How bumblebees manage conflicting information seen on arrival and departure from flowers

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Abstract

Bees are flexible and adaptive learners, capable of learning stimuli seen on arrival and at departure from flowers where they have fed. This gives bees the potential to learn all information associated with a feeding event, but it also presents the challenge of managing information that is irrelevant, inconsistent, or conflicting. Here, we examined how presenting bumblebees with conflicting information before and after feeding influenced their learning rate and what they learned. Bees were trained to feeder stations mounted in front of a computer monitor. Visual stimuli were displayed behind each feeder station on the monitor. Positively reinforced stimuli (CS+) marked feeders offering sucrose solution. Negatively reinforced stimuli (CS-) marked feeders offering quinine solution. While alighted at the feeder station the stimuli were not visible to the bee. The "constant stimulus" training group saw the same stimulus throughout. For the "switched stimulus" training group, the CS+ changed to the CS- during feeding. Learning was slower in the "switched stimulus" training group compared to the constant stimulus" group, but the training groups did not differ in their learning performance or the extent to which they generalised their learning. The information conflict in the "switched stimulus" group did not interfere with what had been learned. Differences between the "switched" and "constant stimulus" groups were greater for bees trained on a horizontal CS+ than a vertical CS+ suggesting bees differ in their processing of vertically and horizontally oriented stimuli. We discuss how bumblebees might resolve this type of information conflict so effectively, drawing on the known neurobiology of their visual learning system.

Introduction

Bees are excellent learners. In nature, their ability to successfully forage relies on their capacity to identify, memorise and return to high quality flowers (Grant 1950). In simple associative paradigms, just three pairings of an odour (Menzel, 1999, Menzel 2001, Giurfa & Sandoz 2012) or colour (Avarguès-Weber and Giurfa 2014, Muth et al. 2015) with sucrose solution reward is sufficient to establish a lifelong memory in a foraging honey bee. In a classical associative task, the conditioned stimulus (CS) precedes and overlaps with the unconditioned stimulus (US) such that bees learn a tight temporal relationship with the conditioned stimulus predicting the unconditioned stimulus (Menzel 1993, Hammer & Menzel 1995). Bees can learn much more than just this temporal contingency, however. Bees can generalise learned relationships (Giurfa et al. 2001, Bernard et al. 2006). Bees are capable of trace conditioning (Menzel 2001, Szyska *et al.* 2011, Paoli et al. 2023), where there is a gap between the presentation of the CS and US. They can learn conditioned stimuli presented after the US (Menzel 2001, Hussaini et al. 2007). They are capable of latent learning where there is no explicit reinforcement (Menzel et al. 1993, Wystrach *et al.* 2023). And they are capable of taste aversion learning in which a tastant causes a malaise after a significant delay (Wright et al. 2010, Hurst et al. 2014). All of these are considered cognitive forms of learning. They give bees great flexibility and capacity to recognise and learn relationships between relevant stimuli, but this flexibility also presents a cognitive challenge. Some relationships between CS and US could be inconsistent, or even contradictory and these could interfere with a bee learning the most useful relationships between CS and US (Menzel 2001, Giurfa *et al.* 2012). In this study, we examined how inconsistent information affected learning in bumblebees to assess how well an insect brain can manage information conflict.

Classical associative learning is typically explained by Hebbian processes and spike-timing dependent neuroplasticity (Hebbian mechanisms: Caporale *et al.* 2008, Johansen et al. 2014, bees neurobiological support: Rath et al. 2011, Galizia *et al.* 2014). Simply put, the connection between neural circuits for the CS and the conditioned response is modified by the co-activated US. In insects, there are several loci for this type of learning, including the antennal lobes and the mushroom bodies (Galizia *et al.* 2014). Other types of learning are considered more complex because something more than this simple type of learning is needed to explain them. For example, in trace conditioning there is a temporal gap between the presentation of the CS and US. In humans and other mammals, trace conditioning is presumed to demand a higher level of cognitive processing, perhaps even involving forms of declarative memory or conscious processing (Clark and Squire, 1998; Lovibond and Shanks, 2002, Birch et al. 2020, Droege et al. 2021). At the very least, it requires some form of enduring neural engram of the CS that persists beyond the presentation of the CS such that it can be related to the later US. Such engrams have been identified in the brains of insects (Menzel 2001, Menzel and Giurfa 2001, Perisse *et al.* 2011).

Lehrer (Lehrer 1991, Lehrer 1993) provided an early and influential demonstration of cognitive flexibility in honey bee learning while questioning of the efficiency of CS before and after US. Lehrer noticed that upon departing a flower on which a bee had just fed, often the bee would pause in flight and "turn back and look at the flower" (Lehrer 1991, Lehrer 1993). This motivated Lehrer to study whether bees were learning the features of a flower on approach or departure or both. By manipulating stimuli seen on arrival and departure from the flower, Lehrer was able to show that bees could learn stimuli seen on both arrival and departure from a rewarded flower (Lehrer 1993). If stimuli seen on arrival and departure where inconsistent, then bees preferred the stimulus seen on arrival over the stimulus seen on departure (Lehrer 1993). Given that bees can learn stimuli that both precede and succeed a food reward, our objective here was to study how conflicting information presented before and after feeding influenced the speed of learning and what bees learned. Bees were trained to feed from Perspex cubes mounted in front of digital displays that allowed stimuli to be instantly changed. While feeding, bees could not see the stimuli, and with this system we could precisely change the stimuli bees saw on arrival and upon departure from the feeder. We used a discriminant learning paradigm in which CS + was rewarded with sugar and CS- was punished with quinine. We compared the learning of bees that saw a consistent CS on arrival and departure from a sucrose feeder with those that experienced the CS + on arrival but the CS- on departure from the feeder.

