1

1 Appetitive learning relies on octopamine and 2 dopamine in ants

3 Maarten Wissink, Volker Nehring

Evolutionary Biology & Ecology, Institute for Biology I (Zoology), University of Freiburg, Freiburg, Germany

Correspondence: volker.nehring@biologie.uni-freiburg.de; Tel.: +49 (0)761 203-2584. Fax: +49 761 203 2544.

4 Abstract

5 Associative learning relies on the detection of coincidence between a stimulus and a
6 reward or punishment. In the insect brain, this process is thought to be carried out in the
7 mushroom bodies under control of octopaminergic and dopaminergic neurons. It was
8 assumed that appetitive learning is governed by octopaminergic neurons, while
9 dopamine is required for aversive learning. This view has been recently challenged:
10 Both neurotransmitters seem to be involved in both types of memory in bees and flies.
11 Here, we test which neurotransmitters are required for appetitive learning in ants. We
12 trained *Lasius niger* ant workers to discriminate two mixtures of linear hydrocarbons
13 and associate one of them with a sucrose reward. We analysed the behaviour of the
14 trained ants using machine learning and found that they preferred the rewarded odour
15 over the other, a preference that was stable for at least 24 hours. We then treated the ants
16 before learning with either epinastine, an octopamine receptor blocker, or with
17 flupentixol, a dopamine receptor blocker. Ants with blocked octopamine receptors did
18 not remember the rewarded odour. Octopamine signalling is thus necessary for the
19 formation of appetitive memory. In contrast, ants with blocked dopamine receptors
20 initially learned the rewarded odour but failed to retrieve this memory 24 hours later.
21 Dopamine is thus required for long-term memory consolidation during appetitive
22 conditioning, independent of short-term memory formation. Our results show that
23 appetitive learning depends on both octopamine and dopamine signalling in ants.

24 **key words:** associative learning, *Lasius niger*, long term memory, neurotransmitters,
25 short term memory

26 Introduction

27 There is nothing stable in the world; those that can adapt to changes will prevail. When
 28 the profitability of food sources changes over time, it would be advantageous to quickly
 29 learn which ones are currently most rewarding. Individuals can do so by associating the
 30 food with other stimuli such as odours or colours, i.e. they establish a predictive
 31 relationship between two independent cues from the environment (Giurfa, 2007). When
 32 a neutral stimulus, like an odour, is paired with a biologically relevant stimulus (food,
 33 the unconditioned stimulus – US), the animal may in future react to the previously
 34 neutral odour (the now conditioned stimulus – CS), as if it were the US. In the case of a
 35 bee searching for food, CS and US are processed by two different pathways. The CS-
 36 pathway starts at the odorant receptors (ORs) in the antenna. Output from the OR
 37 neurons is first processed in the antennal lobe (Giurfa, 2007). From there, projection
 38 neurons carry the information to the mushroom bodies, where learning occurs. The US-
 39 pathway begins at gustatory receptors on the mouth parts. Neurons then project to the
 40 subesophageal ganglion, from where the octopaminergic VUMx1-neuron projects to the
 41 antennal lobe and the mushroom bodies (Giurfa, 2007; Galizia and Sachse, 2010; Perry
 42 and Barron, 2013). CS and US converge in the Kenyon Cells of the mushroom body,
 43 which act as coincidence detectors. In their normal state, they would not relay a specific
 44 odour to the higher brain centres through mushroom body output neurons. However,
 45 after an odour coincided with an US, the Kenyon cells will relay the odour as a now
 46 conditioned stimulus, even in absence of the US. The reliance of the US signalling on
 47 the octopaminergic VUMx neuron suggests that without octopamine (OA) signalling,
 48 the US cannot reach the MB and thus insects cannot learn. Indeed, blocking OA
 49 receptors in crickets, flies, and honeybees prevented appetitive memory formation and
 50 memory retrieval (Schwaerzel et al., 2003; Galizia and Sachse, 2010), while OA
 51 injections could replace the US (Hammer and Menzel, 1998). Similar experiments
 52 blocking dopamine (DA) receptors had no effect on appetitive learning but prevented
 53 individuals from associating a punishment, such as an electric shock, with odours or
 54 colours (aversive conditioning; Schwaerzel et al., 2003; Vergoz et al., 2007; Mizunami
 55 and Matsumoto, 2017). It was thus assumed that aversive and appetitive memory are
 56 formed in different modules of the antennal lobes that use different neurotransmitters

(Hige et al., 2015). To this day, this seems to hold true for crickets (Mizunami and Matsumoto, 2017), but newer data suggest that aversive conditioning relies on both OA and DA signalling in honeybees and fruit flies (Agarwal et al., 2011; Claßen and Scholz, 2018; Manicini et al., 2018). The use of *Drosophila* GAL4 lines that can be used to knock out specific neurons in the MB has allowed to paint a much more detailed picture of learning and memory retrieval: In flies, learning in the MB appears to be organized in modules that each consist of Kenyon cells, mushroom body output neurons, and dopaminergic neurons that modulate the valence of the modules (Berry et al., 2012; Hige et al., 2015.) Some of the modules organize attraction to odours, and others repulsion (Rohwedder et al., 2016). The modules further differ in how long the memory lasts – from a few seconds to several hours (also in honeybees: Menzel, 2014). The control of both appetitive and aversive learning by dopaminergic neurons also means that without dopamine signalling, both types of learning should be hampered, which matches the behavioural evidence from pharmacological receptor blocking and knockouts in *Drosophila* and honeybees (Berry et al., 2012; Perisse et al., 2013; Perry and Barron, 2013; Mancini et al., 2018; Sabandal et al., 2020).

One way to hinder dopamine- and octopamine-related signalling is to block their respective receptors pharmacologically. In insects, epinastine is a specific OA-receptor blocker which abolishes the OA related cAMP production in the mushroom bodies (Roeder et al., 1998). Epinastine seems to specifically target the AmOA1 receptor in honey bees (Beggs et al., 2011), the OA1 receptor in crickets (Awata et al., 2015), the DmOA3 receptor in *Drosophila* (Qi et al., 2017), and the NcOA2B2 receptor in the green rice leafhopper *Nephotettix cincticeps* (Xu et al., 2020). In contrast, flupentixol largely targets dopamine receptors, such as the AmDOP2 receptor in the mushroom bodies of honey bees (Mustard et al., 2003; 2010; Beggs et al., 2011).

