between components in compound signals requires accurate control of the stimuli presented to an individual, which is difficult to do using free-flight protocols (Leonard and Masek, 2014). Such accurate control can be achieved under restrained conditions, enabling variation of stimulus intensity, time of exposure to single or compound cues, synchrony among components and a more precise quantification of components of behaviour (e.g. decision time; Wright et al., 2009). For example, in free-flight experiments, differences in decision time might be obscured, as the pollinator's perception of the respective stimuli remains uncertain because of differences in flight speed, angle of approach, etc. (Wright et al., 2009). Such time differences might affect phenomena such as attention, overshadowing or configural learning. Hence, supplementing free-flight experiments (reflecting more natural conditions) with experiments on restrained individuals should yield important insights into how compound signals, such as multimodal signals, are interpreted and used by pollinators.

Here, we tested the hypothesis that compound bimodal signals enhance learning and memory as well as the speed of response. This

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hypothesis builds on previous accounts and the idea that a compound signal provides individuals with more redundant information, thus leading to better acquisition (Mackintosh, 1974) and retention, and to faster and more accurate decisions. For example, in tasks of discrimination, individuals might enhance their performance if adding more information to the signals leads to better contrast between signals. Moreover, if individuals can more rapidly identify a compound stimulus, their latency of response might decrease. When learning a single stimulus, multimodal stimuli may support faster recognition as a result of redundancy across sensory channels. Also, we explored whether different bimodal combinations of components would lead to similar performance. Differences between combinations might originate from sensory bias (e.g. short wavelength colours) and might determine whether or not a bimodal compound signal is better than its individual components.

Unlike previous attempts, we tested these hypotheses under restrained conditions, relying on conditioning of the proboscis extension response (PER) in bumble bees. The PER is a natural feeding response of bees and other insects characterized by the extension of the proboscis upon stimulation of sensory organs (antennae, tarsi) with a sweet substance (nectar in flowers; Bitterman et al., 1983; Frings, 1944). In an experimental context, the PER can be conditioned by pairing presentation of the unconditioned stimulus (US; e.g. sucrose solution) with a conditioned stimulus (CS; e.g. colour, odour). After a variable number of pairings, a trained individual responds to the CS by exhibiting the PER even in the absence of the US. The PER protocol can be adapted to investigate learning under different tasks to reveal how bees use information. During absolute conditioning tasks, individuals learn the predictive value of a single stimulus presented over consecutive trials. In contrast, tasks of discrimination are more complex as the individual must learn to discriminate the features of a predictive (rewarded) stimulus from a stimulus that is not predictive (unrewarded). Predictive and non-predictive stimuli are presented sequentially in a randomized form. These tasks lead to different use of information by bees in free flight (e.g. better perceptual discrimination of similar stimuli; Giurfa, 2004) and may potentially also lead to differences in learning performance under restrained conditions.

For the bumble bee *Bombus impatiens*, both odour and colour can be used as the CS (Riveros and Gronenberg, 2009a,b, 2012) but have not been used together as a complex signal using the PER protocol. In experiments on the European honey bee *Apis mellifera*, a model comprehensively studied in its olfactory learning using the PER protocol, visual and olfactory stimuli are not commonly combined (but see Mota et al., 2011; Manzur et al., 2018) as classical conditioning to visual stimuli is more difficult to achieve under restrained conditions in honey bees (Avarguès-Weber and Mota, 2016; Dobrin and Fahrbach, 2012; Jernigan et al., 2014; Hori et al., 2006; Mota et al., 2011; Niggebrügge et al., 2009; Sakura et al., 2012; but see Lichtenstein et al., 2018). Hence, *B. impatiens* provides an excellent alternative to study the use of multimodal information under restrained conditions using the PER conditioning protocol.

Our results suggest that: (1) bumble bee foragers exhibit a broad variation in performance when using bimodal signals, apparently depending on the specific identity of signal components; (2) latency of response is not affected by the presentation of a compound signal; and (3) the olfactory component plays a more significant role within the compound signal, such that individual bees are more likely to respond to the olfactory component than to the configuration.

MATERIALS AND METHODS

Experimental subjects

Three colonies of the bumble bee *B. impatiens* Cresson 1863 (Biobest, Inc.) were simultaneously connected to a foraging cage (0.86 m×1.17 m×1 m) with sugar water (15% w/w) provided *ad libitum* from a single feeder. Bees were maintained under a 12 h:12 h light:dark photoperiod, 35% relative humidity and 19°C. Pollen and water were supplied *ad libitum* inside the nest box. Bees were not identified by colony of origin as bees from all colonies were constantly foraging on the same feeder in the same cage. Thus, potential variation among colonies cannot be evaluated but representation of individuals from all colonies was assured as we collected until all three colonies were exhausted and observations of the feeder revealed similar rates of forager arrivals from the three colonies.

On the day they were used in the experiment, bees were collected as they landed on the feeder and chilled on ice for 10 min. They were then harnessed as described in Riveros and Gronenberg (2009a). After 20 min, bees were fed 15 µl of sugar water (50% w/w) and maintained for 3 h in a plastic box until being transferred to the training apparatus.