Material and methods

Bumblebees (*Bombus terrestris audax*) from seven colonies provided by BIOBEST (Biobest Belgium N.V., Westerlo, Belgium) were used. Each colony was housed in a wooden nest box (28 cm L × 16 cm W × 11 cm H). The nest box was connected to a Perspex tunnel leading to a flight arena (60 cm L × 60cm W × 40cm H). Within the flight arena, workers could freely forage for 30% sucrose solution (w/w) from eight transparent feeding cubes (rectangular cuboids to be exact with the following measures 1.5cm^2 0.8 cm H, with a hole 0.6cm ⌀ and 0.3cm deep). These feeding stations were fixed vertically to a transparent Perspex wall in front of a computer screen displaying eight blue circles set against a red environment (Fig. 1a). The walls of the flight arena were covered with a laminated pink and white Gaussian dot pattern to provide optic flow for the bees and create contrast between the bee body and the background for

video tracking. The arena was illuminated using high-frequency fluorescent lighting (TMS 24F lamps with HF-B 236 TLD ballasts, Phillips, Netherland and fitted with Activa daylight fluorescent tubes, Osram, Germany). Both lights operated at a frequency of approximately ~ 42kHz. The high-resolution LCD monitors (Acer Predator GN246HLB) employed to display the visual stimuli boasted a refresh rate of 144Hz significantly suppressing the flicker fusion frequency known for bees (Srinivasan and Lehrer 1984, Skorupski and Chittka 2010). Flight trajectories of bees were recorded by an iPhone camera (iPhone 6, Apple) placed at the rear of the arena, filming at 120 frame per second (fps). Lehrer (1993) used a binary choice apparatus. Our approach used a multiple-choice apparatus to provide more natural foraging environment for bees. Previous work (Chandra et al. 1998), shown that multiple-choice paradigms consistently yield more precise behavioural results, and faster learning rate (Guiraud et al. 2022).

The small volume of sugar solution (10 µL) deposited onto each Perspex cube, was well under the crop capacity of bumblebees, which encouraged bees to visit multiple feeders during a single foraging trip. Workers successfully using the feeders were marked with coloured number tags (Opalithplättchen, Warnholz & Bienenvoigt, Ellerau, Germany).

Stimuli

Stimuli were generated and displayed on the monitor using custom MATLAB (Mathworks) code in conjunction with the PsychToolbox (Brainard 1997, Pelli 1997, Wilson, Tresilian et al. 2011). Each stimulus consisted of a red RGB (255, 0, 0) bar measuring 6.5 cm in length and 1.5 cm in width (adjusted for the screen size), situated within an 8 cm diameter blue disk RGB (0, 0, 255), with a dominant wavelength at 450 nm, all set against a red background. These bars could be individually switched between horizontal and vertical orientations through key presses. The centre of the bars was aligned with the feeding stations (supplementary Fig. S1). In pilot studies, these colours were identified as the most easily discernible by bumblebees and allowed for effective video tracking of the bee against the background.

Training and testing

Pre-training with only blue stimuli (no bars) was used to train the bees to go to the different feeders. The eight blue disks were displayed against the red background, with each disk providing 10µl of 30% sucrose solution (w/w). Once the bee successfully visited each of the feeder locations we began differential conditioning. In a training trial four horizontal stimuli and four vertical stimuli were displayed on the screen. One type of stimulus (horizontal or vertical) was rewarded with 10µL of sucrose solution (50% w/w; CS+), while the other was punished with 10µL of saturated quinine solution (0.12% w/w; CS-). To ensure that bees relied solely on the visual cue for learning, the position of the stimuli was randomised between trials. Additionally, to prevent the potential influence of odour cues on the learning process, the entire arena and screen were cleaned with 70% ethanol in between each trial and test.

Bees were divided into four training groups: Constant Horizontal (CH), Constant Vertical (CV), Switching Horizontal (SH) and Switching Vertical (SV). In the "constant stimuli" groups, the orientation of the stimuli remained unchanged throughout each training trial, while in the "switching stimuli" groups the orientation of the stimuli was switched between the bees' arrival and departure (Fig. 1b). In the Constant Horizontal (CH) group (N = 13), bees were trained with the horizontal stimulus as rewarding (CS+, providing sucrose solution) and vertical stimulus as punishing (CS- providing quinine solution). In the Constant Vertical (CV) group (N = 16), bees were trained to associate the vertical visual stimulus to the sucrose water (CS+) and the horizontal visual stimulus to saturated quinine solution (CS-). In the switching groups, the orientation of the CS + bar was changed as soon as bee landed on the feeder. This change was manually controlled by the experimenter using the keyboard (Fig. 1b). For example, when the bee alighted at a rewarded feeder with a vertical bar, the stimulus was switched to an horizontal bar so that the bee experienced different stimuli on arrival and upon departure from the rewarded stimuli (Fig. 1b). In the Switching Horizontal (SH) group (N = 10), bees were trained on the horizontal visual stimulus as rewarding and the vertical bar as non-rewarding, but, as soon as the bee landed the horizontal stimulus was switched to the vertical stimulus. Finally, in the Switching Vertical (SV) group (N = 14) bees were trained on the vertical stimulus as rewarding and the horizontal stimulus as non-rewarding, but, as soon as the bee finished feeding the vertical stimulus was replaced by the horizontal stimulus. Note that the CS- remained constant in the switching groups. Once the bee left the vicinity of the stimulus it was reset to its original condition prior to the bees next choice.

During a training trial, the bumblebee, typically visited between three and ten feeders. Landings were counted as visits. Feeders were replenished once the bee had fed on three of the four rewarding feeding stations (the bee was caught and put into an opaque cup so she would not see what platforms were replenished). The training phase concluded when a bee exhibited ≥ 80% correct choices in the last twenty choices. It usually took between 5 to 20 trials to train a bee to reach the criterion and identify that one of the stimuli was a consistent indicator of reward.

Following training, non-rewarded tests were performed replacing quinine or sugar with distilled water in the feeding stations. During tests, the number of correct and incorrect choices were recorded during 2 minutes. Trials with the training stimuli and the presence of sucrose reward and quinine solutions were interspersed (in a randomised fashion) among the non-rewarded tests to maintain the bees' motivation. The bees had to reach ≥ 80% correct choices before performing another test, with one to five inter-tests trials typically performed.

In the conflict test, stimuli with angles of 45°, 315° along with the trained stimuli (horizontal and vertical) were presented to the bees to evaluate whether bees in the constant and switching stimuli groups used the pre-landing or post-landing visual features in their choices. In the generalisation test bees were presented with stimuli of the following angles: 22.5°, 67.5°; 112.5° 337.5°, two stimuli of each angle were presented (supplementary Fig S1). This allowed us to assess if generalisation of the CS + differed between the switching and constant training groups.

Statistical analysis

For each test, all contacts with feeders within a two-minute period were counted as choices. Statistical analysis was conducted using MATLAB (2021). To assess and compare the learning of bees during the training phase, we employed a Generalised Linear Mixed Model (GLMM). Bee performance through the training procedure was quantified as the percentage of correct choices in consecutive blocks of 10 visits. In the model, we included the blocks of 10 visits,

the type of training groups (switching or consistent), the rewarding stimuli (horizontal and vertical), and the interaction between the choice block and training groups as explanatory variables. The model's parameters were estimated using the Maximum Likelihood method within MATLAB.