Just like honey bees, ants are social insects for whom learning is important in many contexts (Bos et al., 2010). Ants can learn odours of rewarding food sources and also have to learn the specific odour of their own colony (Neupert et al., 2018). Indeed, ants can perform similar learning tasks as bees and other insects (Bos et al., 2010; Guerrieri and d’Ettorre, 2010; Fernandes et al., 2018; Piqueret et al., 2019). In addition to floral odours that might signal food sources, ants were successfully conditioned to

hydrocarbons that play an important role in social interactions (Bos et al., 2012, Sharma et al., 2015). For example, hydrocarbons can serve as alarm and queen pheromones, and are used to discriminate nestmates from non-nestmates (Leonhard et al., 2016). While it has been found that protein synthesis is important for the formation of the long term memory in *Camponotus* ants (Guerrieri et al., 2011), we know nothing about the neural circuits of learning in the ants, nor of the role different neurotransmitters play in ant learning.

Here, we study reward learning the black garden ant *Lasius niger*. First, we established an assay that trained the ants to associate a mixture of linear hydrocarbons to a sugar reward. We tested the memory retrieval after 5 minutes and after one day using a deep learning algorithm on videos of reward-searching ants. In a second step, we administered epinastine or flupentixol via either the food or topical application, to block OA and DA receptors and to test if a breakdown of the respective neural pathways would prevent the ants from forming short- and long-term memories of the odour-reward association.

103 **Material and Methods**

104 We trained ants to discriminate two mixtures of n-alkanes, one of which was presented
 105 with a sugar reward and the other was unrewarded, during six rounds of training trials.
 106 After that, we ran retention tests without sugar reward to see whether the ants now
 107 preferred the previously rewarded odour (conditioned stimulus - CS+) over the
 108 unrewarded odour (CS0). A first retention test was conducted ten minutes after the final
 109 learning trial to test the short term memory capabilities. The second retention test was
 110 performed one day later to test the (early) long term memory (Menzel, 1999).

111 Before the learning trials, we submitted the ants to treatments with blockers of
 112 octopamine receptors, dopamine receptors, or controls. The drugs were administered
 113 either with food (set 1) or by topical application on the mesothorax (set 2). The two
 114 experimental sets also differed in the way we recorded the data (manual vs. automated
 115 recording, see Experimental Setup).

116 **Experimental Organism**

117 Colonies of black garden ants *Lasius niger* (Linnaeus, 1758) were collected in the years
 118 2017 and 2019 in Freiburg, Germany. They were transported to the laboratory and kept
 119 in plastic boxes at 20°C, a 12:12 light:dark cycle, and were fed with meal-worms
 120 (*Tenebrio molitor*) and honey. Test tubes filled with water, cotton wool, and an
 121 aluminium foil wrapping were placed in the boxes as a refuge and a water source. The
 122 boxes' walls were lined with Fluon® (AGC Chemicals Europe, Ltd.). Around two
 123 weeks before an experiment, the ants were deprived of honey and only fed mealworms,
 124 in order to motivate them to forage for carbohydrates. We used four different source
 125 colonies for the experiments in set 1, and 11 different source colonies for set 2.

126 **Stimuli**

127 Two mixtures of three long-chain linear alkanes each were used as the two conditioned
 128 stimuli (CS). The first mixture (CS-mixture A) was n-octadecane (n-C18), n-
 129 heneicosane (n-C21), and n-heptacosane (n-C27). The second (CS-mixture B) was n-
 130 eicosane (n-C20), n-docosane (n-C22), and n-pentacosane (n-C25; all Sigma–Aldrich,

Steinheim, Germany). The hydrocarbons were solved in n-pentane (10 µg/ml) that would evaporate before the experiments. In a preliminary experiment, we used the retention test setup (see below) with untrained ants to see if they preferred any of the two odour mixtures over the other. The untrained ants spent similar times with the two odour mixtures (n = 30, wilcoxon test V = 253, p = 0.69; Figure S1). A 0.5 µL drop of sugar water (50% w/w) or pure water were used as a positive unconditioned stimulus (US+) and neutral unconditioned stimulus, respectively.

Experimental Setup

For the first set of experiments (feeding experiment) the test arena was the top of a petri dish (90 mm in diameter). Another petri dish lid was used as a roof, and a hole (40 mm in diameter) was cut into the roof to allow placing the ants into the arena. The ants were recorded from a 45° angle by a Nikon D90 with a 35mm lens, which was placed in front of the arena on a tripod. For the second set of experiments (topical application), an acrylic glass cylinder (100 mm diameter – 100 mm height) was used as an arena. The arena was covered by a cardboard box lined with aluminium foil to exclude external stimuli and to allow for even illumination by two light sources above the arena. The ants were videotaped with a SM-P600 tablet computer at 1.5x magnification, the lens of which was placed directly above the centre of the arena.

For both sets of experiments, the walls of the arena were coated with Fluon to prevent the ants from climbing. The floor was lined with filter paper that was evenly divided into quarters. A microscope cover slip (24x24 mm) was placed in the centre of each quadrant (Fig. 1A). In each learning and test trial, one of the cover slips was coated with 20 µL of odour A, another with odour B, and the remaining two with pure pentane. The solutions were always applied to the edges of the cover slips. After every trial, the filter paper and cover slips were replaced with new ones.

7

156

Figure 1 A) Design of the experimental arena used for the learning experiments. The arena was divided into four quadrants, each with a cover slip in it. One of the cover slips was coated with odour A, another one with odour B. During the learning experiments, one of the odours was paired with sucrose solution and the other with water. B, C) Traces of ants during the retention tests with the rewarded odour in the top right quadrant. The ant in B inspected the CS+, the ant in panel C did not show any particular interest in the previously rewarded odour. Colours show how the ants moved from the beginning of the test (red) to the end (blue).

157

158 Learning Trials

159 The learning procedure was adapted from Bos et al.'s (2010, 2012) work on ant
 160 learning. From the main colony, around 30 ants that were moving outside of the nest
 161 tubes were separated into a box equipped with nest tubes like the source colonies were.
 162 Here, ants could perform trophallaxis between the trials, to reduce their sugar storage
 163 and increase the motivation to forage again. The ants were marked on the gaster with a
 164 small dot of paint (Edding No. 751), so that it was possible to individually track them
 165 between the learning trials.

166 For each learning trial, 0.5 μ l of sugar solution was added to the cover slips with one of
 167 the odours (CS+), while water was added to the slip with the CS0. For each ant, the
 168 same odour was rewarded in each learning trial, but which odour was rewarded differed
 169 randomly between ants. An individual ant was then lifted gently into the arena and the
 170 time was measured until it began drinking the sugar solution. Then, the ant was

removed to prevent it from consuming too much sugar solution, and placed back into the experimental colony where it could perform trophallaxis with the nestmates. After six minutes, when trophallaxis was always complete, the ant was brought back into the arena for the next learning trial. In total, there were six learning trials per individual.

