

RESEARCH ARTICLE

Temporal configuration and modality of components determine the performance of bumble bees during the learning of a multimodal signal

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ABSTRACT

Across communicative systems, the ability of compound signals to enhance receiver's perception and decoding is a potent explanation for the evolution of complexity. In nature, complex signaling involves spatiotemporal variation in perception of signal components; yet, how the synchrony between components affects performance of the receiver is much less understood. In the coevolution of plants and pollinators, bees are a model for understanding how visual and chemical components of floral displays may interact to influence performance. Understanding whether the temporal dimension of signal components impacts performance is central for evaluating hypotheses about the facilitation of information processing and for predicting how particular trait combinations function in nature. Here, I evaluated the role of the temporal dimension by testing the performance of bumble bees under restrained conditions while learning a bimodal (olfactory and visual) stimulus. I trained bumble bees under six different stimuli varying in their internal synchrony and structure. I also evaluated the acquisition of the individual components. I show that the temporal configuration and the identity of the components impact their combined and separate acquisition. Performance was favored by partial asynchrony and the initial presentation of the visual component, leading to higher acquisition of the olfactory component. This indicates that compound stimuli resembling the partially synchronous presentation of a floral display favor performance in a pollinator, thus highlighting the time dimension as crucial for the enhancement. Moreover, this supports the hypothesis that the evolution of multimodal floral signals may have been favored by the asynchrony perceived by the receiver during free flight.

KEY WORDS: Configural learning, Multimodal learning, Signal evolution, Floral signals

INTRODUCTION

From mate choice to predator–prey and plant–pollinator interactions, a generalized enhancement of receiver's response is invoked as a potent force for the evolution of compound signals (Buehlmann et al., 2020a; Guilford and Dawkins, 1991; Hebets and

Papaj, 2005; Mitoyen et al., 2019; Rojas et al., 2018; Rowe, 1999). Multicomponent signals may facilitate detection, discrimination and memorization by the receiver (Mackintosh, 1974; Rowe, 1999). Multimodal signals are a special case, which may provide additional, modulatory or redundant information through different sensory channels (Hebets, 2005; Mota et al., 2011; Ronald et al., 2017). For example, plants display multimodal signals (through flowers) that are used by pollinators while assessing the floral market and optimizing their foraging (Giurfa et al., 1995; Raguso, 2004). Consistent with the hypothesized enhancement, flying pollinators generally benefit from using multimodal relative to unimodal signals (Goyret et al., 2007; Kulahci et al., 2008; Kunze and Gumbert, 2001; Leonard et al., 2011a,b,c; Mota et al., 2011; Raguso and Willis, 2005). Similarly, territorial responses in the dart poison frog are elicited only if the bimodal components are presented together (Narins et al., 2003). Also, in mimic warning signals emitted by passion-vine butterflies, subtle components such as locomotor patterns are added to the complex multimodal cue, indicating the value of enriching the message from a prey to its predator (Srygley, 1999). Lastly, during navigation, arthropods, including ants, beetles and whip spiders, benefit from integrating multiple sources of information to enhance performance during short and long distance displacements (Buehlmann et al., 2020a,b; Dacke et al., 2019; Flanigan et al., 2021). These last examples highlight the importance of multimodal integration even outside of a sender–receiver interaction.

However, increasing evidence suggests that other factors, such as the internal structure of the signal (e.g. the components of the cue and their order, timing and duration of presentation), the reliability of the components and the spatiotemporal relationships between them must be considered for a more accurate explanation of performance (Caldart et al., 2022; Goyret et al., 2007; Halfwerk et al., 2019; Mitoyen et al., 2019; Narins et al., 2005; Reş, 2018; Riveros et al., 2020). This is particularly critical for multimodal signaling, which is bound to physical constraints in transmission and physiological differences in detection, as well as separate and integrated processing. Animals seem to cope with this challenge by exhibiting processing windows of 'tolerance' for spatiotemporal disparities. For example, in the dart-poison frog, auditory and visual information must overlap to elicit aggressive behavior; yet, there is a short temporal window of tolerance to the asynchronous presentation of these components (Narins et al., 2005). In the interaction between plants and pollinators, floral signals are displayed constantly, but visitors may acquire some components asynchronously depending upon neural constraints (e.g. olfactory versus visual processing), environmental conditions (e.g. light availability, wind direction) or simply due to the form of approaching flowers (Wright et al., 2009). Further spatiotemporal differences may be expected from diverging strategies of species.

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Flowers may release dynamic odor plumes that reach long distances and are complemented by static visual displays. Alternatively, large floral displays or floral patches may be perceived from afar and complemented by scents at short distance. How these differences may impact the learning of complex signals or their components is not fully understood.

Here, the goal was to test whether the temporal dimension of a bimodal signal may affect learning of the overall stimulus and/or its components. Previous accounts have shown that pollinators benefit from using multimodal relative to unimodal signals, leading to higher performance in learning and memory tasks (Goyret et al., 2007; Kulahci et al., 2008; Kunze and Gumbert, 2001; Leonard et al., 2011a,b,c; Mota et al., 2011; Raguso and Willis, 2005). However, those experiments mostly relied on free-flying pollinators (Goyret et al., 2007; Kunze and Gumbert, 2001; Leonard et al., 2011b,c; Raguso and Willis, 2005), constraining the control of the temporal presentation of training cues (Leonard and Masek, 2014; Wright et al., 2009). Moreover, a synchronous bimodal signal presented to restrained bumble bees did not consistently lead to higher performance, suggesting the potential relevance of other variables, such as stimulus intensity (Gil et al., 2022) and asynchrony of the components (Riveros et al., 2020).