Training apparatus

The training apparatus was adapted from previous designs used for odour (Riveros and Gronenberg, 2009a) and colour conditioning (Riveros and Gronenberg, 2012). Briefly, the apparatus included a carousel of 12 individual plastic chambers separated by 10 cm. Each chamber was painted black on the outside and covered with aluminium foil inside to increase light reflectance within the container and minimize stray light to other chambers. An opening at the front of each chamber allowed access to each bee during training. A mobile platform supported a ring of LEDs used for colour stimulation (see below), a glass tube connected the chamber to the air current used for olfactory stimulation, and a funnel connected the chamber to a vacuum for removal of residual odour through an opening in the rear side of the chamber (Fig. 1; modified from Riveros and Gronenberg, 2012).

Stimuli

Colour stimulation was provided by 4 green (peak wavelength λ=523 nm) or 4 blue (peak wavelength λ=470 nm) LEDs connected to a power supply and controlled by individual switches. During training, the intensity of the LEDs was alternated every trial across three values to keep bees from learning light intensity instead of colour wavelength. For olfactory stimulation, a syringe containing a piece of filter paper with 15 µl of pure odour (linalool 97%, L2602; 1-hexanol 98%, H13303; Sigma, St Louis, MO, USA) was placed between two, one-way solenoid valves. A manual switch controlling both valves allowed odour volatiles to be injected into a constant airstream directed at the bee antennae.

We connected each individual switch-controlling colour and odour to a single ‘master’ switch. The master switch was used to provide simultaneous bimodal colour and odour stimulation after adjusting the desired combinations (see below) using the individual switches.

Training and testing procedure

We trained the bees in a classical conditioning paradigm. During training, one of the chambers containing a single bee was moved above the mobile platform and the bee was allowed 15 s to acclimate. Then, the conditioned stimulus (CS+) was presented. Ten seconds after onset of the CS+, we introduced a syringe with sugar

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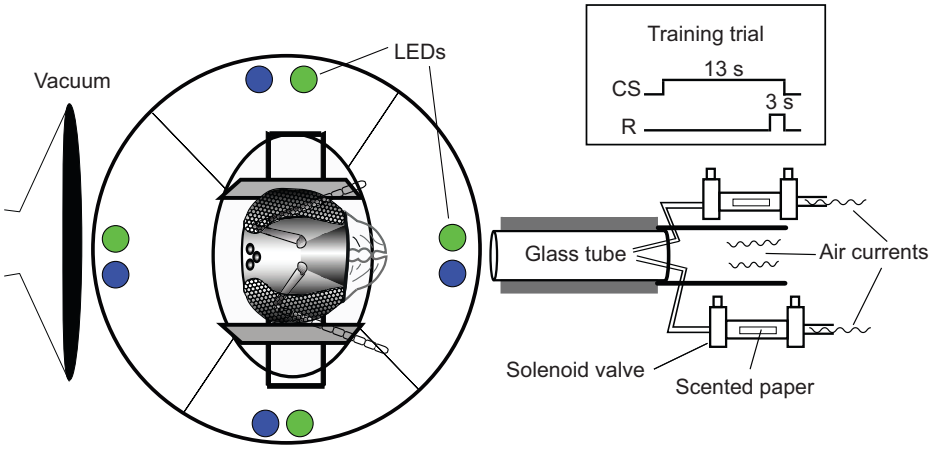


Fig. 1. Experimental setup showing the presentation of visual and olfactory components. Inset: sequence of presentation of the conditioned stimulus (CS) and the reward (R) during a training trial.

water (50% w/w; unconditioned stimulus US) and gently touched the tip of the bee antennae, thus eliciting the unconditioned response (PER; UR); the bee was allowed to drink the reward for 3 s. Hence, the CS+ was presented for 13 s and the US overlapped for 3 s. This procedure, referred to as a training trial, was repeated at intervals of 10 min and the number of trials varied across the tasks (see below). In tasks involving discrimination between two stimuli, the unrewarded trials followed the same sequence, but the bee was not stimulated with sucrose or the presence of a syringe to get any reward. The presentation of rewarded (R) and unrewarded (U) trials followed a pseudorandom sequence (R, U, U, R, U, R, R, U…).

After 24 h, bees were presented with the CS+ used for training, but not the reward. For bees trained using a bimodal stimulus, the bees that responded with PER to the presentation of the CS+ (e.g. blue+linalool; see below), were subsequently presented with the individual components (e.g. blue, linalool) in an alternating, random order after the memory retention test. This last test aimed to determine whether the bees would equally respond to the individual components or to the compound signal. Thus, during the test the bees could respond to: (1) one individual component (either odour or colour), (2) both components separately (odour as well as colour) or (3) only the simultaneous presentation of both components. In all cases, latency was measured using a sound signal (metronome) to a resolution of 1 s.

Experimental conditions

We contrasted the performance of bees trained to unimodal and bimodal stimuli in two tasks: (1) absolute conditioning and (2) differential conditioning. All treatments (unimodal and bimodal, absolute and differential conditioning) were run simultaneously within each experimental cycle but are presented separately for clarity. The treatments involved in each experiment are described below.