To further analyse the performance of bees during the non-rewarding tests, we employed various statistical tests based on our hypothesis. The non-parametric Kruskal-Wallis H test was used to determine if there were statistically significant differences between the four groups of bees during tests. The Wilcoxon signed-ranked test was utilised to compare two related samples to assess whether their population mean ranks differ. Also, the Mann-Whitney U test also called Wilcoxon rank-sum test was used to compare two independent samples means, and test whether two sample means are equal or not. In all figures, means are presented along with standard errors of the mean.

Results

Effect of training treatment (constant versus switching) on learning

We used a Generalised Linear Mixed Model (GLMM) to explore factors influencing the proportion of correct choices made during training. The dependent variable was the number of correct choices from a block of 10 choices. Bee index was included in the model as a random factor (Table 1). Bees from all four groups learned the task (Fig. 2a) since their likelihood of selecting the rewarded stimuli increased over trials: GLMM, P = 7.00e-07 (Table 1). In an unrewarded learning test, bees preferred the rewarded stimulus and avoided the punished stimulus (Fig. 2b, Table S1).

Table 1. Summary of the Generalised Linear Mixed Model (GLMM) examining factors in relation to proportion of rewarded choices during the training. Formula: response ~ 1 + trials + stimulus*protocol + (1 | bee_index). Model fit statistics: AIC = 1789.5, BIC = 1813.8, LogLikelihood=-888.75, Deviance = 1777.5.

Fixed factors	Estimate	SE	tStat	DF	P-value	Lower	Upper
Intercept	2.0562	0.21472	9.5763	420	8.84e-20	1.63	2.47
Trials	0.02	0.005	5.037	420	**7.00e-07**	0.01	0.03
Stimulus (horizontal and vertical	-0.14	0.13	-1.06	420	0.28	-0.39	0.11
Protocol (control versus switching	-0.28	0.13	-2.08	420	**0.03**	-0.56	-0.01
Stimulus : protocol	0.13	0.08	1.64	420	0.10	-0.02	0.30

Training of bees stopped when an individual made 80% (or more) correct choices within the last 20 choices, therefore the number of training trials differed for each bee. Bees in the Switching Horizontal stimuli group (in which the rewarded stimulus was horizontal on approach and vertical on departure from a stimulus) took more training trials to reach criterion than bees from the Constant Horizontal stimuli group (Mann-Whitney U test: U = 31.5, z=-2.047, P = 0.04, Fig. 2b). Bees from the Switching Vertical and Constant Vertical stimuli groups did not differ in number of training trials to reach criterion (Mann-Whitney U test CV *versus* SV U = 101, z=-0.436, P = 0.66). We compared performance of bees in the last 50 training trials until each bee reached the 80% correct criterion (Fig. 2a). Groups differed in their learning rate (GLMM P = 0.03, Table 1, Fig. 2). Switching stimuli groups were slower than constant stimuli groups, with the greatest difference between the Switching Horizontal and Constant Horizontal stimuli groups.

Conflict test

In the unrewarded conflict test, bees were presented with horizontal and vertical bars as well as two intermediate stimuli of angled bars at 45° and 315° (Fig. 3a and b). Bees from all groups exhibited a preference for the stimulus they were trained on: vertical for the Constant Vertical and Switching Vertical stimuli groups (Kruskal-Wallis N = 112, H = 27.54, P < 0.001) and horizontal for the Constant Horizontal and Switching Horizontal stimuli groups (Kruskal-Wallis N = 80, H = 29.08, P < 0.001). Bees in the Switching Horizontal stimuli group were more likely to choose the two novel stimuli (Table S2, Wilcoxon-signed ranked test: P = 0.73 for 45°and P = 0.26 for 315°) and less likely to choose the horizontal stimulus than bees in the Constant Horizontal stimuli group (Table S2), but no difference in choices were seen between the Switching Vertical and Constant Vertical stimuli groups (Fig. 3b, Table S3).

Generalisation test

In the unrewarded generalisation test, bees were presented with two stimuli close to horizontal (67.5° and 112.5°) and two stimuli close to vertical (22.5° and 337.5°). Switching Horizontal and Constant Horizontal stimuli groups did not differ in their preference, and both groups preferred the two stimuli close to horizontal (Table S4). Switching Vertical stimuli group had a stronger preference for 337.5 than Constant Vertical stimuli group but no other differences were detected (Table S5).

Switching and constant stimuli groups showed minimal differences in their preferences in the generalisation test (Table S5). Interestingly, although bees exposed to the vertical bars in their forward inspection (CV and SV) were not as good as bees exposed to the horizontal bar in their forward inspection (CH group and SH group) in selecting the patterns with similar feature to the approach stimuli, the performance of bees were improved by experiencing different pattern in the post-landing inspection (*i.e.* SV group; Table S5).

Discussion

Our study illustrates the robustness of bumblebee learning. We challenged bees with a free-flight conditioning task. This was learned quickly and presenting bees with conflicting information seen on arrival and departure from the feeder had minimal impact on either the rate of learning (Fig. 2a), or on the specificity of what had been learned. Learning of a horizontal CS + was more affected by conflicting information than a vertical CS-.

Our assay had features of trace conditioning, since our stimuli could not been seen by bees when they were feeding, hence the CS did not overlap with attaining the US. Trace conditioning is considered a cognitive form of learning and is even considered by some as evidential of conscious processing (Clark and Squire, 1998; Lovibond and Shanks, 2002, Birch et al. 2020, Droege et al. 2021), but it is a robust finding in insects (Menzel 2001, Dylla et al. 2013, Perisse *et al.* 2011, Klappenbach et al. 2021, Paoli et al. 2023). Lehrer was the first to show bees learn information seen on both arrival and departure from a sucrose solution feeder (1991, 1993). She found that if bees were presented with different stimuli on arrival and departure from a feeder their learning rate slowed. While there was evidence bees could learn a stimulus seen on departing a feeder, they showed a prioritisation of the stimulus seen before feeding (Bitterman and Couvillon 1991, Lehrer 1993). Our work differs from Lehrer (1993) in that while she presented bees with two different stimuli on arrival and departure (essentially two CS+), we used a discriminant learning paradigm and presented some bees with a conflict situation (CS + seen on arrival, CS- seen on departure). In this case, we saw no reduction in learning rate when compared to learning a consistent CS + for a vertical CS + stimulus, and only a minor reduction in learning rate for learning a horizontal CS+. Similarly, in generalisation tests the conflicting information had minimal impact. It is clear, therefore, that, when presented with both the CS + flipped to the CS- on departure from a sucrose feeder bumblebees did not generalise between the two stimuli, nor was there interference between the two stimuli. Bees in the switching groups appear to prioritise the relevant CS + information and entirely disregard the conflicting CS- information, but we may not need to invoke cognitive concepts such as "prioritisation" to explain our findings.