Here, I relied on foragers of the bumble bee *Bombus impatiens*, which are readily trained to learn associations between unimodal or bimodal stimuli and a reward (Riveros and Gronenberg, 2009; Riveros and Gronenberg, 2012; Riveros et al., 2020). I used conditioning of the proboscis extension response (PER) to favor a more accurate control of stimulus delivery (Fig. 1A). I compared the learning performance and latency of conditioned responses across 10 treatments where the olfactory and visual components of a bimodal conditioned stimulus varied from full temporal synchrony to full asynchrony (Fig. 1A). I then compared the acquisition of the individual components by presenting them separately in a sequential, random fashion. This second phase aimed to determine whether acquisition of each component was equally enhanced as a result of the structure of the bimodal stimulus.

I addressed three questions. (1) Do bimodal cues lead to better performance? I hypothesized that bumble bees trained to a bimodal cue would exhibit higher performance as a result of the more reliable information. (2) Does synchrony between components matter? I hypothesized that increasing synchrony would lead to higher performance (Mackintosh, 1974). This hypothesis builds upon the assumption that a higher synchrony would produce a stronger stimulation of a central integrator (stronger sensory binding; Narins et al., 2005) within the nervous system and might contain more information about the contingency between the stimulus and the reward. Within the bee brain, the mushroom bodies function as areas of multimodal integration for olfactory and visual information (Fahrbach, 2006; Gronenberg and López-Riquelme, 2004; Strausfeld et al., 1998, 2009), as well as for information on appetitive rewards. Thus, a higher coincidence should presumably enhance the association. (3) Does identity (modality) within the sequence of components matter? I hypothesized that the performance would be higher when the olfactory component overlapped with the reward and had the longest exposure for the bumble bees ('Odor primary' in the partial synchrony condition and 'First color' in the full asynchrony condition; Fig. 1A). I built my hypothesis upon previous evidence showing that olfactory stimuli lead to higher performance during classical conditioning in bumble bees when used as a unimodal stimulus (Riveros and Gronenberg, 2009, 2012; Riveros et al., 2020).

MATERIALS AND METHODS

Collection and maintenance of bumble bees

Three colonies of the North American bumble bee *Bombus impatiens* Cresson 1863 (Koppert Biological Systems, Howell, MI, USA) were maintained indoors at 20°C on a 12 h:12 h photoperiod. The three colonies were attached to a single foraging flight cage (L×W×H: 100×95×90 cm) where the bumble bees could collect sucrose water (0.5 mol l⁻¹) from an artificial feeder. Pollen was supplied *ad libitum* inside the nest boxes. Bumble bees were collected from the feeders using a customized aspirator and chilled on ice for 10 min, before being harnessed (Riveros and Gronenberg, 2009; Riveros et al., 2020) and left to rest for 4 h until training.

Training apparatus

The training apparatus consisted of 12 individual chambers (each containing a harnessed bumble bee) attached to a rotating platform (Riveros et al., 2020). During training, a chamber was rotated over a platform that contained blue LEDs ($\lambda=470$ nm). Light from the LEDs illuminated the chamber from below and was diffused by aluminium foil coating the inside of each chamber. Thus, bumble bees did not receive light stimulation directly. The LEDs were connected to a power supply and were controlled by individual switches.

Olfactory signals were provided by injecting a stream of 1-hexanol (Sigma-Aldrich; Riveros et al., 2020) into a stream of clean air that was directed into the chamber. A switch that turned on two one-way solenoid valves controlled injection of 1-hexanol. A first valve allowed air to flow from an aquarium pump into a syringe with a piece of filter paper impregnated with 10 μ l of 1-hexanol. A second valve allowed the injection of the odor into the clean stream and toward the training chamber. A series of switches allowed either simultaneous (synchronized) or asynchronous presentation of the olfactory and/or visual stimulus during training.

Training procedure

General protocol

Bumble bee workers were trained to an absolute-conditioning learning task using the PER protocol. In this protocol, the extension of the proboscis in response to antennal stimulation with a sweet solution (unconditioned stimulus, US) is conditioned to the presentation of a stimulus (conditioned stimulus, CS+). Thus, learning is reflected by the extension of the proboscis upon the presentation of the CS alone (i.e. the exhibition of a conditioned response, CR). Specific conditions used in these treatments are described below and illustrated in Fig. 1A. In all cases, bumble bees were presented with six pairings (inter-trial interval: ~10 min) of the US and the CS, with an overlapping reward of sucrose water (1.5 mol l⁻¹) for 2 s at the end of the presentation of the CS. In all training conditions, bumble bees were presented sequentially with the individual components (i.e. light or odor) 2 h after the final training trial: 50% of the bumble bees was presented with the blue light first and 50% with odor first. The second component was presented 10 min after the presentation of the first component.

Training conditions

Bumble bees were randomly assigned to one of the following treatments before starting a training session. (i) Unimodal conditioning: bumble bees received a single rewarded unimodal stimulus (1-Hexanol+ or Blue+) that lasted 12 s. (ii) Fully synchronous signal: bumble bees were presented with a bimodal signal (1-Hexanol/Blue+) that lasted 12 s. (iii) Fully asynchronous

