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Flexible visual learning in nectar-foraging hornets

Mathilde Lacombrade^{1,2,3,4} · Monica Doblas-Bajo⁵ · Naïs Rocher² · Zoé Tourrain² · Emmanuel Navarro⁶ · Christian Lubat⁶ · Fanny Vogelweith³ · Denis Thiéry² · Mathieu Lihoreau¹

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Abstract

Pollinators, such as bees, develop flexible memories of colors, patterns, and shapes, for efficient flower recognition. Here we tested whether other flower-foraging insects have evolved similar cognitive abilities underpinning flexible visual learning. We trained wild hornets from two species commonly found in Europe, the invasive yellow-legged hornet (*Vespa velutina nigrithorax*) and the European hornet (*Vespa crabro*), to associate sucrose solution rewards to color stimuli in a Y-maze. Hornets from both species succeeded in differential and reversal learning and developed short-term memories of the learnt associations. Thus, just like bees, hornets can learn various visual cue-reward associations and remember them for at least 1 h for selecting flowers. Our study in non-model species illustrates how standard conditioning approaches can be used to explore and compare the cognitive abilities of animals sharing similar foraging ecologies.

Significance statement

Bees can learn an impressive diversity of visual cues to recognize the best rewarding flowers. This can be studied using Y-maze in which individual insects must learn to associate visual stimuli to the presence or absence of nectar rewards. Here, using the same approach, we show that hornets are also capable of these associations. We compared the learning abilities of hornets from two common species in Europe and found that they could similarly learn to discriminate two colors and keep this information in memory for at least 1 h. Hornets thus evolved visual cognition facilitating robust flower foraging, like bees and many other pollinators.

Keywords Asian hornet · European hornet · Appetitive conditioning · Reversal learning · Comparative cognition

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✉ Mathilde Lacombrade
lacombrademathilde@gmail.com

- ¹ Research Center on Animal Cognition (CRCA), Center for Integrative Biology (CBI), CNRS, University Paul Sabatier, Toulouse III, France
- ² UMR 1065 Santé et Agroécologie du Vignoble, INRAE, F-33883 Villenave d'Ornon, France
- ³ M2i Biocontrol, 370 route de Cauzenil, 46140 Parnac, France
- ⁴ Laboratory for Analysis and Architecture of Systems (LAAS), Toulouse University, CNRS, INPT, Toulouse, France
- ⁵ Department of Biodiversity and Environmental Management, Universidad de León, Campus de Ponferrada, 24401, Ponferrada, León, Spain
- ⁶ BeeGuard SAS, Labège, France

Introduction

Nectar-foraging animals, such as bees, butterflies, birds, and bats, have evolved a rich cognitive repertoire for flower recognition (Healy and Hurly 2003; von Helversen and von Helversen 2003; Srinivasan 2010; Cepero et al. 2015). Bees, for instance, develop accurate visual memories of shapes, colors, and patterns to exploit the best rewarding flowers in their environment (Wehner 1967; Giurfa et al. 1999; Collett and Collett 2002; Dyer and Chittka 2004; Srinivasan 2006; Chittka and Raine 2006). Some of these information can last in memories for days or weeks (Chittka and Thomson 2001), allowing for flower specialization (i.e., flower constancy, Chittka et al. 1999). At the most basic level, foragers can discriminate flowers by learning associations between visual cues and a sugar reward or the absence of it (differential learning, Giurfa 2007). However, bees also exhibit some cognitive flexibility in order to update these learnt associations

and learn new ones, for instance, if the profitable plant species become unavailable and others start blooming (reversal learning, Raine and Chittka 2012). Consequently, we expect flexible visual associative learning and memory to be broadly observed across nectar-foraging species.