Absolute conditioning

In absolute conditioning, a single CS+ is associated with a reward; there is no unrewarded conditioned stimulus (CS−). In our experiment, we used four unimodal CS+ [colours: blue (B), green (G); odours: linalool (Lin), 1-hexanol (Hex)] and four bimodal CS+ corresponding to the possible combinations across the unimodal CS+ [blue+linalool (BLin), blue+1-hexanol (BHex), green+linalool (GLin), green+1-hexanol (GHex)]. Bees were presented with eight training trials on one type of CS+ with an intertrial interval of 10 min. Memory retention was tested 24 h after training.

Differential conditioning

In differential conditioning, a CS+ is associated with a reward and a CS− is associated with the absence of a reward. In our experiments, bees received either unimodal or bimodal pairs of CS+ and CS−, balanced across treatments (e.g. B+ versus G− and G+ versus B−). Thus, we used four unimodal (colours: B+ versus G−, G+ versus B−; odours: Lin+ versus Hex−, Hex+ versus Lin−) and four bimodal differential tasks (BHex+ versus GLin−, BLin+ versus GHex−, GLin+ versus BHex−, GHex+ versus BLin−). Bees were presented with a pseudorandom sequence of seven rewarded (CS+) and seven unrewarded (CS−) training trials (CS+, CS−, CS−, CS+, CS−, CS+, CS+, CS−…) with an intertrial interval of 10 min. Memory retention was evaluated 24 h after training.

Quantification of variables

Performance at the group level was measured as the percentage of individuals responding to the CS+ by extending their proboscis. Performance at the individual level was calculated as the number of conditioned PERs across trials.

Retention was measured as the percentage of individuals responding to the sole presentation of the CS+ after 24 h following the last training trial.

Latency of response was measured as the time (in seconds) between the onset of the CS presentation and the start of a PER. Based on previous results (Riveros and Gronenberg, 2012), we calculated the average latency response for each individual that responded at least three times.

Statistical analyses

Overall changes of responses across trials were evaluated using repeated-measures MANOVA. Differences of responses among treatments within trials, including comparison of memory retention, were evaluated using a χ^2 test (results presented in Tables S2 and S5); comparisons of response latencies were evaluated using ANOVA. Error associated with multiple comparisons was controlled using the false discovery rate (FDR) procedure (Verhoeven et al., 2005). All the analyses were done using JMP v.11.0 (SAS Institute). The FDR analyses were done using the *P*-values determined by JMP. As the repeated measures MANOVA requires consistent responses across the entire training phase, we excluded 31 bees out of the 372 (bees not exhibiting PER in a CS+ trial and not responding to stimulation with the reward).

RESULTS

Experiment 1: absolute unimodal/bimodal conditioning

We trained a total of 180 worker bees distributed across eight treatments. Memory retention was tested after 24 h in 117 of those bees because of individuals escaping or dying before testing.

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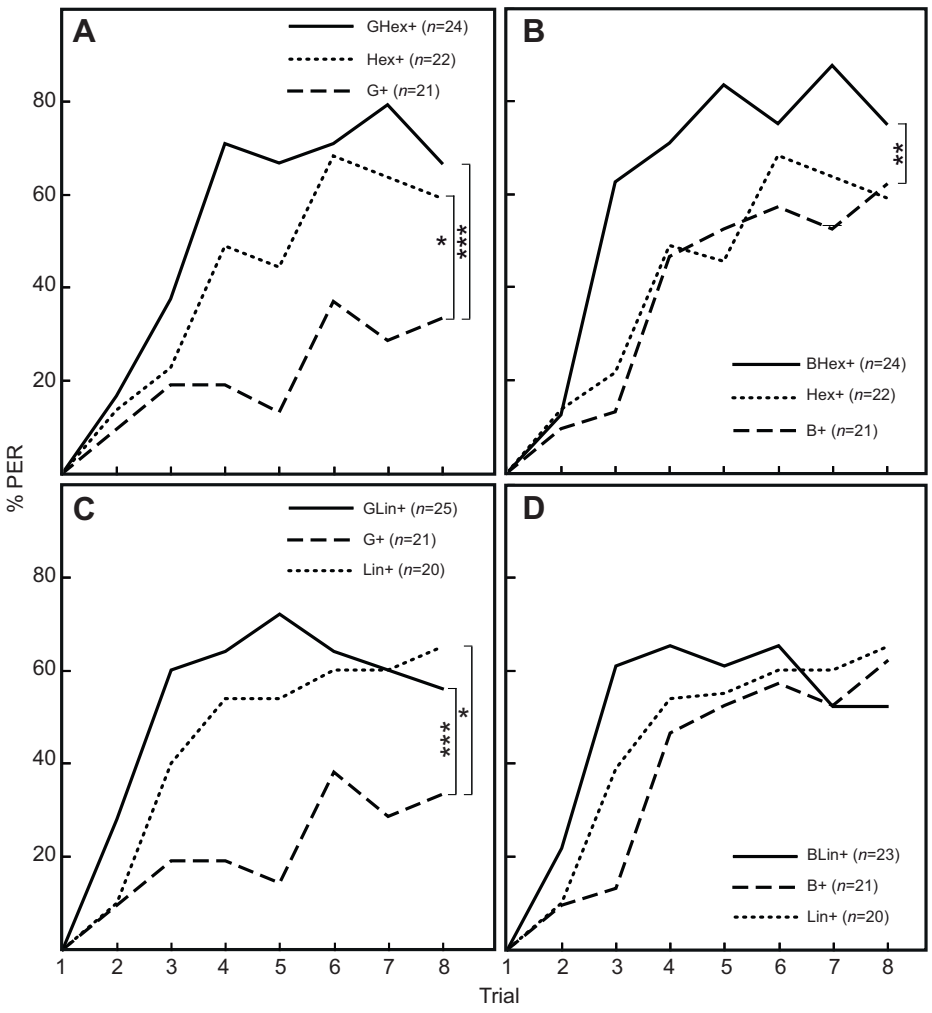