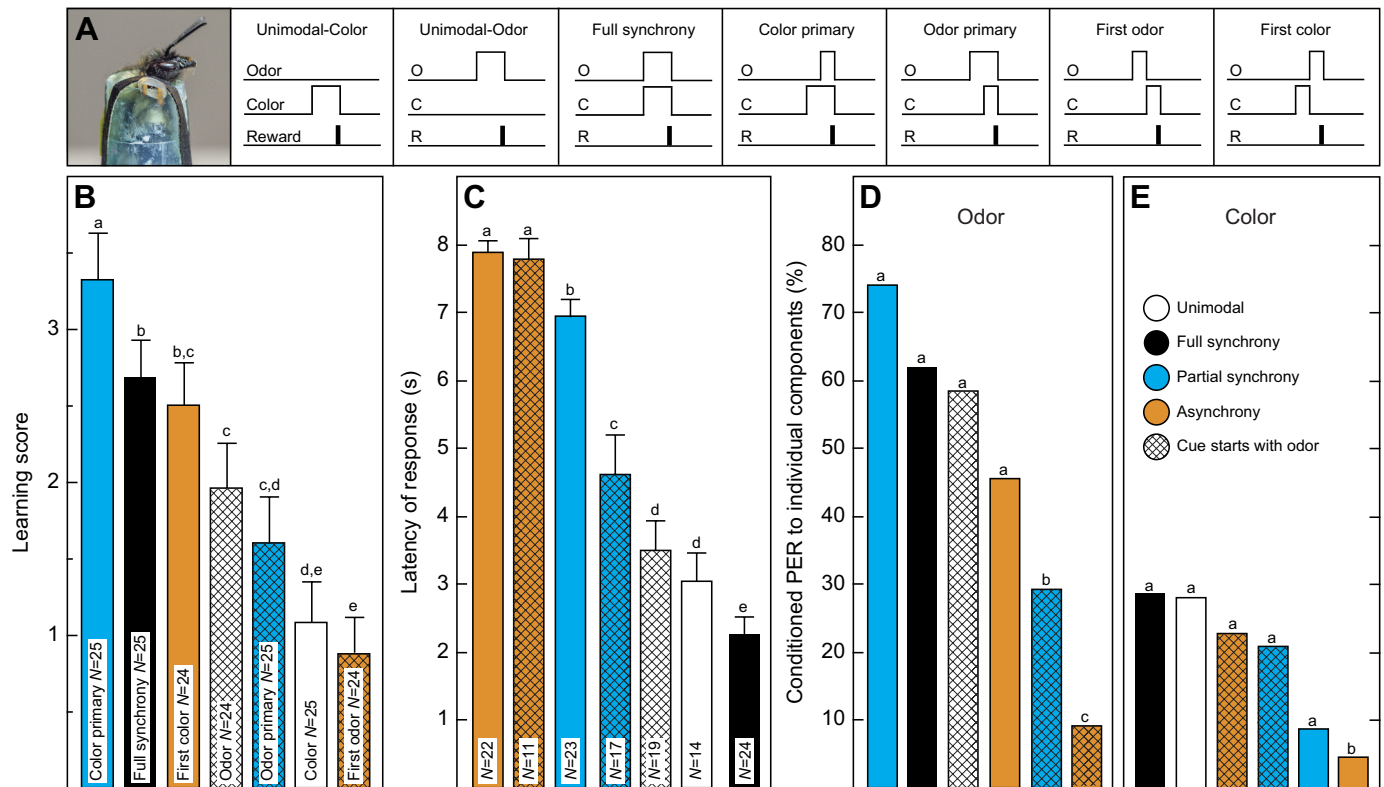


Fig. 1. Performance of foragers and acquisition of individual color and odor components during unimodal and bimodal conditioning.

(A) A harnessed bumble bee (left) and stimulus configurations used for training (right). In all cases, the total exposure time to conditioned stimuli was 12 s. (B) Performance during classical conditioning using the structures presented in A. (C) Latency of response during a conditioned proboscis extension response (PER). (D) Percentage of conditioned PERs after the sole presentation of the olfactory component (1-hexanol) of the stimulus. (E) Percentage of conditioned PER after the sole presentation of the visual (blue light) component of the stimulus. Bars displays means \pm s.e.m. Different letters indicate statistically significant differences ($P < 0.05$) after correction for multiple comparisons (two-stage sharpened false discovery rate) of one- or two-sided tests (see Materials and Methods). Picture in A by Wulfila Gronenberg.

signal: bumble bees were presented with a first stimulus (either 1-Hexanol or Blue) for 6 s followed by the immediate sequential presentation of the second stimulus (either 1-Hexanol or Blue) for 6 s. Thus, the reward only overlapped with the second stimulus presented. Treatments are referred to as ‘First color’ and ‘First odor’ indicating the component that was presented first. (iv) Partially synchronous signal: bumble bees were presented with a first stimulus (either 1-Hexanol or Blue) for 12 s. After 6 s from the onset, a second stimulus (either 1-Hexanol or Blue) was introduced. Thus, the reward overlapped with the bimodal signal. Treatments in this procedure are referred to as ‘Color primary’ and ‘Odor primary’ indicating the component that was presented first.

Data analyses

For analyses, only bumble bees exhibiting a response to the sucrose solution across all training trials and test trials were included. For each bumble bee a learning score between 0 (no conditioned PER across the five trials) and 5 (conditioned response across all five trials) was calculated. The first training trial was not considered in the score and was used to determine that bumble bees did not have a spontaneous response to the conditioned stimulus. I conducted comparisons between the scores of bumble bees in unimodal and bimodal treatments using a one-sided non-parametric test with control (odor, color; Steel method) with the prediction that bumble bees in the bimodal group would exhibit a higher score. The scores between the rest of the planned comparisons were evaluated using the non-parametric Wilcoxon test (one- or two-sided P -values

depending upon predictions as indicated in the Introduction) following a Shapiro–Wilk W -test for normality. Latency of response was recorded as a continuous variable between 0.5 and 12 s using a sound signal emitted by a metronome set at 2 Hz. At least two conditioned responses were averaged (i.e. bumble bees with only a single conditioned PER were not included in the latency analyses). Comparisons of response latencies were performed using a Wilcoxon test. Body size, as estimated from head width, was compared across groups using an ANOVA following a test of normality using a Shapiro–Wilk W -test. Acquisition of individual components of the stimuli was derived from the percentage of conditioned responses to presentations of either color or odor. I compared the probabilities of a conditioned response between pairs of treatments using a two-sample test for proportions (adjusted Wald test). In all cases, error due to multiple comparisons was controlled using the classical one-stage false discovery rate method (Pike, 2011; Verhoeven et al., 2005) and the corrected P -values (q -values) are presented. All the analyses were done using JMP v.16.2.0 (SAS Institute).