Wasps constitute a large group of Hymenopteran insects that is phylogenetically close to bees. Like bees, many species of wasps are generalists and frequently forage on floral nectar for carbohydrates (Richter 2000; Brock et al. 2021). Wasps are well known to use visual cues for place learning (Tinbergen 1932) and nestmate recognition (Sheehan and Tibbetts 2011). Recent studies using appetitive conditioning also reported their ability to learn pictures of human faces (Avarguès-Weber et al. 2018), patterns (D'Adamo and Lozada 2011), and colors (Balamurali et al. 2021). However, little is known about the ability of wasp foragers to exhibit flexibility in these visual learning, in order to update preferences with changes in resource quality and keep these information in memory for optimizing nectar-foraging trips, as reported in bees (Gegear and Thomson 2004).

To address this question, we investigated visual learning and memory in the two main hornet species found in Europe: the European hornet (*Vespa crabro*) and the invasive yellow-legged hornet (*Vespa velutina nigrithorax*). *Vespa crabro* is present in Europe for at least two centuries (Janet 1895), while *V. velutina* was first recorded in France in 2004 and spread across Western Europe (Monceau et al. 2014). Because these social hornets occasionally forage on flowers (Ueno 2015), we hypothesized that they should exhibit flexible visual learning. We tested this hypothesis by adapting visual appetitive conditioning protocols previously developed for bees (Howard et al. 2019), using a semi-automatic Y-maze in which hornets from captive nests could associate a colored light to a sucrose reward and another colored light to an absence of reward during 10 trials, after which the

task was reversed. We measured all the hornets to test for a potential confounding effect of body size on learning performances recently reported in wasps and bees (van der Woude et al. 2018; Monchanin et al. 2021a). We also tested for a potential influence of inter-trial interval (Menzel et al. 2001).

Methods

Hornets

We caught wild nests of *V. crabro* ($n=1$) and *V. velutina* ($n=3$) in June–July 2022 (see details about collection sites and dates in Table S1). Colonies were in their developing phase (Monceau et al. 2014) and thus highly motivated to forage. We selected colonies that were comparable in size and number of individuals to make sure that they had similar nutritional needs and motivation. Once returned to the lab, we cooled the colonies in a cold chamber (4 °C) for 24 h and paint-marked all adults in each nest with a unique color code on their thorax and abdomen for individual identification. We then transferred the nests into plastic boxes (24 cm h × 32 cm l × 32 cm d (Fig. 1A)) kept in an experimental room at ambient temperature (20–22 °C). We provided hornets with ad libitum defrosted honeybees (source of proteins) and 40% (v/v) sucrose solution directly into the boxes. The colonies were tested in these controlled conditions one after the other within a 2-month period (see Table S1 for more details).

Y-maze

We connected the nest box to a homemade Y-maze using a clear transparent plastic tunnel (52 cm l, 2 cm Ø) with shutters to manually control the flux of foragers (see details in

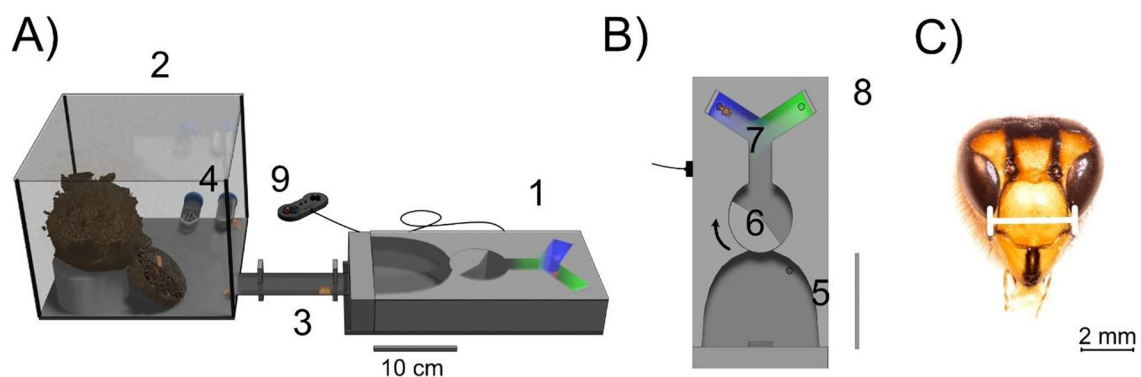


Fig. 1 **A** Overall view of the setup. **B** Detailed view of the Y-maze. (1) Y-maze connected to (2) nest box through a (3) tunnel with shutters. (4) Feeders containing ad libitum food (dead honeybees and sucrose solution). The Y-maze was dug in Styrofoam and covered with transparent plexiglass. (5) Pre-training feeder. (6) Turnstile