Fig. 2. Performance of bumble bee foragers trained using different combinations of unimodal (olfactory and visual) or compound bimodal signals in an absolute conditioning task. (A–D) Responses to the four different stimuli. G, green; B, blue; Hex, 1-hexanol; Lin, linalool. Asterisks indicate significant differences for the distributions based on the effect of treatment from MANOVA analyses (detailed statistics are presented in Table S1). *P<0.02; **P<0.01; ***P<0.001.

Overall, we did not find significant differences in performance between bees trained to different bimodal signals (Fig. 2; mean performance±s.e.m.: BHex+=4.7±0.5, BLin+=3.8±0.5, GHex+=4.1 ±0.5, GLin+=4.0±0.5; $F_{3,92}$=0.62, P=0.61). We did not find significant differences between bees trained to different colours (B+=3.0±0.5, G+=1.6±0.5; $F_{1,40}$=4.02, P=0.05) or between bees trained to different odours (Hex+=3.2±0.5, Lin+=3.4±0.5; $F_{1,40}$=0.082, P=0.77; Fig. 2). However, we found a significantly higher performance of bees trained to odours than bees trained to colours (colour+=2.3±0.4, odour+=3.3±0.4; $F_{1,82}$=4.22, P=0.04). We next compared the performance of bees across the three distinctive groups [colour (visual), odour (olfactory) and bimodal; Fig. 3]. We found a significant effect on performance of the signal used to train the bees (colour=2.3±0.4, odour=3.3±0.4, bimodal=4.1±0.2; signal: $F_{2,177}$=9.6, P=0.0001; trial×signal: $F_{14,342}$=2.39, P=0.003). Bees trained to the bimodal signal exhibited the highest performance, which, however, was not significantly different from the performance of bees receiving the unimodal olfactory signal (odour+=3.3±0.4, bimodal+=4 0.1±0.2; signal: $F_{1,36}$=3.52, P=0.06; trial×signal: $F_{7,130}$=1.69, P=0.12). Yet, the performance of bees trained to the bimodal signal was significantly higher than the performance of bees trained only to the unimodal colour signal (colour+=2.3±0.4, bimodal+=4.1±0.2; signal: $F_{1,136}$=19.46, P<0.0001; trial×signal: $F_{7,130}$=4.29, P=0.0003; Fig. 3A).

We further conducted specific comparisons of unimodal and bimodal combinations (Table S1). In three of the four cases, the bees trained to the bimodal signal exhibited a significantly higher performance than those trained to colour, but we did not find differences in performance between bees trained to the bimodal signal and bees trained to the olfactory signal (Table S1). In one case (using B and Lin) we did not find significant differences in performance of bees trained to bimodal or unimodal signals ($F_{2,61}$=0.62, P=0.54; Table S1) and further tests were not conducted (Table S1).

For the measure of memory retention after 24 h, the sample sizes were not the same as those during training because of some bees escaping and some dying. Thus, these results should be considered with caution. Based on the bees that were available during the retention test, we compared the percentage of bees exhibiting a conditioned PER. Overall, the pattern was similar to that observed during learning. The bees trained to the bimodal signal exhibited the highest memory retention, which, however, was not significantly different from the memory retention of bees trained to the olfactory component alone (percentage of conditioned PER after 24 h: BHex+=43.8%, BLin+=53.8%, GHex+=35.3%, GLin+=43.8%, Hex+=6.2%, Lin+=50%; Table S2). Further, the bees trained to the colour component exhibited the lowest retention (percentage of conditioned PER after 24 h: B+=9.1%; Table S2). Interestingly, none of the bees trained to G as CS+ showed retention after 24 h.