RESULTS

A total of 180 individuals were trained but eight bumble bees that did not consistently exhibit a PER to the sucrose solution were excluded. Thus, analyses were conducted using 172 bumble bees distributed between seven treatments: Color: $N=25$; Odor: 24; Full synchrony: $N=25$; First color: $N=24$; First odor: $N=24$; Color primary: $N=25$; Odor primary: $N=25$. Mean body size (head width)

did not significantly differ across groups (mean±s.e.m. Color: 3.74±0.04 mm; Odor: 3.76±0.05 mm; Full synchrony: 3.80±0.06 mm; First color: 3.74±0.05 mm; First odor: 3.78±0.06 mm; Color primary: 3.78±0.06 mm; Odor primary: 3.79±0.05 mm; Shapiro–Wilk: $W=0.99$, $P=0.25$; ANOVA: $F_{6,165}=0.22$, $P=0.97$), hence ruling out this factor as potentially explanatory of the observed differences.

Do bimodal signals lead to better acquisition?

First, I evaluated whether the bimodal stimuli led to better performance than the unimodal stimuli. To address this question, I compared the learning score of bumble bees exposed to the unimodal stimuli (color, odor) with the performance of bumble bees trained under the five bimodal treatments. I found that the mean score of bumble bees trained only with the olfactory component did not generally differ from that of those trained with the bimodal cue (Fig. 1B). Only in the treatment Color primary did bumble bees exhibit learning scores that were significantly higher (Steel method, Odor versus Color primary: $Z=3.0$, $P=0.03$; Fig. 1B). Also, only in the treatment First odor did bumble bees exhibit learning scores that were significantly lower (Steel method, Odor versus First odor: $Z=-2.66$, $P=0.04$; Fig. 1B). When compared with the performance of bumble bees trained only to the visual component, I found significant differences in three out of the five comparisons. Bumble bees in the unimodal (Color) group exhibited significantly lower performance than bees in the Color primary (Steel method, Color versus Color primary: $Z=4.24$, $P=0.0002$; Fig. 1B), Full synchrony (Steel method, Color versus Color primary: $Z=3.82$, $P=0.0008$; Fig. 1B) and First color groups (Steel method, Color versus Color primary: $Z=-3.40$, $P=0.003$; Fig. 1B).

Also, training with the bimodal cue did not generally lead to faster conditioned responses or to better acquisition of the visual or olfactory elements. On the one hand, the fastest and slowest conditioned responses were observed in bumble bees trained using a bimodal cue (mean±s.e.m.: First color: 7.9±0.18 s; First odor: 7.8±0.3 s; Full synchrony: 2.2±0.26 s; Fig. 1C) with a broad variation across the rest of treatments (Fig. 1C). On the other hand, the acquisition of the color component by bumble bees was not significantly different across groups (Fig. 1E), except for the bumble bees in the ‘First color’ group, which barely responded to the color during the test (Fig. 1E). Similarly, the acquisition of the olfactory component was not generally different in bumble bees across groups, although it was significantly lower than the unimodal in the bumble bees trained with the ‘Odor primary’ (adjusted Wald test, Odor versus Odor primary: $P=0.05$; Fig. 1D) and ‘First odor’ treatments (adjusted Wald test, Odor versus First odor: $P=0.0005$; Fig. 1D).

Thus, bumble bees trained with the bimodal cues did not generally perform better than bumble bees trained with the unimodal components. One may argue that the stimuli with full asynchrony (First odor, First color), with bumble bees exhibiting the highest or lowest performance (Fig. 1B), are not strictly bimodal as they lack temporal overlap between the components, and the reward is offered only while one component is present (Fig. 1A). However, the latency of response was significantly different from that of unimodal treatments (Fig. 1C). Also, in both cases the performance of the bumble bees was significantly affected relative to the unimodal stimuli; yet, the effect followed opposite directions, leading to an enhancement (First color; Fig. 1B) or a depression (First odor; Fig. 1B) of the acquisition. These significant effects suggest an interaction of the visual and olfactory components even without any overlap. Hence, together these results support a rejection of the hypothesis that bimodal signaling generally

enhances performance relative to unimodal signaling (Riveros et al., 2020). Rather, as shown next, these results suggest that the combination between synchrony and the internal structure of the olfactory and visual components determines performance.

Does synchrony between components matter?

I hypothesized that learning the more synchronous stimuli would lead to better performance under the assumption that higher coincidence should enhance the strength of the association (Mackintosh, 1974). Thus, one would expect the bumble bees in the Full synchrony group to exhibit the best performance in score, speed of conditioned response and acquisition of components. Another point to consider is that one might focus the comparisons based on whether the ‘lead’ component (i.e. the one presented first) occurs during the reward delivery. For example, bumble bees in the Color primary and First color groups had in common that they received the color component first. Yet, bumble bees in the latter group received the reward while exposed only to the olfactory component (Fig. 1A). Alternatively, one might consider the comparisons based on which component is rewarded. For example, bumble bees in the Color primary and First odor groups had in common that they received the reward while being stimulated with the color component. Yet, the latter received the olfactory component before receiving the visual component (Fig. 1A). For the following analysis, I focused on the first view because my interest was also to evaluate the support provided across modalities. For instance, by addressing the comparison Color primary versus First color, I could test the importance of color as the lead component in learning the odor even if the color was not present during the reward delivery (and vice versa).