If an ant had not found the sugar solution within 3 minutes in set 1, she was removed from the learning arena and brought back into the experimental colony until the next learning trial. Ants that could not find the sugar solution in two or more learning trials were excluded from the analysis. The process was similar for set two, but we gave the ants only two minutes for the second through sixth learning trial, because the results from set 1 showed that times longer than 2 minutes are very rare in the later trials. We began set 1 with 180 ants across the six treatments and excluded 14 of those. In set 2, we had to exclude 9 out of 189 ants. The highest proportion of excluded ants came from flupentixol treatments in both sets (Table S1). There was no difference between treatments when we fed the receptor blockers ($\chi^2_5 = 8.5$, $p = 0.13$, p-value calculated by Monte Carlo simulation). In the topical application experiment, the treatments differed in the likelihood of ants failing ($\chi^2_3 = 10.7$, $p = 0.02$), although overall only 2 ants were excluded, both from the flupentixol treatment.

We tested whether the ants became quicker to find the sugar solution during the consecutive learning trials with a poisson MCMC glmm, with time until finding the sugar as the dependent variable and trial number as a continuous predictor, for each treatment separately. We entered the colony ID and individual ant ID as random factors into the model using the R package MCMCglmm with 50000 MCMC iterations and otherwise default settings (R version 4.0.3 – R Core Team ,2020; Hadfield, 2010). In set 2, we observed the ants for only 2 minutes in learning trials 2-6, as opposed to 3 minutes in trial 1. To exclude that the few ants taking longer than 2 minutes in trial 1 already cause a downward trend in the learning times across trials, we set the times of all ants that took longer than 2 minutes to 2 minutes for the glmm but left all times shorter than two minutes as they were.

200 Retention Tests

201 When the ants had finished trophallaxis after the sixth learning trial, a first retention test
 202 was conducted. Retention tests resembled training trials but no sugar solution or water
 203 was added to any of the cover slips. The cover slips with the CS+ and CS0 odours were
 204 in opposite quadrants (Fig. 1A). Each ant was recorded for two minutes. Then, a drop of
 205 sugar water was placed on the cover slip with the learned odour to prevent extinction
 206 before the retention trials on the following day. When the ant had finished to eat, it was
 207 lifted back into the test colony. The position of the cover slips was changed and the ant
 208 was recorded a second time. The duration spent in the different quadrants was averaged
 209 over the two tests for each ant. On the next day, the same ants were tested again to see if
 210 they could still remember the CS+. The marked ants were kept in isolation over night
 211 together with the other ants from the same colony that were treated on this day. The
 212 interval between the two test trials was 15-23 hours.

213 The time the ants spent in the four quadrants was measured from the video recordings.
 214 In set 1, times were measured manually using the program etholog (Ottoni, 2000). For
 215 the second set, the deep learning algorithm DeepLabCut (Mathis et al. 2018) was used.
 216 The algorithm was trained to spot the ant and record its coordinates using 200 frames
 217 from each of 20 pre-recorded videos, in which the position of the ant was marked
 218 manually. Then, the algorithm went over these frames 200,000 times to perfect its ant
 219 recognition performance. After this, the first videos from the experiments were
 220 processed with the algorithm and manually checked for errors. Only few errors were
 221 detected and then used to further improve the algorithm, which was virtually perfect
 222 after ca. 10 videos. In total, 756 videos were used for data analyses.

223 From the ant's coordinates, we could calculate in how many frames the ant was
 224 identified in each of the four quadrants, and thus how much time it spent there. We
 225 directly compared whether the ants spent more time on one of the two odour mixtures
 226 using paired wilcoxon tests. In addition, we calculated and visualized (ggplot2 –
 227 Wickham, 2016) a preference index as an intuitive measurement of preference and
 228 hence learning performance based on the times (t) on the different odours (Bos et al.
 229 2010, 2012):

10

$$PI = \frac{t_{CS+} - t_{CS-}}{t_{CS+} + t_{CS-}}$$

231 The index ranges from 1 (preference for the CS+) to -1 (preference for the CS0). If the
232 ant spent an equal amount of time on both odours, the PI would be 0.

233 **Pharmalogical Manipulations**

234 To test in how far neurotransmitters are involved in appetitive learning, the ants were
235 treated with one of two different receptor blockers before the learning trials: epinastine,
236 an octopamine receptor blocker (Kamhi et al., 2015), or flupentixol, a dopamine
237 receptor blocker (Agarwal et al., 2011).

238 For the first set of experiments, ants were fed honey infused with the blocker (20 mM of
239 epinastine hydrochloride or 50 mM of flupentixol hydrochloride) either 1-3 hours, 5-8
240 hours, or 17 hours prior to the experiment. The ants were individually fed with a 1 µL
241 drop of this solution. The different intervals were chosen because it was not clear how
242 long it takes for the drugs after feeding to become effective. The honey with the drug
243 was coloured with neutral red to control if the ants had indeed ingested the honey. Only
244 ants with a red staining in their gaster were chosen for the experiments. When the ants
245 had finished eating, they were kept in groups with other ants that had received the same
246 drug, until the start of the learning trials. Immediately before the beginning of its
247 learning trial, each ant was marked with the paint marker and moved back to a
248 subcolony of ca. 30 ants set up for this purpose. Control ants were fed pure honey
249 instead of blocker-infused honey.

250 For the second set of experiments, the drugs were dissolved in dimethylformamide
251 (DMF), and a 0.5µl droplet directly applied to the ant's cuticula on the mesothorax
252 (Barron et al., 2007). The ants were allowed to recover from the treatment for 15
253 minutes in isolation, after which they were paint-marked and returned 5 minutes later to
254 the subcolonies as described above. Another 10 minutes later, the first learning trial
255 began. For epinastine, the two dosages were 20mM or 100 mM in DMF, corresponding
256 to doses of 1.8ng/µg and 9.1ng/µg of ant body weight. For flupentixol, the dosage was
257 250 mM (39.3ng/µg). Control ants received pure DMF.

11

258 We compared the preference indices (PI) among treatments with MCMC glmms with
 259 treatment as the only predictor, so that we could test for each treatment whether it
 260 differed from the control. Colony ID was entered into the model as a random factor. In
 261 the same manner, we compared the walking speed of ants, calculated from the
 262 coordinates generated during set 2, among the different treatments.