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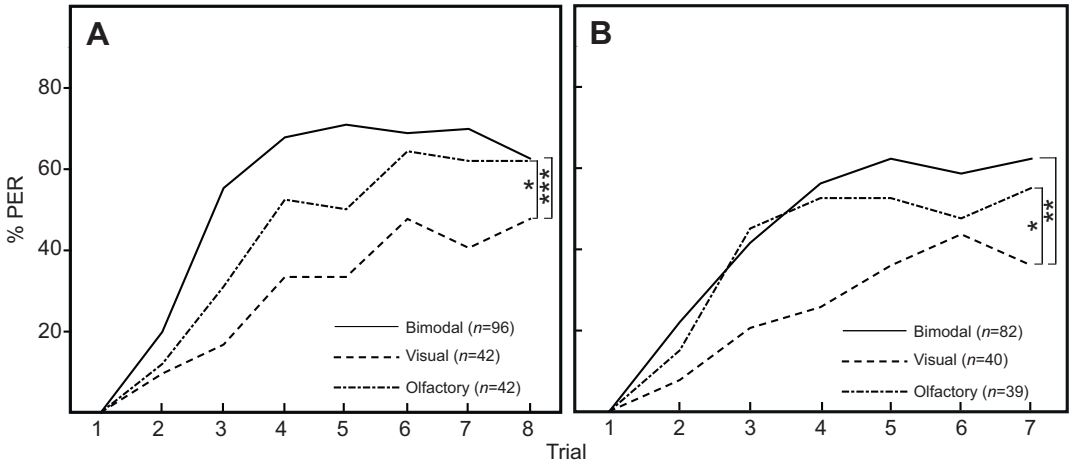


Fig. 3. Performance of bumble bee foragers trained to a bimodal (pooled for four combinations), olfactory (pooled for two odours) and visual signal (pooled for two colours). (A) Absolute conditioning. (B) Differential conditioning (only the curves for responses to rewarded stimuli are presented). Asterisks indicate significant differences for the distributions based on the effect of treatment from MANOVA analyses (bimodal versus olfactory: $F_{1,120}$=0.86, P=0.355; bimodal versus visual: $F_{1,119}$=12.45, P=0.0006; visual versus olfactory: $F_{1,177}$=5.08, P=0.027). *P<0.04; **P<0.001; ***P<0.0001.

Thus, it seems that the odour signal plays a significant role not only in learning but also in memory.

This last inference is further supported by the test of individual components (i.e. colour, odour) in some of the bees trained to the bimodal signal and that also responded after 24 h. This test aimed to identify whether bees that exhibited a conditioned response to the bimodal signal after 24 h had used the compound signal, or whether they used the components independently or even preferentially used one of the components. For this purpose, 23 bees that exhibited a conditioned PER during the retention test were used (Table 1). Following the presentation of the CS+ during the retention test, bees received each component separately (olfactory, visual). The order of presentation was randomly assigned to each bee and occurred with an interval of 10 min. Out of those 23 bees, two responded exclusively to colour alone, five exclusively to odour alone, eight exclusively to both stimuli presented independently (colour or odour) and eight exclusively to the compound signal (colour+odour; i.e. despite responding with PER when tested for memory retention using the bimodal signal, they did not respond with PER when presented with each component separately; Table 1). Thus, individuals appeared to exhibit diverse strategies when faced with the task of learning a bimodal signal. However, this pattern may have been affected to some extent by extinction, as the bees included in this second test were first tested for the bimodal signal without providing a reward, which may have produced some decrease in response to individual components.

Finally, latency of response did not significantly vary between individuals across the treatments (visual, olfactory or bimodal; mean latency±s.e.m.: bimodal+=2.2±0.1, colour+=2.4±0.3, odour+=1.9±0.2; ANOVA: $F_{2,111}$=0.98, P=0.38). Thus, adding more signal components or information through more than one sensory channel did not affect the speed of response.

Table 1. Responses to single components of the bimodal signal or to the compound signal in individuals exhibiting a conditioned response after 24 h

Conditioned response	Absolute conditioning	Differential conditioning
Only if components were presented simultaneously	8	4
Odour component	5	5
Colour component	2	4
Colour & odour independently	8	5
Total	23	18

Experiment 2: differential unimodal/bimodal conditioning

We trained a total of 161 workers distributed across eight treatments. Memory retention was tested after 24 h only in 124 bees because of individuals escaping or dying before testing. In all cases, bees readily discriminated between rewarded and unrewarded stimuli (Table S3). Indeed, all responses to unrewarded signals were not significantly different from zero, as reflected by the null effect of trial in the MANOVA model (B−: $F_{6,15}$=0.12, P=0.99; G−: $F_{6,15}$=0.12, P=0.99; Hex−: $F_{6,18}$=0.00, P=1.00; Lin−: $F_{6,14}$=0.12, P=0.99; BLin−: $F_{6,12}$=0.00, P=1.00; GHex−: $F_{6,17}$=0.13, P=0.99). Thus, to test the potential difference in performance between unimodal and bimodal treatments, we only used the responses to rewarded signals (Fig. 4).