I found that the effect of synchrony varied between the conditions. For example, within the ‘color conditions’ (when color was presented first), increasing synchrony did not enhance performance. In fact, it was the intermediate level of synchrony (Color primary) that rendered the highest learning score while there was no difference in performance between the bumble bees using full synchrony versus full asynchrony (Fig. 1B). Moreover, the acquisition of neither the olfactory nor the color components was affected by the synchrony (Fig. 1D,E). In contrast, the results followed the hypothesis within the ‘odor conditions’ (when odor was presented first), such that increasing synchrony led to higher performance (Fig. 1B) and higher acquisition of the odor component (see First odor, Odor primary and Full synchrony groups; Fig. 1D). Remarkably, while the bumble bees in the Full synchrony group did not exhibit the highest performance (Fig. 1B), they had the fastest conditioned PERs (Fig. 1C). In contrast, bumble bees in both groups of full asynchronies exhibited the slower conditioned PERs (Fig. 1C).

Hence, synchrony was a factor that significantly impacted performance, particularly overall learning, and latency of response. For these two variables, increasing synchrony generally enhanced performance (higher scores and shorter latencies of response). A remarkable exception was the bumble bees in the Color primary group, which had the highest learning scores and intermediate levels of latency of response. Moreover, these results suggest that for the bumble bees it was not a mere presentation of a sequence of independent components; rather, the presentation of a first component clearly set the acquisition of the second component (Mota et al., 2011). Also, occurrence during the reward delivery was essential for a component to be learned, but did not necessarily imply its acquisition. For instance, bumble bees in the Color primary group did not learn the color component as well as the odor

component, despite the color being presented for a longer time and occurring during the reward (Fig. 1B).

Does identity within the sequence of component presentation matter?

For this last question, the data should be differentiated across comparisons within ‘partial synchrony’ and ‘full asynchrony’ conditions to rule out the effect of synchrony. I hypothesized that the performance would be favored when the olfactory component overlapped with the reward (First color, Color primary, Odor primary) and had the longest exposure for the bumble bees (Odor primary). Thus, bumble bees in the Odor primary group were expected to perform the best. Interestingly, I found support for this hypothesis only within the full asynchrony condition, with a higher performance (Wilcoxon test, First odor versus First color: $Z=-3.78$, $P=0.0001$; Fig. 1B) and acquisition of the odor component exhibited by the bumble bees receiving first the color followed by a rewarded presentation of odor (adjusted Wald test, First odor versus First color: $P=0.003$; Fig. 1D). Surprisingly, the bumble bees in the Color primary treatment exhibited significantly higher performance than bumble bees in the Odor primary group, despite not having the longest exposure of odor (Wilcoxon test, Color primary versus Odor primary: $Z=-3.48$, $P=0.0005$; Fig. 1B). Yet, the bumble bees in the Odor primary treatment exhibited significantly faster conditioned responses than bumble bees in the Color primary treatment, indicating faster responses but lower acquisition (Wilcoxon test, Color primary versus Odor primary: $Z=-2.89$, $P=0.004$).

Interestingly, the bumble bees in the two asynchronous treatments exhibited the longest latencies of responses (Fig. 1C). A particular case are the bumble bees in the First odor group, which exhibited the lowest learning score (Fig. 1B) and the slowest latency of responses (Fig. 1C). In this case, the bumble bees were rewarded while receiving the color component (Fig. 1A), but even bumble bees in the unimodal color treatment exhibited faster responses (Fig. 1C).

DISCUSSION

Taken together, the analyses of the three questions posted above indicate that it is the interaction between synchrony and the internal structure of the signal (olfactory and visual components) that determines the enhancement led by the bimodal stimuli. Asynchronous presentation of a visual component followed by an overlapped olfactory component (Color primary) is the most effective bimodal structure for the overall performance. Most remarkably, this bimodal structure further enhances the acquisition of the olfactory, but not the visual, component.

The enhanced learning through more synchrony and the presentation of a color component first may result from increased attention and a higher probability of sensory binding (Leonard et al., 2011c; Mota et al., 2011; Rowe, 1999). First, presenting color may activate the neural network and facilitate the acquisition of the olfactory component, or of the full configuration (Mota et al., 2011). As a caveat to this argument, the olfactory component failed to boost such an attentional effect for the acquisition of color or of the overall bimodal stimulus (Fig. 1B). In fact, presenting the odor first even had a negative effect on acquisition of the overall bimodal stimuli. However, the low acquisition of the visual component is not surprising as harnessed bumble bees generally have lower performance during color learning conditioning (Riveros and Gronenberg, 2012). Indeed, even the bumble bees trained to the unimodal color exhibited low percentages of response to color after

training (Fig. 1E). Not surprisingly either, bumble bees in the First color treatment exhibited the lowest response to color as the reward was offered while the odor, not the color, was presented (Fig. 1E). Second, higher synchrony would produce a stronger stimulation of a central integrator (stronger sensory binding; Halfwerk et al., 2019) within the nervous system (e.g. mushroom bodies) and might contain more information about the contingency stimulus–reward (Rowe, 1999). Clearly, the Color primary treatment represents an exception that deserves further research. Thus, these results suggest that the effect of synchrony depends on the identity of the first component presented and that the interactions between components are synergistic and complex rather than simply additive.