263 Results

264 Set 1: Feeding of receptor blockers

265 Over the successive learning trials, control-treated ants became quicker in finding the
 266 sugar reward (Fig. 2, poisson glmm $p = 0.04$, $n = 30$ ants), indicating that they learned
 267 to associate the rewarded odour with the sugar reward. This was also true for all
 268 treatments with receptor blockers (epinastine 1-3h $p < 0.001$, $n = 27$ ants; epinastine 5-
 269 8h $p < 0.001$, $n = 29$ ants; epinastine 17-26h $p = 0.02$, $n = 25$ ants; flupentixol 1-3h $p =$
 270 0.04 , $n = 26$ ants, Fig. 2), with the exception of the ants that were treated with
 271 flupentixol 5-8h before the first learning trial ($p = 0.50$, $n = 21$ ants).

272 In the first retention test 10 minutes after the last learning trial, the ants were able to
 273 differentiate between CS+ and CS0, independent of treatment (Figure 3A, wilcoxon-
 274 tests $p < 0.05$). Only the ants that were fed with flupentixol 5-8h before the treatment
 275 had difficulties to differentiate between CS+ and CS0 (wilcoxon test $p = 0.052$). In most
 276 treatments, the preference indices were indistinguishable from that of control ants
 277 (glmm $p > 0.16$; Tab. S2 for details), but the PIs of ants treated with flupentixol 1-3
 278 hours before the learning trials were lower than those of control ants (glmm $p = 0.045$),
 279 and there was a similar trend for the preference indices of ants treated with epinastine 5-
 280 8 hours before the learning trials ($p = 0.07$). On the second day (Figure 3B), the control
 281 ants and some of the epinastine-treated (5-8 hours and 17-26 hours before the learning
 282 trials) ants still differentiated between CS+ and CS0 (wilcoxon test $p < 0.05$). In
 283 contrast, ants that were treated with epinastine 1-3 hours before the experiment, and the
 284 ants from both flupentixol treatments were not able to differentiate between CS+ and
 285 CS- (wilcoxon tests $p > 0.05$). However, there was no difference in the preference
 286 indices between the treatments (glmm $p > 0.5$; Tab. S2), with the exception of ants
 287 treated with epinastine 1-3 hours before the learning trials, for which there was a trend
 288 of the PIs being lower than those of control ants ($p = 0.06$).

Figure 2: The time it took ants to find the sugar reward in six consecutive learning trials. Ants were either control treated (a), or received epinastine (b-d) or flupentixol (e, f) with their food at different intervals before the first learning trials (1-3 hours, 5-8 hours, 17-26 hours). Individual data points are plotted; the lines are poisson regressions.

Figure 3: Preference for the previously rewarded odour (CS+) during retention tests with ants that received control treatment, epinastine, or flupentixol through the food. Each ant completed two retention tests without reward, one five minutes after the sixth learning trial (A), and a second test one day later (B). The ants could move in an arena in which one quadrant was marked with the CS+ odour and another one with CS0. The dotted lines indicate the null hypothesis of ants spending equal time on the CS+ and the CS0; positive values indicate that the ants spend more time in the quadrant marked with the CS+. Numbers above the boxes are sample sizes, * means that the times spent on CS+ and CS0 differed (wilcoxon test $p < 0.05$). For each treatment, standard boxplots and indices of individual ants are plotted.

15

289 Set 2: Topical application

290 The exact dosage of the receptor blockers is difficult to control when feeding the drugs,
 291 because the ants may sometimes ingest more and sometimes less of the food. We thus
 292 performed a second set of experiments where we topically applied precise doses of the
 293 blockers 30 minutes before the learning trials. During the learning trials, the time it took
 294 the ants to find the sugar solution decreased for the control-treated ants (poisson glmm p
 295 $= 0.010$, $n = 31$ ants; Fig. 4A). This effect was also evident for ants that had been treated
 296 with 100mM epinastine ($p < 0.001$, $n = 10$ ants) or flupentixol ($p = 0.001$, $n = 30$ ants)
 297 previous to the learning trials (Fig. 4B,C). Note that we could only analyse ten of the
 298 epinastine learning trials with high dose and none of the ones with low dose.

Figure 4: The time it took ants to find the sugar reward in six consecutive learning trials. Ants were either control treated (a), or topically treated with 100mM epinastine (b) or 250mM Flupentixol (c). Lines are from poisson regressions.

16

300 In the first retention test, the ants that had been treated only with DMF control preferred
 301 the CS+ over the CS0 (Fig. 5A; wilcoxon test $p < 0.001$). The same was true for ants
 302 that had been treated with 20 mM epinastine (wilcoxon test $p < 0.001$) or flupentixol
 303 (wilcoxon test $p < 0.001$). The ants that had been treated with 100 mM epinastine did
 304 not differentiate between the CS+ and CS0 on the first day (wilcoxon test $p = 0.83$). The
 305 preference indices of the 100mM epinastine treatment were lower than those of the
 306 control ants (glmm $p < 0.001$, Tab, S3), but those of the 20mM epinastine treatment ($p =$
 307 0.45) or the flupentixol treatment ($p = 0.18$) were not.

Figure 5: Preference for the CS+ during retention tests with ants that received control treatment, epinastine, or flupentixol through topical application. Each ant completed two retention tests without reward, one five minutes after the sixth learning trial (A) and a second test one day later (B). The ants could move in an arena in which one quadrant was marked with the CS+ odour and another one with CS0. The dotted lines indicate the null hypothesis of ants spending equal time on the CS+ and the CS0; positive numbers indicate that the ants spend more time in the quadrant marked with the CS+. Numbers above the boxes are sample sizes, stars indicate significant differences between the time spent on CS+ and CS0 (wilcoxon test; *** $p < 0.001$).

17

309 One day later, the preference for CS+ was still evident for control ants and ants treated
310 with 20mM epinastine (wilcoxon tests $p < 0.001$), and still absent in ants treated with
311 100mM epinastine (wilcoxon test $p = 0.77$). There was only a trend for flupentixol-
312 treated ants to discriminate CS+ and CS0 ($p = 0.092$; Figure 5B). However, all ants
313 appeared to be affected by the receptor blockers, as the preference indices of all ants
314 treated with receptor blockers were lower than those of the control ants (glmm, all $p <$
315 0.05, Tab. S3).