In two cases, we did not find significant differences in performance between bees trained to the bimodal signal and those trained to the unimodal components (mean performance±s.e.m.: BHex+=3.7±0.4, B+=2.4±0.4, Hex+=3.0±0.4; $F_{2,59}$=2.57, P=0.09; BLin+=3.0±0.4, B+=2.4±0.4, Lin+=2.2±0.4; $F_{1,39}$=0.03, P=0.86; Table S4). In one case (GLin+=3.4±0.4), the bees trained to the bimodal signal exhibited the highest performance, which was not significantly different from the performance of bees trained to the olfactory component (Lin+=2.2±0.4), but was significantly higher than the performance of bees trained to the colour component (G+=1.0±0.4; Fig. 4C; Table S4). Interestingly, in one case the highest performance was achieved by bees trained to the olfactory component (Hex; Fig. 4A). However, there were no significant differences relative to the performance of bees trained to the bimodal signal (GHex+=1.8±0.5, G+=1.0±0.4, Hex+=3.0±0.4; $F_{1,34}$=3.22, P=0.082; Table S4).

Similar to the results with the absolute conditioning protocol, for the measure of retention after 24 h, the sample sizes were affected by some bees escaping and some dying. Based on the bees that were alive during the retention test, we compared the percentage of bees exhibiting a conditioned PER. We found that the bees trained to the bimodal and the olfactory signals exhibited the highest retention (percentage of conditioned PER after 24 h: B+=13.3%, G+=8.3%, Hex=40.0%, Lin=22.2%, BHex+=37.5%, Blin=42.1%, GLin=40%;

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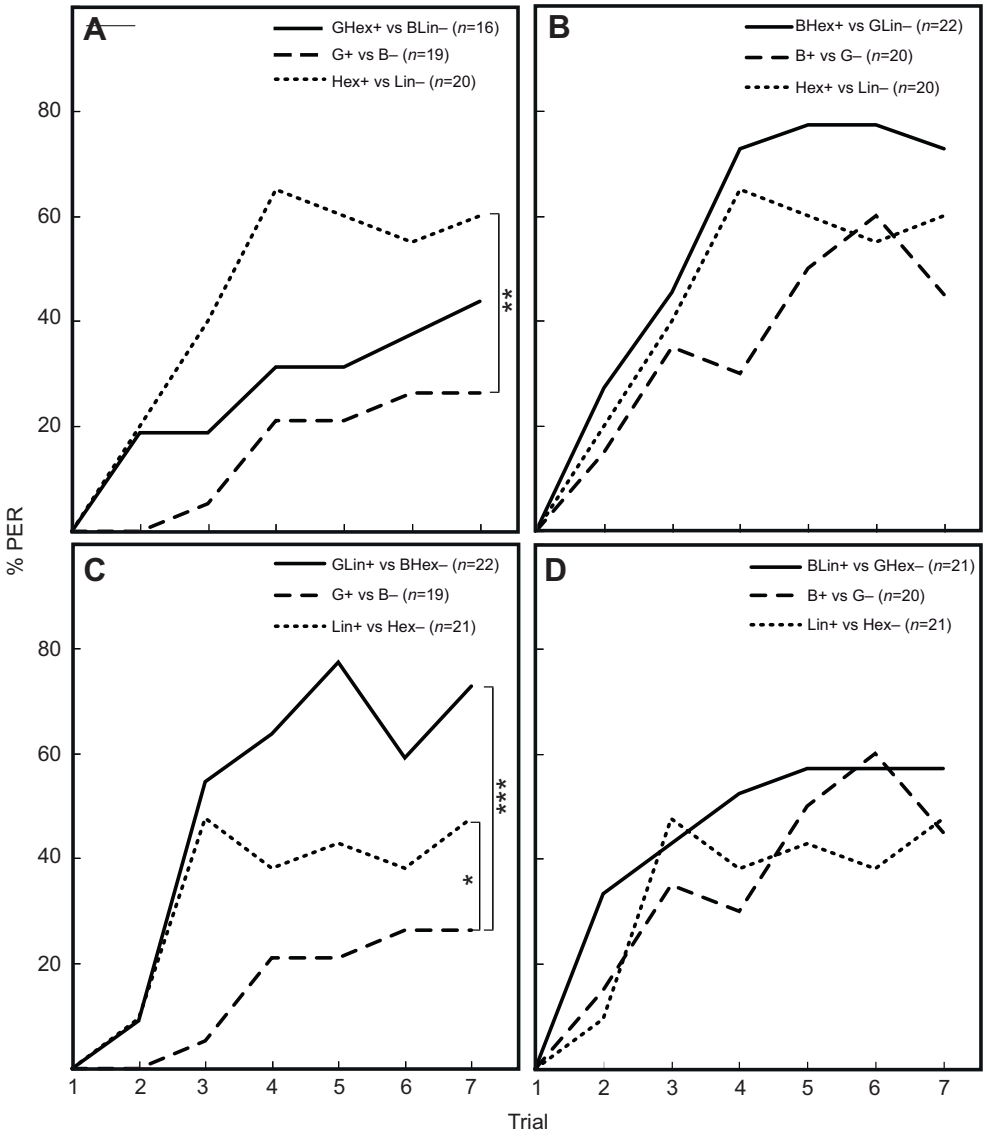


Fig. 4. Performance of bumble bee foragers trained using different combinations of unimodal or bimodal signals in a differential conditioning task. Only curves for responses to rewarded stimuli are presented and used for analyses (see Materials and Methods). (A–D) Responses to the four different stimuli (bimodal signal: solid line, olfactory signal: dotted line, visual signal: dashed line). Asterisks indicate significant differences for the distributions based on the effect of treatment from MANOVA analyses (detailed statistics are presented in Table S4). *P<0.05; **P<0.01; ***P<0.001.