The most plausible anatomical locus for the associative learning phenomena studied here are the mushroom bodies (Barth *et al.* 1997, Li L. et al. 2017). The Kenyon cells of the mushroom bodies receive processed sensory input, and output from premotor regions (Mobbs 1982; Fahrbach 2006). There is experience-dependent neuroplasticity at both the input and output of the Kenyon cells that is sensitive to neurochemicals released in response to appetitive or aversive reinforcers (Barnstedt et al. 2016). It is theoretically possible for the mushroom body to support trace conditioning (Menzel 2001, Menzel & Giurfa 2001). Certainly, an enduring "trace" of neural activation can be held by the mushroom body structure for a short period of time. The Kenyon cells have a prolonged accommodation property (Strausfeld et al. 2009), and in *Drosophila*, recurrent connections have been detected between Kenyon cells (Dylla et al., 2013, Lyutova et al. 2019, Chandra et al. 2010, Aso et al. 2014, Bennett et al. 2021). These could, in theory, support a reverberation of neural activity in the Kenyon cell populations. Either or both mechanisms could maintain a trace of neural activity that persists beyond the presentation of a stimulus. This could support elementary forms of trace conditioning.

Learning stimuli on departure from the feeder most likely also involves the mushroom bodies. In classic associative learning theory, a CS that comes after the US is typically not learned since it is not predictive of the occurrence of the US. And yet, bees demonstrate a specific behaviour – the turn back and look – at a feeder on departure and learn features of a feeder during this behaviour. This form of learning could either be a form of secondary reinforcement or latent learning (Menzel 2001). Secondary reinforcement would assume that the feeder station and/or feeder location has become a reinforcer following pairing with food reward, in which case the feeder could now act as a conditioned reinforcer for any view directed at the feeder. Latent learning is simply learning with no explicit reinforcer and is presumed to be important for many forms of spatial learning. Both secondary reinforcement and latent learning are believed to involve the mushroom bodies in conjunction with the spatial systems of the lateral accessory lobes (Wystrach *et al.* 2023).

If mushroom bodies are involved in learning the stimuli seen both before and after feeding, how is it that learning performance is largely unchanged even if this information conflicts? In terms of the robustness of bees to learning conflicted information, here we should consider the mechanisms of decision making in bees as well as the learning mechanisms. Ultimately, the outcome of learning is to influence a decision of whether a bee should land at a feeder marked by a horizontal or vertical stimulus. The mushroom body alone is not a decision maker (Galizia 2014, Bhazenov *et al.* 2013, Huerta et al. 2004, 2009). It can perhaps best be thought of a as a classifier – learning to associate presented stimuli with different outcomes which are conveyed by mushroom body output neurons to premotor regions (Galizia 2014, Maboudi et al. 2023). The punished stimuli were consistent in all groups therefore the rate of learning to avoid the CS- would be the same in all groups. In both the switching and constant groups, the CS + was seen on approach to the feeder, therefore in all groups the CS + was reinforced for approach behaviour only, whereas the CS- stimulus would be reinforced for avoidance of punished stimuli in all groups and departure from the CS + in the switching groups. If we consider the mushroom body as classifying stimuli by behavioural response, this alone is sufficient to resolve any conflicting information associated with a feeder. In our paradigm, the CS + was only associated with approach responses, regardless of training groups.

In this experiment, learning of a horizontal CS + was more disrupted by the switching manipulation than learning of a vertical CS+. Why this might be is not clear, but there are other reports of insects responding differently to vertical and horizontal stimuli or learning them at different rates (Srinivasan et al. 1999, Wang, Tie et al. 2014, Wolf et al. 2015). It is possible these are processed differently by the visual system or have different innate responses.

In summary, our study demonstrates remarkable speed and proficiency for bumble bees learning a trace conditioning paradigm. Their learning was rapid, specific and largely unaffected if the CS + feeder was linked with conflicting information. Our study speaks to the remarkable efficacy of the bee brain for learning food related stimuli.

Declarations

Declarations

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval

Our research involved bumblebees from commercially available colonies dedicated to research for which an approval of an ethical committee is not mandatory. The protocols comply with standard welfare practice in our field and a minimum number of individuals were used to study our scientific question. The animals were not harmed during the experimental procedures.

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References

1. Aso Y., Sitaraman D., Ichinose T., Kaun K.R., Vogt K., Belliart-Guérin G., Plaçais P-Y., Robie A.A., Yamagata N., Schnaitmann C., Rowell W.J., Johnston R.M., Ngo T-T B., Chen N., Korff W., Nitabach M.N., Heberlein U., Preat T., Branson K.M., Tanimoto H., Rubin G.M. (2014). "Mushroom body output neurons encode valence and guide memory-based action selection in Drosophila". eLife 3:e04580. https://doi.org/10.7554/eLife.04580

2. Avarguès-Weber A. and M. Giurfa (2014). "Cognitive components of color vision in honey bees: how conditioning variables modulate color learning and discrimination." J Comp Physiol A Neuroethol Sens Neural Behav Physiol 200(6): 449- 461. https://doi.org/10.1007/s00359-014-0909-z

3. Barnstedt O., Owald D., Felsenberg J., Brain R., Moszynski J.P., Talbot C.B., Perrat P.N., Waddell S. (2016). "Memory-Relevant Mushroom Body Output Synapses Are Cholinergic." Neuron. Mar 16;89(6):1237-1247. Epub 2016 Mar 3. PMID: 26948892; PMCID: PMC4819445. https://doi.org/10.1016/j.neuron.2016.02.015

4. Barth M, Heisenberg M. (1997). "Vision affects mushroom bodies and central complex in Drosophila melanogaster." Learn & Mem.; 4:219–229. http://www.learnmem.org/cgi/doi/10.1101/lm.4.2.219