316 To test whether ant learning or memory retrieval may be prevented by a general lethargy
317 caused by the receptor blockers, we tested whether they affected the walking speed of
318 the ants. Indeed, during the first retention test, ants treated with both concentrations of
319 epinastine were slower than control ants (Fig. 6A; glmm $p < 0.01$; Tab. S4), but
320 flupentixol did not affect walking speed ($p = 0.29$). However, the speed reduction was
321 only in the range of 20% and should not have prevented the ants from encountering the
322 odours. Indeed, only a single ant, treated with 100mM epinastine, did not enter the
323 quadrant with the rewarded odour during the retention test. One day later, only the
324 walking speed of epinastine 100mM ants was reduced (glmm epinastine $p < 0.05$; both
325 other treatments $p > 0.59$; Tab. S4; Fig. 6B).

Figure 6. Walking speed during retention tests of control ants and ants that topically received either epinastine or flupentixol. Stars indicate treatments that changed the average speed significantly compared to the control treatment (glmm * $p < 0.05$; ** $p < 0.01$).

326 **Discussion**

327 Using a classical conditioning protocol, we could show that black garden ant workers
 328 can learn to associate blends of linear hydrocarbons with a sucrose reward, and that the
 329 memory is stable for at least one day. Exposing the ants to the octopamine receptor
 330 blocker epinastine prevented memory formation or retrieval. In contrast, when we
 331 exposed the ants to the dopamine receptor blocker flupentixol, the short term memory
 332 persisted but the long-term memory was affected. Both octopamine and dopamine
 333 signalling are thus involved in the formation of appetitive memory in ants. Our results
 334 further suggest that the active period of the receptor blockers is limited in that learning
 335 is possible again a few hours after blocker ingestion.

336 **Associating hydrocarbons with a sucrose reward**

337 During the test trials, the ants spent more time on the quadrant that contained the
 338 conditioned stimulus (CS+) than that containing a neutral stimulus not previously
 339 associated with a sucrose reward (CS0). The increase in time spent in the CS+ quadrant
 340 is likely a result of the ants searching for the reward. The ants are thus able to learn and
 341 discriminate blends of linear hydrocarbons. Most appetitive conditioning assays in
 342 hymenoptera rely on volatile odours that are typically emitted from flowers, to closely
 343 resemble the natural situation in which learning can lead to flower constancy in bees
 344 (Koethe et al., 2020). However, many pheromones of social insects are less volatile
 345 hydrocarbons. Our study confirms previous work on Carpenter Ants and Argentine Ants
 346 that demonstrates hydrocarbons are also adequate stimuli for appetitive conditioning,
 347 and that the meaning of pheromones and signature mixtures could potentially be
 348 changed through learning (Bos et al., 2012; van Wilgenburg et al., 2012; di Mauro et al.,
 349 2015; Sharma et al., 2015).

350 **Involvement of octopamine in appetitive learning**

351 Unlike control treated ants, ants that were treated with 100mM epinastine topically to
 352 the thorax did not prefer the conditioned hydrocarbon odour over a non-conditioned
 353 odour within five minutes after the learning trials, and neither did they prefer the
 354 conditioned stimulus after a break of one day. As epinastine blocks octopamine

receptors, this shows that octopamine signalling is required for the formation of short and long term memory of appetitive stimuli in ants, potentially because octopaminergic neurons relay the unconditioned stimulus to the Kenyon cells (e.g. VUMx1 in bees, Rein et al., 2013). Our findings confirm previous work demonstrating such an effect in fruit flies, honey bees, and crickets (Schwaerzel et al., 2003; Giurfa, 2007; Mizunami and Matsumoto, 2017).

Epinastine does not only affect memory but potentially also other traits. Under the influence of epinastine, silkmooths were less sensitive for pheromones (Pophof 2000), the metabolism of hungry honeybees was reduced (Buckenmüller et al., 2017), and aggression was modulated in crickets and red wood ants (Rillich and Stevenson 2011, 2018; Yakovlev, 2018). In our study, the mobility of ants treated with epinastine was reduced during the first retention test. For the high dose of epinastine, this effect was still visible one day later, which may indicate that epinastine is only slowly degraded. However, the ants still visited all quadrants of the arena during the retention tests, and they also reacted to the sucrose solution during the learning trials. We thus argue that the failure to identify the CS+ in the retention tests is not due to a decrease of motivation or even an inability to walk, but due to a lack of memory formation or retrieval. In fact, in all our learning trials with epinastine-treated ants, the time until the ants found the sucrose solution decreased. A simple explanation would be that the ants do learn to expect sucrose in the arena and search for it, which decreases search time, but they do not associate it with the odour and thus do not spend more time with the previously rewarded odour in the non-rewarded retention trials.

Ants topically treated with a lower dose of epinastine were not impaired in their learning ability. More interestingly, the effects were still visible when we fed epinastine with the food, but weaker than when we directly applied the drug to the thorax. It is possible that only part of the dose is entering the hemolymph when the drugs are taken up with the food. Ant workers possess a crop used to store food that is later regurgitated to feed nestmates (Greenwald et al., 2018). Some epinastine entering the crop may thus never reach the octopamine receptors in the brain, exposing them to lower doses than intended. It may take a while until epinastine is taken up into the hemolymph and reaches the brain, meaning that it will take action only after a certain delay (but see

Barron et al., 2007: octopamine delivered through the food reaches the honey bee brain in less than one hour). We thus fed the ants epinastine at different intervals before subjecting them to the learning trials. However, longer intervals only reduced the effect of epinastine, and ca. 20 hours after being fed the receptor blockers, the preference indices of epinastine-fed individuals were no different than those of control ants. Finally, when ants were fed epinastine, we found only weak effects on the short term memory retention. Given that there was a clear effect on the short term memory when epinastine was topically applied, this might have to do with long and short term memory being formed in different parts of the brain (e.g. different populations of Kenyon cells, Sachse and Galizia, 2003) and potentially at different times. If short term memory formation is quicker than long term memory formation, epinastine may have reached the brain before the long term memory formation, but after short term memory formation was complete.

Involvement of dopamine

In our experiments, flupentixol strongly affected the long term memory as well, but not necessarily the short term memory. After topical application, we did not find any evidence of flupentixol affecting the short term memory. Flupentixol blocks dopamine receptors, so that our observation suggest that the dopaminergic neurons are probably not required in ants to relay the unconditioned stimulus to the Kenyon cells (as it is in fruit flies and bees, Schwaerzel et al., 2003; Giurfa, 2007). However, we found weak effects of flupentixol on the short term memory when we fed it to the ants, which may indicate that a functional dopaminergic system may improve learning performance.

