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1 **Degradation of an appetitive olfactory memory *via***
2 **devaluation of sugar reward is mediated by 5-HT**
3 **signaling in the honey bee**

4

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25 **Abstract**

26 Conditioned taste aversion (CTA) learning induces the devaluation of a preferred food through its
 27 pairing with a stimulus inducing internal illness. In invertebrates, it is still unclear how this aversive
 28 learning impairs the memories of stimuli that had been associated with the appetitive food prior to
 29 its devaluation. Here we studied this phenomenon in the honey bee and characterized its neural
 30 underpinnings. We first trained bees to associate an odorant (conditioned stimulus, CS) with
 31 appetitive fructose solution (unconditioned stimulus, US) using a Pavlovian olfactory conditioning.
 32 We then subjected the bees that learned the association to a CTA training during which the antennal
 33 taste of fructose solution was contingent or not to the ingestion of quinine solution, which induces
 34 malaise a few hours after ingestion. Only the group experiencing contingent fructose stimulation and
 35 quinine-based malaise exhibited a decrease in responses to the fructose and a concomitant decrease
 36 in odor-specific retention in tests performed 23 h after the original odor conditioning. Furthermore,
 37 injection of dopamine- and serotonin-receptor antagonists after CTA learning revealed that this long-
 38 term decrease was mediated by serotonergic signaling as its blockade rescued both the responses to
 39 fructose and the odor-specific memory 23 h after conditioning. The impairment of a prior CS
 40 memory by subsequent CTA conditioning confirms that bees retrieve a devaluated US representation
 41 when presented with the CS. Our findings further highlight the importance of serotonergic signaling
 42 in aversive learning in the bee and uncover mechanisms underlying aversive memories induced by
 43 internal illness in invertebrates.

44 **Key Words:** Learning, Conditioned Taste Aversion, Malaise, US Devaluation, CS-US Contingency
 45 Degradation, Honey Bee, PER Conditioning.

46

47 **Abbreviations**

48 **CTA:** conditioned taste aversion

CS: conditioned stimulus

49 **US:** unconditioned stimulus

PER: proboscis extension response

50 **SER:** sting extension response

PQ: paired quinine group

51 **UQ:** unpaired quinine group

PW: paired water group

52 **UW:** unpaired water group

5-HT: serotonin

53 **DA:** dopamine

54

55 1. Introduction

56 Among the different forms of associative learning, Pavlovian learning has received wide
57 attention due to the universality of its principles across species (Fanselow and Wassum, 2015). In
58 this learning form, individuals learn the association between a conditioned stimulus (CS) and an
59 unconditioned stimulus (US). The latter is a biologically relevant stimulus that triggers an inborn
60 response while the former is initially a neutral stimulus that does not elicit a response (Pavlov,
61 1927). Forward pairing of the CS with the US results in the acquisition of a predictive relationship
62 between CS and US, and thus, of a conditioned response to the CS.

63 A well-established form of Pavlovian learning is taste aversion learning (or conditioned
64 taste aversion, CTA), which was established by Garcia and colleagues (1955). This form of aversive
65 learning relies on associating a preferred food or its taste with a gastric malaise usually produced
66 by intraperitoneal injections of lithium chloride (LiCl), or by irradiations delivered immediately after
67 food ingestion. In this way, animals associate food taste as a CS with gastric illness as a US, and
68 avoid in consequence consuming the previously preferred food. This learning is extremely robust
69 and can induce long-lasting aversive memories after just one forward pairing of food with gastric
70 malaise (Reilly and Schachtman, 2008). It is a ubiquitous phenomenon, which has been observed in
71 many species, from invertebrates to humans (Reilly and Schachtman, 2008), and which has a clear
72 ecological relevance (Garcia, Ervin, and Koelling, 1966) as it mediates avoidance of substances that
73 are potentially toxic and may result in an individual's death.

74 As CTA relies on the devaluation of a preferred food, a relevant question refers to the
75 transfer of this aversive learning to stimuli that have been previously paired with this appetitive
76 food. Prior Pavlovian associations may have been established in which the same food acted as a US
77 for other types of CS. For instance, an animal may have first learned the contingency between an
78 auditory CS and a type of food, which will later be devaluated via injection of LiCl. In this case, the
79 US devaluation results in a reduction in CS responses (Holland and Straub, 1979). This also shows
80 that the subject mentally recalls the devaluated US when presented with the CS (Fanselow and
81 Wassum, 2015).

82 An interesting animal model to study this problem is the honey bee *Apis mellifera*. Honey
83 bees have been widely studied to understand behavioral and neural principles governing Pavlovian
84 conditioning based on the existence of a laboratory Pavlovian protocol, which allows the study of
85 olfactory appetitive learning (Bitterman, Menzel, Fietz, and Schäfer, 1983; Giurfa, 2007; Giurfa and
86 Sandoz, 2012). In this protocol, termed the olfactory conditioning of the proboscis extension
87 response (PER), harnessed bees are exposed to an odorant (CS) followed by a reward of sucrose
88 solution (US), which is delivered to their antennae and then to the proboscis. The antennal

89 stimulation with sucrose elicits PER in hungry bees. After successful learning, the odorant acquires
90 the capacity to elicit PER *per se*. Olfactory PER conditioning leads to robust long-term memories
91 that are stabilized in time (Menzel, 1999; 2001; Muller, 2012), even after a single trial conditioning
92 (Villar, Marchal, Viola, and Giurfa, 2020), thanks to the process of protein synthesis.