Table S5). However, in one case (GHex+) none of the bees trained to the bimodal signal remembered after 24 h. This cannot be attributed to the bees not being tested, as only two bees died or escaped in this group.

A subgroup of bees that responded with a PER to presentation of the bimodal signal was then presented with the individual components following the same protocol used for the test during the absolute conditioning protocol (see above). Of 18 bees analysed, four bees responded exclusively to colour only, five bees exclusively to odour only, five bees to both stimuli presented independently (colour as well as odour), and five bees exclusively to the compound signal (not to the individual components) (Table 1). Thus, similar to the responses in our absolute conditioning protocol, individual bees exhibited different strategies of responses to the cues.

Finally, we did not find differences in latency of response between workers across any of the treatments (olfactory, visual or bimodal signals; mean latency±s.e.m.: bimodal+=2.3±0.2, colour+=2.7±0.4, odour+=2.2±0.3; ANOVA: $F_{2,61}$=0.79, P=0.46).

DISCUSSION

We aimed to compare the performance of harnessed bumble bees using a synchronously bimodal signal versus a unimodal signal. We further aimed to investigate whether individuals trained using a bimodal signal attend independently to different components, or whether they use the bimodal signal as a unitary composite. Overall, our results show that providing individual bumble bees with compound bimodal signals leads to a broad variation in performance, such that individuals receiving a bimodal signal do not necessarily perform better than those receiving unimodal signals, and that the speed of their responses is not significantly different. Although the acquisition response was numerically higher when bees were trained to compound bimodal signals, it did not consistently differ from responses to training using either olfactory or visual components alone. These patterns were similar for both absolute and differential conditioning tasks.

Our results therefore are not fully consistent with previous accounts comparing the performance of bees in free-flight protocols (e.g. Kulahci et al., 2008) in which compound bimodal signals offered higher performance than unimodal ones, as predicted (Leonard et al., 2011a,b). What could explain the discrepancy? First, and probably foremost, our protocol differs from free-flight protocols in the temporal presentation of the components of the bimodal signal. During free flight, bees will most likely first perceive components of the multimodal signals at different times. In

nature, whether visual or olfactory stimuli would be detected first might vary depending upon ambient conditions and floral composition. In either case, detection of the first signal may have several effects on signal processing. For instance, a first component may increase attention, favouring learning of the second component, or defining a context that facilitates how the second component is learned (Rowe, 1999; Goyret et al., 2007; Leonard et al., 2011a; Mota et al., 2011). In our more synchronized presentation, bees might lack this benefit, thus having to select one of the components. Testing whether synchronization of stimulus components is essential for the benefit of bimodal signals can be achieved using the PER approach to precisely vary the order of presentation of the olfactory and visual components. Under such circumstances, a variation of synchrony in components leads to variable performance and suggests that the component presented first may enhance or decrease the process of acquisition (A.J.R. and A.S.L., unpublished). However, even under our more controlled setup, some variation in synchrony would be expected because of the nature of the stimuli presented (light turned on versus odour pumped). The extent to which this variation would affect the whole presentation during 10 s of exposure to the CS+ before the reward cannot be determined here and requires further investigation.

A second possibility relates to the difference in energy investment by bees under protocols of free flight versus PER. Free-flight protocols, and more importantly foraging bouts in nature, are associated with energetic costs that favour accuracy of choice. In contrast, tasks of absolute or differential conditioning, using the PER protocol, imply that the individual receives a reward either if it exhibits a conditioned response (in which case it is rewarded for a 'correct' decision) or if it does not (in which case is it rewarded with the goal of increasing the strength of the association). Although similar mechanisms may underlie learning in free flight and under restrained conditions, it is important to consider the costs and benefits of responses in each, as these may account for differences in results.

A second goal of our study was to test whether using a compound bimodal signal would modify the latency of responses. Traditionally, latency of response has not been the primary focus of studies on learning (Chittka et al., 2009). This is particularly true for analysis of the conditioned response in the PER protocol as it requires videotaping to precisely assay the short latency of response in honey bees (the most traditional model). In contrast, latency of response in bumble bees is typically longer, enabling an easier estimation of decision speed. Nevertheless, we did not find significant differences between bees trained using unimodal versus bimodal cues, which suggests that adding more information does not favour faster decisions or require longer processing times to make decisions. It remains to be determined whether specific features of the task affect response latency. For example, similarity of stimuli to be discriminated is known to affect both accuracy and speed of response (Chittka et al., 2009), and in such contexts adding more information to a compound signal might favour faster responses.