In contrast to the short term memory, the long term memory was always affected when we administered flupentixol via either food or topical application. It is particularly curious that this even happened after topical application of flupentixol, when ants still clearly preferred the rewarded stimulus in the short term memory retention tests. This indicates a necessary involvement of dopaminergic neurons in the consolidation phase of long term memory formation that is independent of the short term memory and cannot be rescued by octopaminergic neurons, which are still functioning when only flupentixol is administered. The dopaminergic neurons may thus not be relaying the

unconditioned stimulus to the learning centres, but be part of feedback loops or modulation pathways (Eschbach et al., 2020). Long term memory formation is a more complex process than short term memory formation (e.g. Menzel, 1999; Villar, 2020) and memory consolidation might rely on additional neural circuits that could be dopaminergic. For example, some lateral neurons in the antennal lobes are dopaminergic (Galizia and Sachse, 2010) and might be used to improve the perception of relevant odours such as the CS+. In the mushroom body, Kenyon cells receive feedback from the primary protocerebral anterior cluster via dopaminergic neurons in fruit fly larvae (Lyutova et al., 2019).

Blocking dopamine receptors does not only influence appetitive and aversive learning. Blocking the AmDOP2 receptor has led to reduced motor behaviours and an increase of grooming in honey bees (Mustard et al., 2010). In *Drosophila*, social interactions and temperature preferences seem to be affected (Verlinden, 2018). In our study, there was no evidence of effects on motor behaviour, since walking speed of flupentixol-treated ants did not differ from that of control ants. Interestingly though, flupentixol fed 5-8 hours before learning, the treatment that affected both the short and the long term memory, also prevented an improvement of the times the ants needed to find the reward during the learning trials (Fig. 2). Also, in the flupentixol-treated groups, particularly many individuals did not find the sucrose reward during the two minutes of the learning trial (Tab. S1). One possible explanation could be that blocking dopamine signalling interferes with the motivational state of the ants. If lack of dopamine signalling would make them believe they were not hungry, they might not be primed to seek the sucrose reward in the learning trials, but may still be drawn to the conditioned stimulus in the retention tests because of its familiarity. Indeed, dopamine is important for hunger signalling in *Drosophila* (Krashes et al. 2009; Siju et al., 2021). Hunger affects the motivational state, which is important for memory formation and retrieval because satiated flies do not seek food. However, dopaminergic neurons appear to signal satiation rather than hunger in *Drosophila*, so that blocking them should improve motivation and thus memory formation (Krashes et al., 2009). If the neural circuits were similar in ants, hunger could thus not explain the curious pattern caused by blocking dopamine receptors. However, the motivation of ants as social beings is perhaps regulated less by their personal physiological hunger and more by that of their colony,

22

448 so that entirely different regulatory circuits might be involved here (e.g. Cholé et al.,
449 2019). In any case, such a lack of hunger or motivation could only explain an effect of
450 flupentixol on the short term memory in the feeding trial: In the topical application
451 experiment, ants did initially learn in learning trials and remembered in the first
452 retention test, but clearly failed to remember on the next day. This observation indicates
453 an involvement of dopamine in the consolidation of the long term memory, independent
454 short term memory formation and motivation.

455 **Conclusions**

456 *Lasius niger* ants can learn to associate mixtures of linear hydrocarbons to a sugar
457 reward and remember the association for at least 24 hours. Both octopamine and
458 dopamine appear to be involved in memory formation, with strong effects of a
459 pharmacological octopamine knockout on both the short and the long term memory.
460 Dopamine signalling is required for the formation of the long term memory but not
461 necessarily for the short term memory.

462

463 **Acknowledgements**

464 We would like to thank the members of the Department of Ecology and Evolution,
465 University of Freiburg, Germany, and Andrew Straw for fruitful discussions. Thanks to
466 Mélanie Bey for helpful comments on a previous version of the manuscript, and to the
467 German Research Foundation (DFG grant) for funding.

23

468 Literature

469 **Agarwal, M., Guzman, M. G., Morales-Matos, C., Del Valle Diaz, R. A., Abramson,**
 470 **C. I., and Giray, T.** (2011). Dopamine and Octopamine Influence Avoidance Learning
 471 of Honey Bees in a Place Preference Assay. *PLoS ONE* **6(9)**, e25371.

472

473 **Awata, H., Watanabe, T., Hamanaka, Y., Mito, T., Noji, S. and Mizunami, M.**
 474 (2015). Knockout crickets for the study of learning and memory: Dopamine receptor
 475 Dop1 mediates aversive but not appetitive reinforcement in crickets. *Sci. Rep.* **5(1)**,
 476 15885.

477

478 **Barron, A. B., Maleszka, J., Vander Meer, R. K., Robinson, G. E. and Maleszka, R.**
 479 (2007). Comparing injection, feeding and topical application methods for treatment of
 480 honeybees with octopamine. *J. Insect Physiol.* **53(2)**, 187–194.

481

482 **Beggs, K. T., Tyndall, J. D. A. and Mercer, A. R.** (2011). Honey Bee Dopamine and
 483 Octopamine Receptors Linked to Intracellular Calcium Signaling Have a Close
 484 Phylogenetic and Pharmacological Relationship. *PLoS ONE* **6(11)**, e26809.

485

486 **Berry, J.A., Cervantes-Sandoval, I., Nicholas, E.P. and Davis, R.L.** (2012).
 487 Dopamine Is Required for Learning and Forgetting in *Drosophila*. *Neuron* **74(3)**, 530–
 488 542.

489

490 **Bos, N., Guerrieri, F. J. and d'Ettorre, P.** (2010). Significance of chemical
 491 recognition cues is context dependent in ants. *Anim. Behav.* **80**, 839-844.

24

492 **Bos, N., Dreier, S., Jorgensen, C. G., Nielsen, J., Guerrieri, F. J. and d’Ettorre, P.**
 493 (2012). Learning and perceptual similarity among cuticular hydrocarbons in ants. *J.*
 494 *Insect Physiol.* 58(1), 138–146.

495

496 **Buckemüller, C., Siehler, O., Göbel, J., Zeumer, R., Ölschläger, A. and Eisenhardt,**
 497 **D.** (2017). Octopamine Underlies the Counter-Regulatory Response to a Glucose
 498 Deficit in Honeybees (*Apis mellifera*). *Front. Syst. Neurosci.* **11**, 63.

499

500 **Cholé, H., Carcaud, J., Mazeau, H., Famié, S., Arnold, G. and Sandoz, J.-C.** (2019).
 501 Social Contact Acts as Appetitive Reinforcement and Supports Associative Learning in
 502 Honeybees. *Curr. Biol.* **29(8)**, 1407-1413.