5. Bazhenov M., Huerta R., Smith B.H. (2013). "A computational framework for understanding decision making through integration of basic learning rules." J Neurosci. Mar 27;33(13):5686-97. PMID: 23536082; PMCID: PMC3667960. https://doi.org/10.1523/JNEUROSCI.4145-12.2013

6. Bennett J. EM, Philippides A., and Nowotny. T. (2021). "Learning with reinforcement prediction errors in a model of the Drosophila mushroom body." Nature communications 12.1: 2569. https://doi.org/10.1038/s41467-021-22592-4

7. Bernard J, Stach S, Giurfa M. (2006) "Categorization of visual stimuli in the honeybee Apis mellifera". Anim Cogn. 2006 Oct;9(4):257-70. PMID: 16909238. https://doi.org/10.1007/s10071-006-0032-9

8. Birch J., Schnell A.K., Clayton N.S. (2020). D"imensions of animal consciousness." Trends Cogn Sci 2020, 24:789-801. https://doi.org/10.1016/j.tics.2020.07.007

9. Bitterman, M. E. and P. A. Couvillon (1991). "Failures to find evidence of adaptive specialization in the learning of honeybees." The Behaviour and Physiology of Bees. L. J. Goodman and R. C. Fisher. Wallingford (England), C.A.B. International: 288-305. http://www.learnmem.org/cgi/doi/10.1101/lm.029975.112

10. Brainard DH. (1997). "The Psychophysics Toolbox." Spat Vis.10(4):433-6. PMID: 9176952. https://doi.org/10.1163/156856897X00357

11. Caporale N, Dan Y. (2008). "Spike timing–dependent plasticity: a Hebbian learning rule." Annu. Rev. Neurosci.. 2008 Jul 21;31:25-46. https://doi.org/10.1146/annurev.neuro.31.060407.125639

12. Chandra B. C. S., Geetha L., Abraham V. A., Karanth P, Thomas K., Srinivasan M. V. and Gadagkar R. (1998). "Uniform discrimination of pattern orientation by honeybees." Animal Behavior 56: 1391-1398. https://doi.org/10.1006/anbe.1998.0922

13. Chandra S.B., Wright G.A., Smith B.H. (2010) "Latent inhibition in the honey bee, *Apis mellifera*: Is it a unitary phenomenon?" Anim Cogn. Nov;13(6):805-15. Epub 2010 Jun 3. PMID: 20521073. https://doi.org/10.1007/s10071-010-0329-6

14. Clark, R. E., & Squire, L. R. (1998). "Classical conditioning and brain systems: The role of awareness." Science, 280 (5360), 77–81. https://www.science.org/doi/10.1126/science.280.5360.77

15. Droege P., Weiss D.J., Schwob N., Braithwaite V. (2021). "Trace conditioning as a test for animal consciousness: a new approach." Animal Cognition (6), 24:1299-1304, https://doi.org/10.1007/s10071-021-01522-3

16. Dylla K.V., Galili D.S., Szyszka P., Lüdke A. (2013). "Trace conditioning in insects-keep the trace!" Front Physiol. Aug 23;4:67. https://doi.org/10.3389/fphys.2013.00067

17. Fahrbach S.E. (2006). "Structure of the mushroom bodies of the insect brain." Annual Review of Entomology. jan; 51(1):209–232. http://www.annualreviews.org/doi/10.1146/annurev.ento.51.110104.150954

18. Galizia CG. (2014). "Olfactory coding in the insect brain: data and conjectures." European Journal of Neuroscience. Jun;39(11):1784-95. https://doi.org/10.1111/ejn.12558

19. Giurfa M., Zhang S., Jenett A., Menzel R., Srinivasan M.V. (2001) "The concepts of 'sameness' and 'difference' in an insect." Nature. Apr 19;410(6831):930-3. PMID: 11309617. https://doi.org/10.1038/35073582

20. Giurfa, M. and Sandoz J-C. (2012). "Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees." Learning & Memory 19(2): 54-66. http://www.learnmem.org/cgi/doi/10.1101/lm.024711.111

21. Grant V. (1950). "The flower constancy of bees." Bot. Rev. 16: 379-398. https://www.jstor.org/stable/4353438

22. Guiraud M., Roper M., Wolf S., Woodgate J.L. and Chittka L. (2022). "Discrimination of edge orientation by bumblebees." PLOS ONE. https://doi.org/10.1371/journal.pone.0263198

23. Hammer M., Menzel R. (1995). "Learning and Memory in the Honey bee" The Journal of Neuroscience, March, 15(3): 1617-1630. https://doi.org/10.1523/JNEUROSCI.15-03-01617.1995

24. Huerta R., Nowotny T., García-Sanchez M., Abarbanel H.D., Rabinovich M.I. (2004). "Learning classification in the olfactory system of insects." Neural Comput. Aug;16(8):1601-40. PMID: 15228747. https://doi.org/10.1162/089976604774201613

25. Huerta R., Nowotny T. (2009). "Fast and robust learning by reinforcement signals: explorations in the insect brain." Neural Comput. Aug;21(8):2123-51. Erratum in: Neural Comput. 2009 Sep;21(9):2713. PMID: 19538091. https://doi.org/10.1162/neco.2009.03-08-733

26. Hurst V., Stevenson P.C., Wright G.A. (2014). "Toxins induce 'malaise' behaviour in the honeybee (*Apis mellifera*)." J Comp Physiol A Neuroethol Sens Neural Behav Physiol. Oct;200(10):881-90. Epub 2014 Aug 23. PMID: 25149875; PMCID: PMC4169619. https://doi.org/10.1007/s00359-014-0932-0

27. Hussaini S.A., Komischke B., Menzel R., Lachnit H. (2007) "Forward and backward second-order Pavlovian conditioning in honeybees." Learn Mem. Oct 1;14(10):678-83. PMID: 17911371; PMCID: PMC2044558. http://www.learnmem.org/cgi/doi/10.1101/lm.471307

28. Johansen J.P., Diaz-Mataix L., Hamanaka H., Ozawa T., Ycu E., Koivumaa J., Kumar A., Hou M., Deisseroth K., Boyden E.S., LeDoux J.E. (2014). "Hebbian and neuromodulatory mechanisms interact to trigger associative memory formation." Proc Natl Acad Sci U S A. Dec 23;111(51):E5584-92. Epub 2014 Dec 8. PMID: 25489081; PMCID: PMC4280619 https://doi.org/10.1073/pnas.1421304111