entrance/exit door. (7) Feeding holes containing sucrose solution (positive reinforcement) or water (negative reinforcement) associated with (8) LED light displayed on the back wall (visual stimuli). (9) Turnstile and LEDs (on/off, change colors) were remote-controlled using a manual controller. **C** Head width measurement (white line)

Fig. 1B). Hornets could enter and exit the Y-maze through a turnstile door at the entrance. The first branch of the Y-maze led to two identical arms, each containing a feeding cup at its extremity. The feeding cup was a lidless transparent 0.2-ml Eppendorf inserted into the floor of the Y-maze. Depending on the training protocol, the feeding cup could contain sucrose solution, water, or nothing (see below). The back wall of each arm was illuminated by colored LEDs through a light diffuser (blue light: $\lambda = 465\text{--}467$ nm, intensity = 180–200 mcd; green light: $\lambda = 522\text{--}525$ nm, intensity = 660–720 mcd). Activation of the turnstile door and the LEDs was remote-controlled by the experimenter.

Pre-training

We pre-trained hornets to collect 40% (v/v) sucrose solution ad libitum on a feeding cup placed at the entrance of the Y-maze (Fig. 1B). During pre-training, shutters in the transparent tube connecting the colony to the Y-maze were left open so that hornets could visit the feeder ad libitum. We considered all hornets that made at least 3 visits to the feeder within 1 h of observation as regular foragers. Once one or several foragers were identified as regular, we removed the sucrose solution at the entrance to start training on these individuals.

Training

We trained 20 foragers of each species in two visual conditioning protocols routinely used to assess learning and memory in bees (Avarguès-Weber et al. 2011). Based on preliminary observations showing that hornets in this context preferred sucrose solution to water (i.e., we never observed water collection), we used sucrose solution as positive reinforcement and water as negative reinforcement. Water is commonly used by wasps for building nests and thermoregulation (Richter 2000). Since our colonies were maintained in a controlled temperate room, with only little population growth, it is likely that needs for water were reduced compared to field conditions. Each hornet was submitted to two training protocols immediately followed by a memory test. This was achieved on the same day (mean \pm SE: 207.72 \pm 63.23 min, range: 117–418 min).

Differential conditioning

We trained individual hornets to associate color A with a sucrose reward (positive-conditioned stimulus CS+) and color B with unrewarded water (negative-conditioned stimulus CS-) for 10 consecutive trials (Giurfa 2007). In differential conditioning, the conditioned stimuli are unambiguously associated with an unconditioned stimulus or with its absence. This protocol was used to evaluate the learning ability of hornets.

Reversal conditioning

Immediately after differential learning, we trained the same hornets to learn the opposite association, so that the sucrose reward (CS+) was paired to color B and water (CS-) to color A during 10 additional trials (Raine and Chittka 2012). In reversal conditioning, there is a transient ambiguity of stimulus outcome that needs to be overcome by the insect. This protocol was thus used to evaluate the cognitive flexibility of hornets.

At each trial of each learning protocol, CS+ and CS- were randomly assigned in the arms of the Y-maze. Hornets were free to come in the Y-maze when motivated, which means that the inter-trial interval was not controlled ($n=800$ inter-trials, mean \pm SE: 7.35 \pm 6.71 min, range: 1–116 min). We cleaned the Y-maze with 70% ethanol after each trial to remove any potential chemical marks left by the hornets.