503

504 **Claßen, G. and Scholz, H.** (2018). Octopamine Shifts the Behavioral Response From
 505 Indecision to Approach or Aversion in *Drosophila melanogaster*. *Front. Behav.*
 506 *Neurosci.* **12**, 131.

507

508 **di Mauro, G., Perez, M., Lorenzi, M. C., Guerrieri, F. J., Millar, J. G. and**
 509 **d’Ettorre, P.** (2015). Ants Discriminate Between Different Hydrocarbon
 510 Concentrations. *Front. Ecol. Evol.* **3**, 133.

511

512 **Eschbach, C., Fushiki, A., Winding, M., Schneider-Mizell, C. M., Shao, M.,**
 513 **Arruda, R., Eichler, K., Valdes-Aleman, J., Ohyama, T., Thum, A. S., Gerber, B.,**
 514 **Fetter, R. D., Truman, J. W., Litwin-Kumar, A., Cardona, A. and Zlatić, M.** (2020).
 515 Recurrent architecture for adaptive regulation of learning in the insect brain. *Nat.*
 516 *Neurosci.* **23(4)**, 544-555.

25

517

518 **Fernandes, A. S. D., Buckley, C. L. and Niven, J. E.** (2018). Visual associative
519 learning in wood ants. *J. Exp. Biol.* **221**(3), jeb173260.

520

521 **Galizia, C. G. and Sachse, S.** (2010). Odor Coding in Insects. In *The Neurobiology of*
522 *Olfaction* (ed. A. Menini), pp. 35-70. Boca Raton (FL): CRC Press.

523

524 **Giurfa, M.** (2007). Behavioral and neural analysis of associative learning in the
525 honeybee: a taste from the magic well. *J Comp Physiol A Neuroethol Sens Neural*
526 *Behav Physiol.* **93**, 801–824.

527

528 **Greenwald, E. E., Baltiansky, L. and Feinerman, O.** (2018). Individual crop loads
529 provide local control for collective food intake in ant colonies. *ELife* **7**, e31730.

530

531 **Guerrieri, F. J. and d’Ettorre, P.** (2010). Associative learning in ants: Conditioning of
532 the maxilla-labium extension response in *Camponotus aethiops*. *J. Insect Physiol.* **56**(1),
533 88–92.

534

535 **Guerrieri, F. J., d’Ettorre, P., Devaud, J. M. and Giurfa, M.** (2011). Long-term
536 olfactory memories are stabilised via protein synthesis in *Camponotus fellah* ants. *J.*
537 *Exp. Biol.* **214**(19), 3300-3304.

538 **Hadfield, J.** (2010). MCMC Methods for Multi-Response Generalized Linear Mixed
539 Models: TheMCMCglmmRPackage *J. Stat. Softw.* **33**(2), 10.18637/jss.v033.i02.

26

540

541 **Hammer, M. and Menzel, R.** (1998). Multiple sites of associative odor learning as
542 revealed by local brain microinjections of octopamine in honeybees. *Learn Mem.* **5(1)**,
543 146-56.

544

545 **Hige, T., Aso, Y., Modi, M. N., Rubin, G.M. and Turner, G. C.** (2015).
546 Heterosynaptic Plasticity Underlies Aversive Olfactory Learning in *Drosophila*. *Neuron*
547 **88(5)**, 985-998.

548

549 **Kamhi, J. F., Nunn, K., Robson, S. K. A. and Traniello, J. F. A.** (2015).
550 Polymorphism and division of labour in a socially complex ant: neuromodulation of
551 aggression in the Australian weaver ant, *Oecophylla smaragdina*. *Proc. Royal Soc. B*
552 **282**

553

554 **Koethe, S., Fischbach, V., Banysch, S., Reinartz, L., Hrncir, M. and Lunau, K. A**
555 (2020). Comparative Study of Food Source Selection in Stingless Bees and Honeybees:
556 Scent Marks, Location, or Color. *Front. Plant Sci.* **11**, 516. .

557

558 **Krashes, M. J., DasGupta, S., Vreede, A., White, B., Armstrong, J. D. and Waddell,**
559 **S.** (2009). A neural circuit mechanism integrating motivational state with memory
560 expression in *Drosophila*. *Cell* **139(2)**, 416–427.

561

562 **Leonhardt, S. D., Menzel, F., Nehring V., Schmitt, T.** (2016). Ecology and Evolution
563 of Communication in Social Insects, *Cell* **164(6)**, 1277-1287.

27

564

565 **Lyutova, R., Pfeuffer, M., Segebarth, D., Habenstein, J., Selcho, M., Wegener, C.,**
 566 **Thum, A.S. and Pauls, D.** (2019). Reward signaling in a recurrent circuit of
 567 dopaminergic neurons and peptidergic Kenyon cells. *Nat. Commun.* **10**, 3097.

568

569 **Mancini, N., Giurfa, M., Sandoz, J. C. and Avarguès-Weber, A.** (2018). Aminergic
 570 neuromodulation of associative visual learning in harnessed honey bees. *Neurobiol.*
 571 *Learn. Mem.* **155.**, 556-567.

572

573 **Mathis, A., Mamidanna, P., Cury, K. M., Murthy, V. N., Mathis, M. W. and Bethge,**
 574 **M.** (2018). DeepLabCut: markerless pose estimation of user-defined body parts with
 575 deep learning. *Nat. Neurosci.* **21**, 1281–1289.

576

577 **Menzel, R.** (1999). Memory dynamics in the honeybee. *J Comp Physiol A* **185**, 323–
 578 340.

579

580 **Menzel, R.** (2014). The insect mushroom body, an experience-dependent recording
 581 device. *J. Physiol. Paris* **108**, 84–95.

582 **Mizunami, M. and Matsumoto, Y.** (2017). Roles of Octopamine and Dopamine
 583 Neurons for Mediating Appetitive and Aversive Signals in Pavlovian Conditioning in
 584 Crickets. *Front. Physiol.* **8**, 1027.

585

28

586 **Mustard, J. A., Blenau, W., Hamilton, I. S., Ward, V. K., Ebert, P. R. and Mercer,**
 587 **A. R.** (2003). Analysis of two D1-like dopamine receptors from the honey bee *Apis*
 588 *mellifera* reveals agonist-independent activity. *Mol. Brain Res.* **113(1-2)**, 67-77.

589

590 **Mustard, J. A., Pham, P. M. and Smith, B. H.** (2010). Modulation of motor behavior
 591 by dopamine and the D1-like dopamine receptor AmDOP2 in the honey bee. *J. Insect*
 592 *Physiol.* **56(4)**, 422–430.