29. Klappenbach M., Agustín E. L., Fernando F. L. (2021) "Honey bees can store and retrieve independent memory traces after complex experiences that combine appetitive and aversive associations." bioRxiv 2021.10.12.464105; https://doi.org/10.1101/2021.10.12.464105

30. Lehrer, M. (1991). "Bees Which Turn Back and Look." Naturwissenschaften 78: 274-276. https://doi.org/10.1007/BF01134357

31. Lehrer, M. (1993). "Why do bees turn back and look?" J. Comp. Physiol. A 172: 549-563. https://doi.org/10.1007/BF00213678

32. Li L., MaBouDi H., Egertová M., Elphick M.R., Chittka L., Perry C.J. (2017). "A possible structural correlate of learning performance on a colour discrimination task in the brain of the bumblebee." Proc Biol Sci. 2017 Oct 11;284(1864):20171323. PMID: 28978727; PMCID: PMC5647297. https://doi.org/10.1098/rspb.2017.1323

33. Lovibond, P. F., & Shanks, D. R. (2002). "The role of awareness in Pavlovian conditioning: Empirical evidence and theoretical implications." Journal of Experimental Psychology: Animal Behavior Processes, 28(1), 3–26. PMID: 11868231. https://doi.org/10.1037/0097-7403.28.1.3

34. Lyutova, R., Selcho, M., Pfeuffer, Segebarth M.D., Habenstein J., Rohwedder A., Frantzmann F., Wegener C., Thum A.S. & Pauls D. (2019). "Reward signaling in a recurrent circuit of dopaminergic neurons and peptidergic Kenyon cells." Nat Commun 10, 3097. https://doi.org/10.1038/s41467-019-11092-1

35. MaBouDi H., Marshall J. A.R., Dearden N., Barron A. B. (2023) "How honey bees make fast and accurate decisions". eLife 12:e86176. https://doi.org/10.7554/eLife.86176

36. Menzel R, Greggers U, Hammer M. (1993). "Functional organization of appetitive learning and memory in a generalist pollinator, the honeybee." In: Papaj D, Lewis AC, editors. Insect learning: Ecological and evolutionary perspectives. New York: Chapman and Hall; pp. 79–125. https://scholar.google.com/scholar_lookup?title=Insect+learning:+Ecological+and+evolutionary+perspectives&author=R+Menzel&author=U+Greggers&author=M+Hammer&publication_year=1993&

37. Menzel, R. (1993). "Associative learning in honey bees". Apidologie, Vol 24, N 3, 1993, 157-168. https://doi.org/10.1051/apido:19930301

38. Menzel, R. (1999). "Memory dynamics in the honeybee". J Comp Physiol A 185, 323–340. https://doi.org/10.1007/s003590050392

39. Menzel, R. (2001). "Searching for the memory trace in a mini-brain, the honeybee." Learning & Memory 8(2): 53-62. https://doi.org/10.1101/lm.38801

40. Menzel R, Giurfa M. (2001) "Cognitive architecture of a mini-brain: the honeybee". Trends Cogn Sci. 1;5(2):62-71. PMID: 11166636. https://doi.org/10.1016/S1364-6613(00)01601-6

41. Menzel R., Manz G., Menzel R., Greggers U. (2001). "Massed and spaced learning in honeybees: the role of CS, US, the intertrial interval, and the test interval". Learning & memory.;8(4):198-208. http://www.learnmem.org/cgi/doi/10.1101/lm.40001

42. Mobbs PG. (1982). "The brain of the honeybee *Apis Mellifera*. I. The connections and spatial organization of the mushroom bodies." Philosophical Transactions of the Royal Society of London B: Biological Sciences. 298(1091). https://doi.org/10.1098/rstb.1982.0086

43. Muth F., Papaj D. R. and Leonard A. S. (2015). "Colour learning when foraging for nectar and pollen: bees learn two colours at once". Biol. Lett.11:20150628.20150628 https://doi.org/10.1098/rsbl.2015.0628

44. Pelli D.G. (1997). "The VideoToolbox software for visual psychophysics: transforming numbers into movies." Spat Vis. 1997;10(4):437-42. PMID: 9176953.

45. Perisse E., Waddell S. (2011). "Associative memory: without a trace." Current Biology : Cb. 21: R579-81. PMID 21820619 https://doi.org/10.1016/j.cub.2011.06.012

46. Paoli M., Macri C., Giurfa M. (2023). "A cognitive account of trace conditioning in insects". Curr. Opinion in Insect Science, vol 57, June 2023, 101034. https://doi.org/10.1016/j.cois.2023.101034

47. Rath L, Giovanni Galizia C, Szyszka P. (2011). "Multiple memory traces after associative learning in the honey bee antennal lobe." European Journal of Neuroscience. Jul;34(2):352-60. https://doi.org/10.1111/j.1460-9568.2011.07753.x

48. Skorupski P., Chittka L. (2010). "Differences in Photoreceptor Processing Speed for Chromatic and Achromatic Vision in the Bumblebee, *Bombus terrestris*". Journal of Neuroscience 17 March 2010, 30 (11) 3896-3903; https://doi.org/10.1523/JNEUROSCI.5700-09.2010

49. Srinivasan, M.V., Lehrer, M. (1984). "Temporal acuity of honeybee vision: behavioural studies using moving stimuli." J. Comp. Physiol. 155, 297–312 (1984). https://doi.org/10.1007/BF00610583

50. Srinivasan M.V., Poteser M., Kral K. (1999). "Motion detection in insect orientation and navigation". Vision Research, Vol 39, Iss 16, P 2749-2766, ISSN 0042-6989 https://doi.org/10.1016/S0042-6989(99)00002-4

51. Strausfeld N.J., Sinakevitch I., Brown S.M., Farris S.M. (2009). "Ground plan of the insect mushroom body: functional and evolutionary implications." J Comp Neurol. Mar 20;513(3):265-291. PMID: 19152379; PMCID: PMC4876875. https://doi.org/10.1002/cne.21948

52. Szyszka P., Demmler C., Oemisch M., Sommer L., Biergans S., Birnbach B. (2011). "Mind the gap: olfactory trace conditioning in honeybees". J Neurosci, 31, pp. 7229-7239 https://doi.org/10.1523/JNEUROSCI.6668-10.2011

53. Wang H., Tie S., Yu D., Guo Y.-H. and Yang C.-F. (2014). "Change of Floral Orientation within an Inflorescence Affects Pollinator Behavior and Pollination Efficiency in a Bee-Pollinated Plant, Corydalis sheareri." PLOS ONE 9(4): e95381. https://doi.org/10.1371/journal.pone.0095381