Remarkably, the present results showing the effect of partial synchrony reconcile with the enhancement of multimodality observed in free flight (Kulahci et al., 2008). The results showing the effect of asynchrony support a more conspicuous effect of multimodality in free-flying protocols (typically showing an enhancement; Kulahci et al., 2008) than under restrained conditions (showing a larger variability; Riveros et al., 2020). Flying pollinators likely receive multimodal stimuli with varying levels of synchrony as some flowers have scents perceived at a distance but visual displays perceived at close range (e.g. citrus flower), while others exhibit the reverse pattern (e.g. sunflower). Pollinators may follow odor plumes in order to arrive at flowers before perceiving visual patterns (Riffell et al., 2014), suggesting that one component may serve first for navigation and then as part of a multimodal signal (Buehlmann et al., 2020a; Halfwerk et al., 2019). During experiments using floral patches, perception of visual information is favored unless odor plumes are offered (Riffell et al., 2014). Nevertheless, while reconciling with the enhancement observed in previous accounts, the present results contrast with observations in the moth *Manduca sexta* suggesting that odor works as the ‘enhancer’ (Goyret et al., 2007). Whether this difference reflects divergent adaptations to crepuscular versus diurnal foraging requires further investigation.

Importantly, functional hypotheses on multimodal signaling also consider the speed of the response (Leonard et al., 2011c; Rowe and Halpin, 2013). In the present case, I measured the latency of the conditioned PER following the onset of stimulus presentation. Efficacy-based hypotheses posit that the latency of response would be shorter when using multimodal signals if detection is enhanced or processing is faster as a result of parallel neural pathways (Kulahci et al., 2008; Leonard et al., 2011c). Considering the current experimental setup that fully exposes individuals to the stimuli, I favor the possibility that processing and not detection was affected by the conditioning treatments. Intriguingly, the latency of response of bumble bees was positively affected (i.e. was shorter) with synchrony and unimodality. Bumble bees in the Full synchrony group exhibited the fastest responses (Fig. 1C), suggesting an efficient parallel processing (Kulahci et al., 2008). In contrast, bumble bees in the First odor and First color treatments exhibited mean latencies that surpassed the sum of the mean processing times of unimodal components (Fig. 1C), most likely reflecting the sequential processing induced by the structure of the signal (Kulahci et al., 2008).

Most interestingly, the latencies of the two treatments with partial synchrony were intermediate and differed from each other and from the rest. The difference between them, along with their learning scores and the acquisition of components, led to a rejection of the hypothesis that individuals trained with a stimulus differing across its presentation (e.g. first color and then odor overlapped) learn the latest part of the stimulus (Mackintosh, 1974). Instead, the latency of

response suggests that bumble bees in these treatments followed a speed–accuracy tradeoff and favors the idea of an increase in attention associated with the presentation of the color but not of the odor. Bumble bees in the Color primary group exhibited slower responses (Fig. 1C) and high performance (Fig. 1B), while bumble bees in the Odor primary group exhibited faster responses (Fig. 1C) and lower performance (Fig. 1B). Moreover, bumble bees in the Color primary group exhibited rapid responses to the odor introduced after 6 s (Color primary: mean±s.e.m. latency 6.9±0.3 s; i.e. 0.9 s after odor introduction), which was even faster than responses exhibited by bumble bees trained only with odor (Odor: latency 3.5±0.4 s). This suggests a degree of sustained attention maintained by the color stimulation.

As a final point, 19% of all the bumble bees tested across bimodal treatments did not respond to the individual components, only to the bimodal cue (i.e. conditioned responses were elicited only by the presentation of the configuration). Also, 11% of all the bumble bees tested across bimodal treatments responded to both components when tested separately. Interestingly, we previously reported this diversity in the responses using different bimodal combinations with restrained bumble bees (Riveros et al., 2020), which suggests that it is not an isolated finding. This behavioral diversity may reflect different learning strategies supported by the specialized unimodal and bimodal responses observed in neurons within the mushroom bodies (Strube-Bloss and Rössler, 2018). Similar diversity in responses using configural learning or components of the signal occurs in a context-dependent fashion during navigation in ants (Buehlmann et al., 2020b). However, in this restricted experimental setup, context dependency cannot explain the results here and in previous accounts (Riveros et al., 2020). Nevertheless, these strategies may have contrasting processing costs. Multimodal neurons within the mushroom bodies may be allocated to learning the bimodal configuration (Devaud et al., 2015; Strube-Bloss and Rössler, 2018), decreasing the cost of separate processing. In contrast, the costs may be higher if separate networks are used every time the stimulus is presented, even if a benefit for redundancy is argued. Moreover, learning a configuration enables individuals to solve non-elemental learning tasks, which cannot be solved if components are learned separately (Devaud et al., 2015; Giurfa, 2003), and enhances discrimination power.

I conclude that the performance of the bumble bee *B. impatiens* while learning a bimodal signal depends on the temporal configuration and the identity of the components within the signal. The demonstration of the relevance of the temporal dimension during the perception of signals under restrained conditions and across multiple sensory channels is central to incorporating mechanistic approaches and to supporting efforts examining the factors that underlie the construction of complex communicative representations during the coevolution between signal senders and receivers (Caldart et al., 2022; Halfwerk et al., 2019; Rowe, 1999).

Acknowledgements

The author thanks Annie Leonard for continuous support during the conceptualization, design and development of the experiments and for comments on earlier versions of the manuscript. The author also thanks Wulfila Gronenberg for comments on an earlier version of the manuscript and the photo in Fig. 1A.

Competing interests

The author declares no competing or financial interests.

Funding

This project was funded by the Leonard's lab at the University of Nevada, Reno. Open Access funding provided by University of Arizona. Deposited in PMC for immediate release.