For each hornet, we computed a learning score for differential learning and reversal learning by summing its first choices during the 10 trials (Monchanin et al. 2021b) (0: only CS-; 5: random; 10: only CS+). A trial began when the hornet entered the Y-maze by the turnstile entrance and ended when it collected the sucrose solution (in first or second choice). First choice was defined as the first feeding cup (CS+ or CS-) antennated by the hornets at each trial and was successful when it chooses CS+. For each protocol, we considered individuals that selected sucrose solution as their first choice for the last trial as “learners,” as this is done in bee experiments (Buatois et al. 2017; Cabirol et al. 2018; Geng et al. 2022).

Memory retention

We tested short-term memory (STM) 1 h after the last trial of reversal learning using the same colors for CS+ and CS- as in reversal conditioning. This analysis was conducted only for motivated learners. The trained hornets were allowed to re-enter the Y-maze and choose between the two visual stimuli without any sucrose or water. We considered that the hornet memorized the association when it chose the arm colored as CS+ and antennated the empty feeding cup.

Morphometry

To control for a potential influence of head size on the learning and memory performances of hornets, we froze-killed the conditioned hornets and made morphological measurements with the ToupView software coupled to a Nikon SMZ 745T dissecting microscope (objective $\times 0.67$) with a Toup-Cam camera model U3CMOS. We measured head width as a proxy of head size (Riveros and Gronenberg 2010). Note that 4 out of the 20 *V. crabro* were physically damaged and removed from these analyses.

Statistics

We analyzed the data in R 4.0.4. From the raw data (available in Dataset S1 and S2), we extracted the first choice of each hornet at each trial (CS+: 1, CS–: 0) of differential and reversal conditioning. We then tested the influence of species (*V. crabro* or *V. velutina*) and trials 1–10 on first choice (CS+), using a generalized linear mixed model (GLMM; R package lme4, Bates et al. 2015), with binomial error structure and identity as a random factor, followed by an ANOVA (R package car, Fox and Weisberg 2019). For each conditioning protocol, we compared the number of learners in the two species using a chi-square test with a continuity correction (R function chisq.test). We analyzed the effect of head width and inter-trial interval on learning scores (0–10) using a linear mixed model (R package lme4) with the colony as a random factor for *V. velutina* and a linear model for *V. crabro* as we tested only one colony for this species.

Results

We first assessed the learning performances of hornets in a differential learning task in which one color was rewarded and the other was not (Fig. 2A). The percentage of individuals that correctly chose the reward increased with the number of trials (Binomial GLMM, trial: $X^2=43.53$, $df=9$, $p<0.001$), and this was similar in the two species (Binomial GLMM, species: $X^2=2.26$, $df=1$, $p=0.133$). The proportion of learners was maximal and identical in *V. velutina* and *V. crabro* (100%, 20/20 of hornets in each species).

We then measured the cognitive flexibility of the learners in a reversal learning task where the previously learnt reward-color associations were inverted (Fig. 2B). Here again the proportion of hornets that chose the rewarded stimulus increased with the number of trials (Binomial GLMM, trial: $X^2=0.53$, $df=9$, $p<0.001$), and this was similar in the two species (Binomial GLMM, species: $X^2=0.59$, $df=1$, $p=0.407$). The proportion of learners was also comparable in *V. velutina* (65%, 13/20 hornets) and *V. crabro* (70%, 14/20