54. Wilson, A.D., Tresilian, J. & Schlaghecken, F. (2011). "The masked priming toolbox: an open-source MATLAB toolbox for masked priming researchers." Behav Res 43, 210–214 (2011). https://doi.org/10.3758/s13428-010-0034-z

55. Wolf, S., Roper M. and Chittka L. (2015). "Bumblebees utilize floral cues differently on vertically and horizontally arranged flowers." Behavioral Ecology 26(3): 773-781. https://doi.org/10.1093/beheco/arv010

56. Wright G.A., Mustard J.A., Simcock N.K., Ross-Taylor A.A., McNicholas L.D., Popescu A., Marion-Poll F. (2010). "Parallel reinforcement pathways for conditioned food aversions in the honeybee." Curr Biol. Dec 21;20(24):2234-40. Epub 2010 Dec 2. PMID: 21129969; PMCID: PMC3011020. https://doi.org/10.1016/j.cub.2010.11.040

57. Wystrach A. (2023). "Neurons from pre-motor areas to the Mushroom bodies can orchestrate latent visual learning in navigating insects." Biorxiv https://doi.org/10.1101/2023.03.09.531867
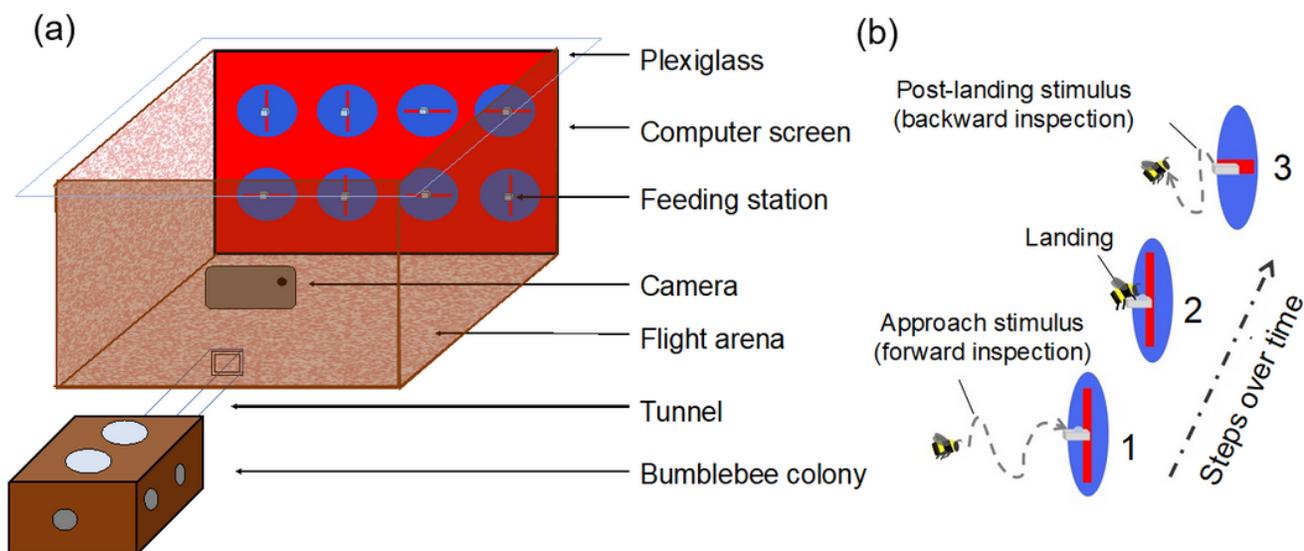
Figures

Figure 1

Experimental setup and switching condition. **(a)** The back of the flight arena displayed eight stimuli, each of which had a feeding station at its centre. Four stimuli provided a sucrose solution (rewarding) and the other four provided quinine solution (punishing). Across training, the location of rewarding stimuli and repellent stimuli changed pseudorandomly. Bees in the constant stimuli groups were exposed to the same rewarded stimulus on approach and post-landing ("constant horizontal stimuli" and "constant vertical stimuli" groups, referred to as CH and CV). Bees in the switching stimuli groups saw different approach and post-landing stimuli ("switching horizontal stimuli" and "switching vertical stimuli" groups, referred to as SH and SV). Example shown here **(b)** Switching vertical group.
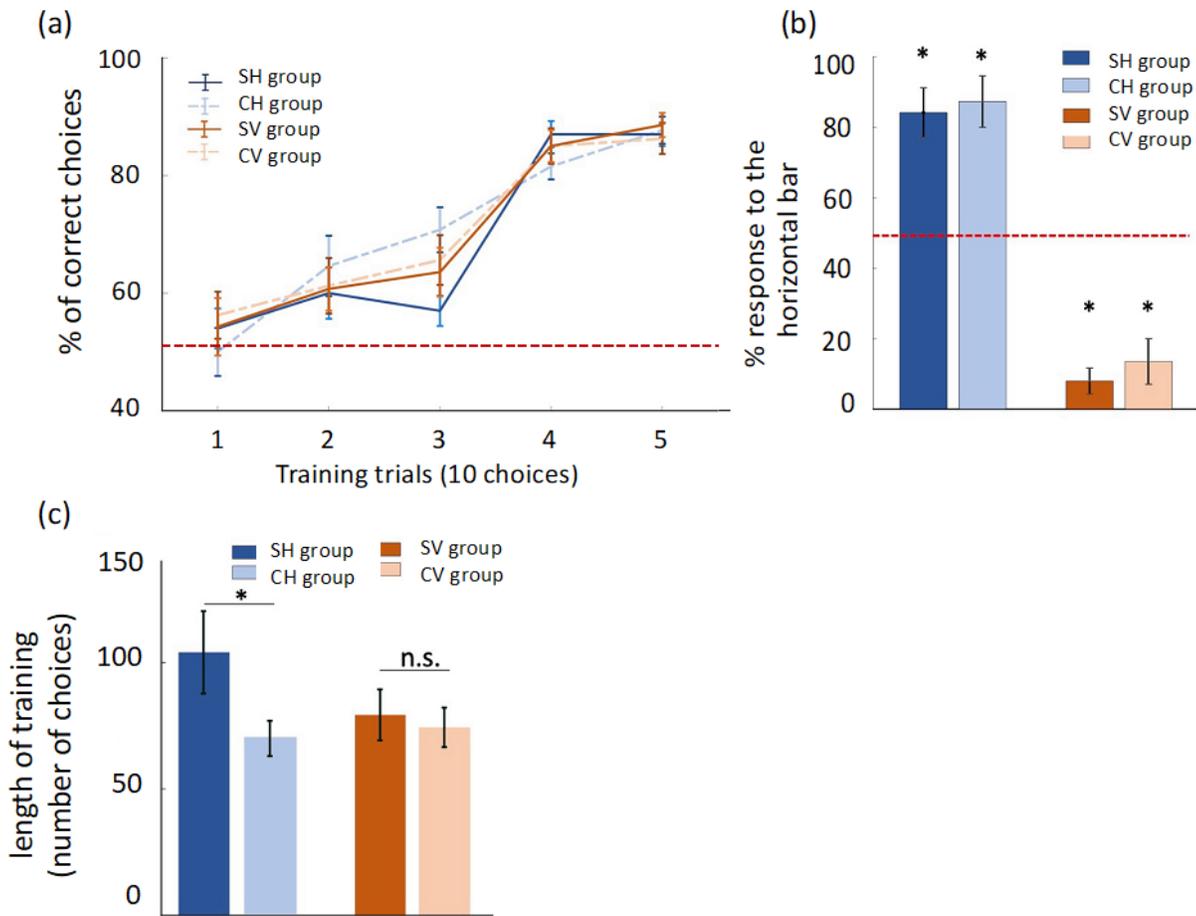
Figure 2