593

594 **Neupert, S., Hornung, M., Grenville Millar, J. and Kleineidam, C. J.** (2018).
 595 Learning Distinct Chemical Labels of Nestmates in Ants. *Front. Behav. Neurosci.* **12**,
 596 191.

597

598 **Ottoni, E. B.** (2000). EthoLog 2.2: A tool for the transcription and timing of behavior
 599 observation sessions. *Behav. Res. Methods Instrum. Comput.* **32(3)**, 446-449.

600

601 **Perisse, E., Yin, Y., Lin, A. C., Lin, S., Huetteroth, W. and Waddell, S.** (2013).
 602 Different kenyon cell populations drive learned approach and avoidance in *Drosophila*.
 603 *Neuron* **79(5)**, 945–956.

604

605 **Perry, C. J. and Barron, A. B.** (2013). Neural Mechanisms of Reward in Insects.
 606 *Annu. Rev. Entomol.* **58**, 543–562.

607

29

608 **Piqueret, B., Sandoz, J.-C., and d’Ettorre, P.** (2019). Ants learn fast and do not
609 forget: Associative olfactory learning, memory and extinction in *Formica fusca*. *R. Soc.*
610 *Open Sci.* **6(6)**, 190778.

611

612 **Pophof, B.** (2000). Octopamine modulates the sensitivity of silkworm pheromone
613 receptor neurons. *J. Comp. Physiol A.* **186(3)**, 307-13.

614

615 **Qi, Y. X., Xu, G., Gu, G. X., Mao, F., Ye, G. Y., Liu, W. and Huang J.** (2017). A new
616 *Drosophila* octopamine receptor responds to serotonin. *Insect. Biochem. Mol. Biol.* **90**,
617 61-70.

618

619 **R Core Team** (2020). R: A language and environment for statistical computing. R
620 Foundation , Vienna, Austria. URL <https://www.R-project.org/>

621

622 **Rein, J., Mustard, J. A., Strauch, M., Smith, B. H. and Galizia, C. G.** (2013).
623 Octopamine modulates activity of neural networks in the honey bee antennal lobe. *J.*
624 *Comp. Physiol. A* **199**, 947–962.

625

626 **Rillich, J. and Stevenson, P.A.** (2011). Winning fights induces hyperaggression via the
627 action of the biogenic amine octopamine in crickets. *PLoS One.* **6(12)**, e28891.

628

629 **Rillich, J. and Stevenson, P.A.** (2018). Serotonin Mediates Depression of Aggression
630 After Acute and Chronic Social Defeat Stress in a Model Insect. *Front. Behav.*
631 *Neurosci.* **12**, 233.

30

632

633 **Roeder, T., Degen, J. and Gewecke, M.** (1998). Epinastine, a highly specific
634 antagonist of insect neuronal octopamine receptors. *Eur. J. Pharmacol.* **349(2–3)**, 171-
635 177.

636

637 **Rohwedder, A., Wenz, N. L., Stehle, B., Huser, A., Yamagata, N., Zlatic, M.,**
638 **Truman, J. W., Tanimoto, H., Saumweber, T., Gerber, B. and Thum, A. S.** (2016).
639 Four Individually Identified Paired Dopamine Neurons Signal Reward in Larval
640 *Drosophila*. *Curr. Biol.* **26**, 661-669.

641

642 **Sabandal, J. M, Sabandal, P. R., Kim, Y.-C. and Han, K.-A.** (2020) Concerted
643 Actions of Octopamine and Dopamine Receptors Drive Olfactory Learning. *J.*
644 *Neurosci.* **40(21)**, 4240-4250.

645

646 **Sachse, S. and Galizia, C. G.** (2003). The Coding of Odour-Intensity in the Honeybee
647 Antennal Lobe: Local Computation Optimizes Odour Representation. *Eur. J.*
648 *Neurosci.* **18**, 2119-2132.

649

650 **Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S. and**
651 **Heisenberg, M.** (2003). Dopamine and Octopamine Differentiate between Aversive and
652 Appetitive Olfactory Memories in *Drosophila*. *J. Neurosci* **23 (33)**, 10495–10502.

653

654 **Sharma, K. R., Enzmann, B. L., Schmidt, Y., Moore, D., Jones, G. R., Parker, J.,**
655 **Berger, S. L, Reinberg, D., Zwiebel, L. J., Breit, B., Liebig, J., Ray, A.** (2015).

31

656 Cuticular Hydrocarbon Pheromones for Social Behavior and Their Coding in the Ant
657 Antenna. *Cell Rep.* **12(8)**, 1261-1271.

658

659 **Siju, K. P., De Backer, J. F. & Grunwald Kadow, I. C.** (2021). Dopamine modulation
660 of sensory processing and adaptive behavior in flies. *Cell Tissue Res.* **383**, 207–225 (.
661

662 **van Wilgenburg, E., Felden, A., Choe, D.-H., Sulc, R., Luo, J., Shea, K. J., Elgar,**
663 **M. A. and Tsutsui, N. D.** (2012) Learning and discrimination of cuticular hydrocarbons
664 in a social insect. *Biol. Lett.* **8**, 17–20
665

666 **Vergoz, V., Roussel, E., Sandoz, J. C. and Giurfa, M.** (2007). Aversive Learning in
667 Honeybees Revealed by the Olfactory Conditioning of the Sting Extension Reflex.
668 *PLoS ONE* **2(3)**, e288.

669

670 **Verlinden, H.** (2018). Dopamine signalling in locusts and other insects. *Insect*
671 *Biochem. Mol. Biol.* **97**,40-52.

672

673 **Villar, M. E., Marchal, P., Viola, H. and Giurfa, M.** (2020) Redefining Single-Trial
674 Memories in the Honeybee. *Cell Rep.* **30(8)**,2603-2613.

675

676 **Wickham, H.** (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York, US:
677 Springer-Verlag.

678

32

679 **Xu, G., Chang, X. F., Gu, G. X., Jia, W. X., Guo, L., Huang, J. and Ye, G. Y. (2020).**

680 Molecular and pharmacological characterization of a β -adrenergic-like octopamine

681 receptor from the green rice leafhopper *Nephotettix cincticeps*. *Insect. Biochem. Mol.*

682 *Biol.* **120**, 103337.

683

684 **Yakovlev, I.K. (2018) Effects of Octopamine on Aggressive Behavior in Red Wood**

685 *Ants. Neurosci. Behav. Physi.* **48**, 279–288.