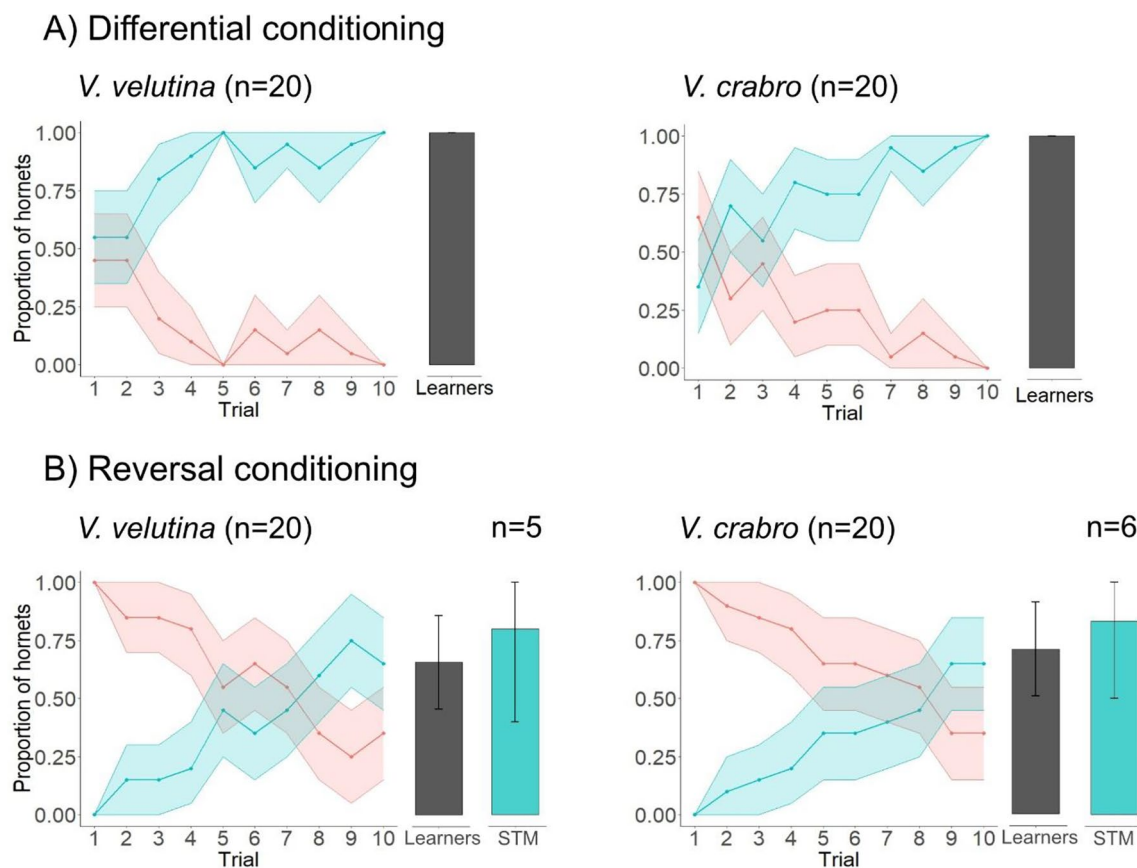


Fig. 2 Learning curves for differential (A) and reversal (B) conditioning of *V. velutina* and *V. crabro*. Blue: responses to CS+. Red: responses to CS–. Learners: percentage of hornets that responded to

CS+ in the last trial. STM: short-term memory test. Error bars are 95% confidence intervals

hornets) ($X^2 < 0.001$, $df=1$, $p=1$). However, these proportions were lower than for differential learning, indicating that overcoming the transient ambiguity of the stimuli following the reversal of contingencies was complex.

For both species, most of the learners developed a short-term memory of the color-reward association when tested 1 h after the reversal learning phase (*V. velutina*: 80%, $n=4/5$; *V. crabro*: 83%, $n=5/6$). Here, however, our sample size was too small to test for statistical differences between species.