Data availability

All relevant data can be found within the article and its supplementary information.

References

- Buehlmann, C., Mangan, M. and Graham, P. (2020a). Multimodal interactions in insect navigation. *Anim. Cogn.* **23**, 1129–1141. doi:10.1007/s10071-020-01383-2
- Buehlmann, C., Aussen, A. and Graham, P. (2020b). Dynamic multimodal interactions in navigating wood ants: what do path details tell us about cue integration? *J. Exp. Biol.* **223**, jeb221036. doi:10.1242/jeb.221036
- Caldart, V. M., Dos Santos, M. B. and Machado, G. (2022). Function of a multimodal signal: a multiple hypothesis test using a robot frog. *J. Anim. Ecol.* **91**, 701–714. doi:10.1111/1365-2656.13620
- Dacke, M., Bell, A. T. A., Foster, J. J., Baird, E. J., Strube-Bloss, M. F., Byrne, M. J. and El Jundi, B. (2019). Multimodal cue integration in the dung beetle compass. *Proc. Natl. Acad. Sci. USA* **116**, 14248–14253. doi:10.1073/pnas.1904308116
- Devaud, J. M., Papouin, T., Carcaud, J., Sandoz, J. C., Grönewald, B. and Giurfa, M. (2015). Neural substrate for higher-order learning in an insect: mushroom bodies are necessary for configural discriminations. *Proc. Natl. Acad. Sci. USA* **112**, E5854–E5862. doi:10.1073/pnas.1508422112
- Fahrbach, S. E. (2006). Structure of the mushroom bodies of the insect brain. *Annu. Rev. Entomol.* **51**, 209–232. doi:10.1146/annurev.ento.51.110104.150954
- Flanigan, K. A. S., Wiegmann, D. D., Hebets, E. A. and Bingman, V. P. (2021). Multisensory integration supports configural learning of a home refuge in the whip spider *Phrynus marginemaculatus*. *J. Exp. Biol.* **224**, jeb238444. doi:10.1242/jeb.238444
- Gil-Guevara, O., Bernal, H. A. and Riveros, A. J. (2022). Honey bees respond to multimodal stimuli following the principle of inverse effectiveness. *J. Exp. Biol.* **225**, jeb243832. doi:10.1242/jeb.243832
- Giurfa, M. (2003). Cognitive neuroethology: dissecting non-elemental learning in a honeybee brain. *Curr. Opin. Neurobiol.* **13**, 726–735. doi:10.1016/j.conb.2003.10.015
- Giurfa, M., Núñez, J., Chittka, L. and Menzel, R. (1995). Colour preferences of flower-naïve honeybees. *J. Comp. Physiol. A* **177**, 247–259. doi:10.1007/BF00192415
- Goyret, J., Markwell, P. M. and Raguso, R. A. (2007). The effect of decoupling olfactory and visual stimuli on the foraging behavior of *Manduca sexta*. *J. Exp. Biol.* **210**, 1398–1405. doi:10.1242/jeb.02752
- Gronenberg, W. and López-Riquelme, G. O. (2004). Multisensory convergence in the mushroom bodies of ants and bees. *Acta Biol. Hung.* **55**, 31–37. doi:10.1556/ABiol.55.2004.1-4.5
- Guilford, T. and Dawkins, M. S. (1991). Receiver psychology and the evolution of animal signals. *Anim. Behav.* **42**, 1–14. doi:10.1016/S0003-3472(05)80600-1
- Halfwerk, W., Varkevisser, J., Simon, R., Mendoza, E., Scharff, C. and Riebel, K. (2019). Toward testing for multimodal perception of mating signals. *Front. Ecol. Evol.* **7**, 124. doi:10.3389/fevo.2019.00124
- Hebets, E. A. (2005). Attention-altering signal interactions in the multimodal courtship display of the wolf spider *Schizocosa uetzi*. *Behav. Ecol.* **16**, 75–82. doi:10.1093/beheco/arl133
- Hebets, E. A. and Papaj, D. R. (2005). Complex signal function: developing a framework of testable hypotheses. *Behav. Ecol. Sociobiol.* **57**, 197–214. doi:10.1007/s00265-004-0865-7
- Kulachi, I. G., Dornhaus, A. and Papaj, D. R. (2008). Multimodal signals enhance decision making in foraging bumble-bees. *Proc. R. Soc. B Biol. Sci.* **275**, 797–802. doi:10.1098/rspb.2007.1176
- Kunze, J. and Gumbert, A. (2001). The combined effect of color and odor on flower choice behavior of bumble bees in flower mimicry systems. *Behav. Ecol.* **12**, 447–456. doi:10.1093/beheco/12.4.447
- Leonard, A. S. and Masek, P. (2014). Multisensory integration of colors and scents: insights from bees and flowers. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **200**, 463–474. doi:10.1007/s00359-014-0904-4
- Leonard, A. S., Dornhaus, A. and Papaj, D. R. (2011a). Flowers help bees cope with uncertainty: signal detection and the function of floral complexity. *J. Exp. Biol.* **214**, 113–121. doi:10.1242/jeb.047407
- Leonard, A. S., Dornhaus, A. and Papaj, D. R. (2011b). Forget-me-not: complex floral displays, inter-signal interactions, and pollinator cognition. *Curr. Zool.* **57**, 113–121. doi:10.1093/czoolo/57.2.215
- Leonard, A. S., Dornhaus, A. and Papaj, D. R. (2011c). Why are floral signals complex? An outline of functional hypotheses. In *The Systematics Association Special Volume 81: Evolution of Plant-Pollinator Relationships* (ed. S. Patiny), pp. 279–300. Cambridge University Press.
- Mackintosh, N. J. (1974). *The Psychology of Learning*. London: Academic Press.
- Mitoyen, C., Quigley, C. and Fusani, L. (2019). Evolution and function of multimodal courtship displays. *Ethology* **125**, 503–515. doi:10.1111/eth.12882
- Mota, T., Giurfa, M. and Sandoz, J. C. (2011). Color modulates olfactory learning in honeybees by an occasion-setting mechanism. *Learn. Mem.* **18**, 144–155. doi:10.1101/lm.2073511