Bees' length of training, training performance and choices during learning test. (a) Bees' last 50 choices (means ± standard error; * P-value < 0.05). The red line represents chance level (50%). **(b)** Choices made by bees during the learning test (means ± standard error; * P-value < 0.05). Bees trained with a horizontal CS+ on approach (CH and SH) have a strong preference for the horizontal stimulus. Bees trained with a vertical CS+ on approach (CV and SV) avoided horizontal stimulus (S2 Table). **(c)** Average length of time taken by bees to reach the threshold of 80% performance during the training phase (means ± standard error; * P-value < 0.05).

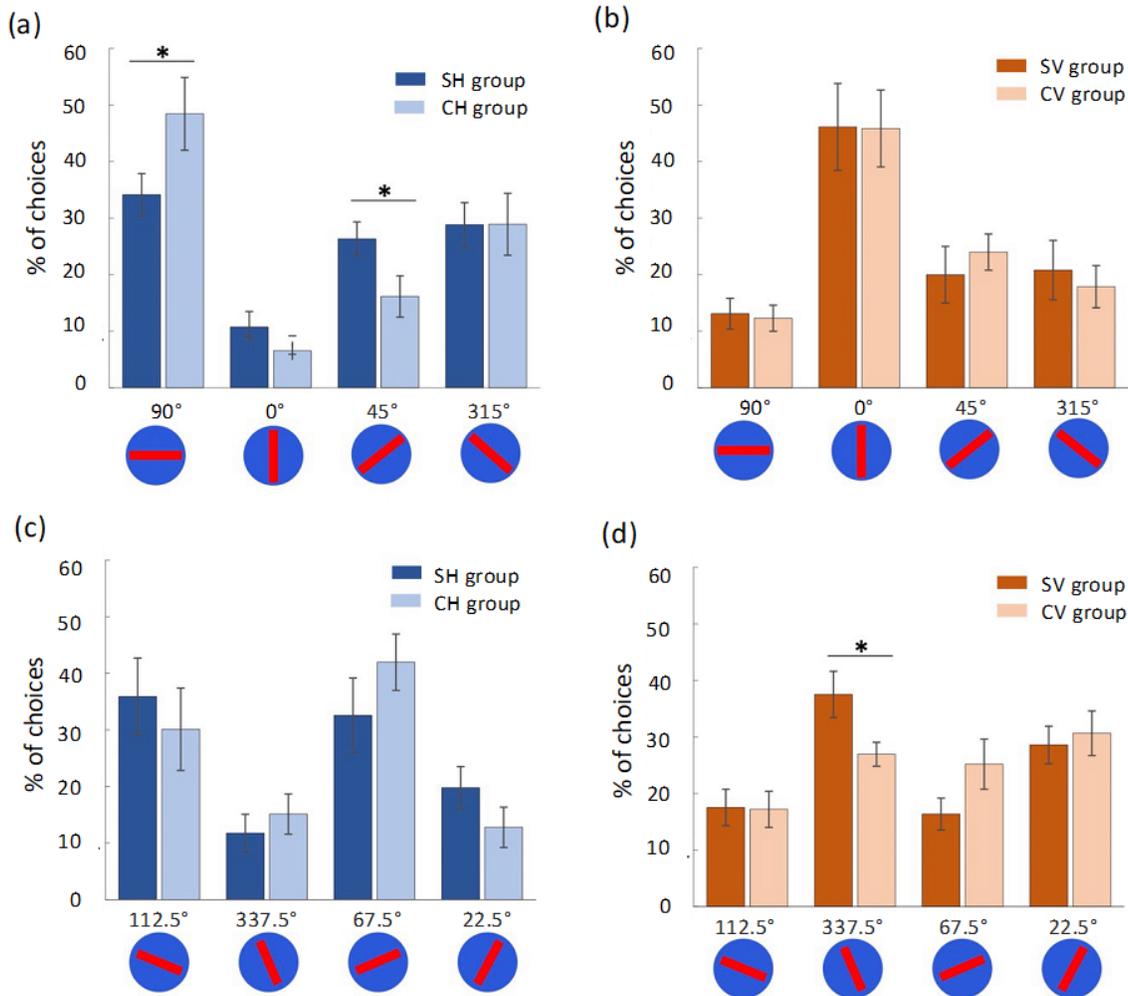**Figure 3**

Fig 4. Bees' performance in the conflict and generalisation tests. (a) and **(b)** Preference for each stimulus in the conflict test for bees trained with horizontal stimuli rewarded on approach (a) or vertical stimuli rewarded on approach (b) (means ± standard error, * P-value < 0.05). The SH stimuli group differed from the CH stimuli group (Table S2), but no differences were found between the SV and CV stimuli groups (Table S3). **(c)** and **(d)**Preference in the generalisation test for bees trained to horizontal (c) or vertical (d) rewarded stimuli on approach (means ± standard error; * P-value < 0.05). SV stimuli group shows a preference towards one angle in comparison with CV stimuli group (see result section).

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