When looking for potential confounding factors, we found important variation in head size (*V. velutina*: mean±SE: 3.37 ± 0.13 mm, range: 3.12–3.54 mm, $n=20$; *V. crabro*: 3.86 ± 0.19 mm, range: 3.33–4.07, $n=20$) and inter-trial intervals (*V. velutina*: differential learning: mean ± SE: 9.36 ± 3.83 min, range: 5.6–21.9 min, reversal learning: 8.03 ± 3.47 min, range: 2.6–13.8 min; *V. crabro*: differential learning: 6.09 ± 1.18 min, range: 4.1–8.5 min, reversal learning: 5.42 ± 1.84 min, range: 3.5–11.2 min) across hornets. However, none of these parameters had a significant influence on the learning scores (LMM differential learning: *V. velutina*: head width: $X^2=3.21$, $df=1$, $p=0.073$, inter-trial interval: $X^2=0.0002$, $df=1$, $p=0.988$; *V. crabro*: head width: $X^2=6.09$, $df=1$, $p=0.149$, inter-trial interval: $X^2=1.42$, $df=1$, $p=0.472$; LMM reversal learning: *V. velutina*: head width: $X^2=0.89$, $df=1$, $p=0.344$, inter-trial interval: $X^2=0.71$, $df=1$, $p=0.398$; *V. crabro*: head width: $X^2=4.25$, $df=1$, $p=0.353$, inter-trial interval: $X^2=0.007$, $df=1$, $p=0.969$).

Discussion

We adapted appetitive conditioning protocols used in bee research (Avarguès-Weber et al. 2011) to explore and compare flexible visual cognition in nectar-foraging hornets. Foragers of European and yellow-legged hornets' species learned equally well the visual-color associations in differential and reversal conditioning and remembered these associations for at least 1 h. This suggests that these forms of visual learning and cognitive flexibility are widespread among flower-foraging animals.

Wasps are known to use visual cues in navigation (Tinbergen 1932; Beier and Menzel 1972; D'Adamo and Lozada 2003; D'Adamo and Lozada 2011) and communication (Sheehan and Tibbetts 2011; Baracchi et al. 2015; Avarguès-Weber et al. 2018). However, the importance of visual learning and memory in flower selection is less clear (Balamurali et al. 2021). While experimental conditions are not identical but only similar, the hornet visual associative performances were comparable to or higher than that of bees (Giurfa 2004; Strang and Sherry 2014). Like nectar-foraging bees, *V. velutina* and *V. crabro* foragers can learn to associate colors with sucrose rewards and store these associations. This memory of color-reward

association likely supports flower constancy, a behavior recently described in hornets (Ueno 2015) and known to improve foraging success in bees (Gegear and Thomson 2004). Importantly, the hornets were also capable of quickly replacing the learnt associations with new ones, indicating that they can adjust their flower preferences to natural fluctuations of flower reward values through time, over the course of their foraging career. Such behavioral flexibility at the individual level may be critical for foragers to adapt nutrient collection to changing colony needs, depending on variations in colony composition (e.g., adult-to-larvae ratio) or external conditions (e.g., ambient temperature) (Simpson et al. 2014).

The fact that we did not detect the difference in the performances of *V. crabro* and *V. velutina* suggests that the cognitive traits we studied are basic abilities shared across nectar-foraging species, irrespective of differences in their morphology (e.g., body size) and invasion history. Y-maze conditioning is a simple, yet powerful, approach for further comparative analyses of the cognitive capacities of insect pollinators. Future studies using this approach in non-model species could explore more the elaborated forms of visual learning such as non-elemental associative learning (Giurfa et al. 2001) or bimodal visual-olfactory learning (Zhang et al. 2014) described in honey bees and bumblebees. In the case of *V. velutina*, a detailed understanding of their cognitive abilities may help better predict their spreading dynamics (e.g., in relation to food type and abundance) or develop new tools for biocontrol of invasive populations in Europe and Western Asia. For instance, visual conditioning could be used to attract hornets into traps or to feed them on contaminated baits that could act as a Trojan horse (Poidatz et al. 2018). Ultimately, comparing cognitive abilities across phylogenetically close species using standard, replicable tests is critical to understand the ecological drivers of the evolution of cognitive traits.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00265-023-03349-z>.

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Author contribution MLa, DT, and MLi designed the study. EN and CL built the Y-maze. MLa performed the experiments and wrote the first draft. MDB, NR, and ZT participated to the experiments. All authors revised the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article (supplementary Table S1, Dataset S1, Dataset S2).

Declarations

Competing interests The authors declare no competing interests. M2i and BeeGuard did not influence the analysis and the conclusions.

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