- Narins, P. M., Hödl, W. and Grabul, D. S.** (2003). Bimodal signal requisite for agonistic behavior in a dart-poison frog, *Epipedobates femoralis*. *Proc. Natl. Acad. Sci. USA* **100**, 577-580. doi:10.1073/pnas.0237165100
- Narins, P. M., Grabul, D. S., Soma, K. K., Gaucher, P. and Hödl, W.** (2005). Cross-modal integration in a dart-poison frog. *Proc. Natl. Acad. Sci. USA* **102**, 2425-2429. doi:10.1073/pnas.0406407102
- Pike, N.** (2011). Using false discovery rates for multiple comparisons in ecology and evolution. *Methods Ecol. Evol.* **2**, 278-282. doi:10.1111/j.2041-210X.2010.00061.x
- Raguso, R. A.** (2004). Flowers as sensory billboards: progress towards an integrated understanding of floral advertisement. *Curr. Opin. Plant Biol.* **7**, 434-440. doi:10.1016/j.pbi.2004.05.010
- Raguso, R. A. and Willis, M. A.** (2005). Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, *Manduca sexta*. *Anim. Behav.* **69**, 407-418. doi:10.1016/j.anbehav.2004.04.015
- Rek, P.** (2018). Multimodal coordination enhances the responses to an avian duet. *Behav. Ecol.* **29**, 411-417. doi:10.1093/beheco/axx174
- Riffell, J. A., Shlizerman, E., Sanders, E., Abrell, L., Medina, B., Hinterwirth, A. J. and Kutz, J. N.** (2014). Flower discrimination by pollinators in a dynamic chemical environment. *Science (80-)*. **344**, 1515-1518. doi:10.1126/science.1251041
- Riveros, A. J. and Gronenberg, W.** (2009). Olfactory learning and memory in the bumblebee *Bombus occidentalis*. *Naturwissenschaften* **96**, 851-856. doi:10.1007/s00114-009-0532-y
- Riveros, A. J. and Gronenberg, W.** (2012). Decision-making and associative color learning in harnessed bumblebees (*Bombus impatiens*). *Anim. Cogn.* **15**, 1183-1193. doi:10.1007/s10071-012-0542-6
- Riveros, A. J., Leonard, A. S., Gronenberg, W. and Papaj, D. R.** (2020). Learning of bimodal versus unimodal signals in restrained bumble bees. *J. Exp. Biol.* **223**, jeb220103. doi:10.1242/jeb.220103
- Rojas, B., Burdfield-Steel, E., De Pasqual, C., Gordon, S., Hernández, L., Mappes, J., Nokelainen, O., Rönkä, K. and Lindstedt, C.** (2018). Multimodal aposematic signals and their emerging role in mate attraction. *Front. Ecol. Evol.* **6**, 93. doi:10.3389/fevo.2018.0009
- Ronald, K. L., Zeng, R., White, D. J., Fernández-Juricic, E. and Lucas, J. R.** (2017). What makes a multimodal signal attractive? A preference function approach. *Behav. Ecol.* **28**, 677-687. doi:10.1093/beheco/ax015
- Rowe, C.** (1999). Receiver psychology and the evolution of multicomponent signals. *Anim. Behav.* **58**, 921-931. doi:10.1006/anbe.1999.1242
- Rowe, C. and Halpin, C.** (2013). Why are warning displays multimodal? *Behav. Ecol. Sociobiol.* **67**, 1425-1439. doi:10.1007/s00265-013-1515-8
- Srygley, R. B.** (1999). Locomotor mimicry in *Heliconius* butterflies: contrast analyses of flight morphology and kinematics. *Philos. Trans. R. Soc. B Biol. Sci* **354**, 203-214. doi:10.1098/rstb.1999.0372
- Strausfeld, N. J., Hansen, L., Li, Y., Gomez, R. S. and Ito, K.** (1998). Evolution, discovery, and interpretations of arthropod mushroom bodies. *Learn. Mem.* **5**, 11-37. doi:10.1101/lm.5.1.11
- Strausfeld, N. J., Sinakevitch, I., Brown, S. M. and Farris, S. M.** (2009). Ground plan of the insect mushroom body: functional and evolutionary implications. *J. Comp. Neurol.* **513**, 265-291. doi:10.1002/cne.21948
- Steel, R. G. D.** (1959). A multiple comparison rank sum test: treatments versus control. *Biometrics* **15**, 560-572.
- Strube-Bloss, M. F. and Rössler, W.** (2018). Multimodal integration and stimulus categorization in putative mushroom body output neurons of the honeybee. *R. Soc. Open Sci.* **5**, 171785. doi:10.1098/rsos.171785
- Verhoeven, K. J. F., Simonsen, K. L. and McIntyre, L. M.** (2005). Implementing false discovery rate control: increasing your power. *Oikos* **108**, 643-647. doi:10.1111/j.0030-1299.2005.13727.x
- Wright, G. A., Carlton, M. and Smith, B. H.** (2009). A honeybee's ability to learn, recognize, and discriminate odors depends upon odor sampling time and concentration. *Behav. Neurosci.* **123**, 36-43. doi:10.1037/a0014040