Our protocol enables a general approach to test the possibility that individuals vary in their use of a bimodal signal. For example, only about a third of the tested bees in the two learning protocols (13 out of 41) appeared to have learned the bimodal signal as a single composite (i.e. responded exclusively to the compound but not to its individual components), which implies that two-thirds of the bees distinguished the different components. Importantly, only about 15% (Table 1) of the bees relied exclusively on colour information. In contrast, 24% (Table 1) of the bees relied exclusively on odour as the conditioned stimulus. This difference, though not statistically different, agrees with the relatively lower performance exhibited when using colour as a conditioned stimulus using the PER protocol (Riveros and Gronenberg, 2012). Moreover, almost 33% (Table 1) responded to either colour alone or odour alone, which suggests that these bees used each as an independent CS+. This apparent behavioural variation might imply important consequences in information use. For example, a capacity to identify a multicomponent signal as a single configuration is required to solve many tasks of non-elemental learning (differential tasks in which the contingency between individual components of a CS and the reward may be ambiguous unless the whole configuration is used to make the discrimination; reviewed by Giurfa, 2003), which, from our results, would be a challenging task for most individuals. A possibility is that longer protocols of training might lead to configural learning, as discussed in early accounts on classical conditioning (Mackintosh, 1974). Also, the fact that about a third of the bees used the two components independently (but not the compound signal) suggests that they might take advantage of an enhanced redundancy of the association, yet given the similar reliability of the two components of the bimodal signal, this strategy would not be economically optimal (Rubi and Stephens, 2015).

A final point is that bees' performance varied considerably depending upon the stimuli used. This variation may be attributed to known bias toward some stimuli (e.g. Gumbert, 2000; Rubi and Stephens, 2015), which may affect the propensity to learn particular cues. The overall difference in performance may be evidence of this unbalanced use of certain colours or odours. However, interpretation must be done with caution as the PER method is more effective with olfactory than visual stimuli in honey bees and bumble bees. Thus, differences across modalities may reflect constraints of the method rather than specific patterns of information use occurring in natural conditions.

Nevertheless, within a single modality, our results reflect significant variation. Specifically, in treatments where green was used as the unimodal signal or as the colour component of the bimodal signal, bees showed significantly lower performance. Green typically characterizes foliage rather than flowers and, accordingly, performance when learning this colour is lower under restrained PER protocols (Jernigan et al., 2014; Riveros and Gronenberg, 2012). Moreover, under restrained conditions, bumble bees tend to readily learn odours, exhibiting higher performance when using this modality (Riveros and Gronenberg, 2009a,b) than when using colours (Riveros and Gronenberg, 2012). This difference may help explain why the bees appeared to prefer the olfactory to the visual component during compound bimodal conditioning. It could also explain why the performance of bees in the bimodal group was closer to that of the bees learning the unimodal olfactory stimulus than to that of bees learning the unimodal colour stimulus. Alternatively, olfactory and colour cues might differ in their salience, which is known to affect compound learning (Mackintosh, 1974) and might have favoured odour learning in our protocol. Further analysis varying light and odour intensity are needed to test this scenario. Overall, these results support the idea that individuals may rely on a compound bimodal signal or its components depending upon several factors that extend beyond the reliability of the components as predictors of an output (e.g. a reward), including individual variation and experience and species bias (Knauer and Schiestl, 2015; Rubi and Stephens, 2015).

Moreover, our findings suggest that the effects of the bimodal signal were not exclusively the result of adding more information, but rather of the interaction between the components. A prediction of our hypothesis was that bees trained to a bimodal signal would exhibit higher performance than bees trained to olfactory and visual

signals alone. Under such a scenario, the interaction of components might lead to additive associative strengths, reflected as higher performance when presented as a compound. Alternatively, one of the components might support learning of the other, thus enhancing overall acquisition. Our results suggest that performance using bimodal stimuli was not significantly affected by such additive interactions between the components. Rather, performance at the population level was more impacted by individual strategies in the use of information (using single components, both components or the compound signal) and the quality of that information (identity of odours and colours), which may explain the broad variation observed.

Finally, our findings in the differential conditioning task (Fig. 3B) reveal an unexpected interaction of components within a compound bimodal configuration. When bees were provided with the compound bimodal signal GHex+, their performance was lower compared with the performance discrimination of Hex alone. This raises the possibility that certain combinations of components in a bimodal signal may have a negative rather than positive impact on the decisions of individuals. Possibly, combinations of components with a positive bias lead to enhanced performance, whereas combinations of components with a negative bias lead to poorer performance. Moreover, this result suggests that the similarity of performance among bees using compound bimodal and unimodal olfactory cues should not be interpreted as an indication that bees ignored the colour component. Instead, it supports the idea that colour may serve as a context for the use of odour information (Mota et al., 2011) and that during compound conditioning one of the components may control the responses (Mackintosh, 1974).

In summary, we conclude that performance using a compound bimodal signal does not reflect a straightforward positive synergistic effect of signal components and that the interaction of components of a multimodal signal may vary widely among individuals and according to the nature of the components (Balkenius et al., 2006). We have shown that using the PER protocol opens new possibilities to study the interaction between components in a compound multimodal signal, given that stimulus features (intensity, duration, synchrony) can be controlled more precisely than in free-flight assays. Tapping the potential of *B. impatiens* to learn colour and odour signals while using the PER protocol will allow us to better understand the proximate mechanisms underlying the use of different sensory modalities by nectar-foraging bumble bees.

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