93 Research on honey bee gustation has shown that although free-flying bees seem to avoid
94 some bitter compounds that are bitter to humans such as quinine solution (Avergues-Weber, de
95 Brito Sanchez, Giurfa, and Dyer, 2010), they respond in a different way to them if they are
96 harnessed and their mobility is reduced (de Brito Sanchez, Serre, Avergues-Weber, Dyer, and
97 Giurfa, 2015). In this case, they may consume different bitter substances without obvious
98 reluctance, thus raising the question of the bees' capacity to detect bitter tastes under these
99 experimental conditions (Ayestarán, Giurfa, and de Brito Sanchez, 2010; de Brito Sanchez, 2011; de
100 Brito Sanchez, Giurfa, de Paula Mota, and Gauthier, 2005). Consumption of these substances (e.g.
101 quinine solution) in a pure state (i.e. non-mixed with other substances) induces significant
102 mortality a few hours after ingestion, thus showing their harmfulness for bees. It was thus
103 suggested that bees experience a malaise-like state short after ingesting these pure substances
104 (Ayestarán et al., 2010), which was confirmed by analyses on motor performances (Hurst,
105 Stevenson, and Wright, 2014). The existence of such a malaise opens, therefore, the possibility of
106 studying CTA in this insect model.

107 Pairing odorants with sucrose devaluated by the addition of bitter substances or toxins
108 results in reduced learning performances in olfactory PER conditioning (Mustard, Dews, Brugato,
109 Dey, and Wright, 2012; Wright, Mustard, Simcock, Ross-Taylor, McNicholas, Popescu, and Marion-
110 Poll, 2010). Yet, these studies using US devaluation did not address the impact of CTA on *prior*
111 appetitive memories. In an experiment that addressed this issue (Ayestarán et al., 2010), bees were
112 first trained to associate an odor with fructose solution. Then they were subjected to paired or
113 unpaired presentations of fructose and quinine solutions, to induce associative fructose
114 devaluation in the paired, but not in the unpaired, group. Ninety minutes later, bees exhibited not
115 only reduced fructose responsiveness, consistent with CTA, but also a reduction in responses to the
116 odor previously associated with fructose (Ayestarán et al., 2010). Yet, the degradation of the
117 previous olfactory memory was observed in both the paired and the unpaired groups, thus
118 indicating that it was not related to an associative process. Given the short time elapsed between
119 the fructose devaluation and the CS-testing phases, the effect was rather due to the permanence
120 of generalized malaise induced by the ingestion of the bitter compound. Therefore, further spacing
121 between US devaluation and CS-testing phases is necessary to determine if the devaluation of
122 fructose via CTA translates into the degradation of prior CS memories.

123 Here we studied this phenomenon and characterized its neural underpinnings using a
124 pharmacological approach. We first determined if the ingestion of a 10 mM quinine solution results
125 in significant mortality consistent with the development of a malaise-like state in surviving bees.
126 Then we performed a second experiment including three phases: 1) a *first phase of olfactory*
127 *conditioning*, 2) a *second phase of CTA* based on quinine ingestion and 3) a *third phase of US and CS*
128 *testing*, performed 23 h and 21 h 30 min after the end of the first and second phases, respectively.
129 The last phase allowed determining if CTA decreased not only US responses (US devaluation) but
130 also degraded the original CS appetitive memory. By introducing a long spacing between the
131 second and the third phase, we ensured that bees that survived the toxicosis induced by quinine
132 ingestion were no longer in a malaise-like state during the test phase. In a third experiment, we
133 reproduced the phases of the previous experiment and injected, in addition, pharmacological
134 antagonists of biogenic amine receptors into the bee brain following the second phase of CTA. We
135 used flupentixol as a dopamine (DA) receptor antagonist (Beggs, Tyndall, and Mercer, 2011;
136 Mustard, Blenau, Hamilton, Ward, Ebert, and Mercer, 2003) because DA signaling has been
137 repeatedly associated with aversive learning (Vergoz, Roussel, Sandoz, and Giurfa, 2007) and
138 mediates the reinforcing properties of aversive stimuli in the bee brain (Tedjakumala, Aimable, and
139 Giurfa, 2014; Tedjakumala and Giurfa, 2013). We also used methiothepin as an antagonist of
140 serotonin (5-HT) receptors as 5-HT signaling is important for aversive responsiveness (Tedjakumala
141 et al., 2014) and was suggested as a key neurotransmitter for malaise states in bees (Wright et al.,
142 2010). We thus aimed at identifying the neural mechanisms mediating changes in US and CS
143 responses due to CTA.

144 Our results reveal that the ingestion of quinine generates a post-ingestive malaise, which
145 devaluates fructose reward and further degrades a prior appetitive contingency between an
146 odorant and fructose *via* 5-HT signaling. They also broaden the spectrum of aversive learning
147 protocols available in the honey bee, and bring new light to the mechanistic study of aversive
148 memories induced by internal illness in invertebrates.

149

150 **2. Materials and Methods**

151 2.1. *Insects*

152 Experiments were performed on honeybee workers (*Apis mellifera* L.) obtained from the
153 experimental apiary located at the University Paul Sabatier. Bees were captured every morning,
154 enclosed in glass vials, and cooled down on ice until they stopped moving. They were then
155 harnessed in individual plastic tubes using tape strips and low-temperature melting wax applied to
156 the back of the head. In this way, they could only move their antennae and mouthparts, including

157 the proboscis. Bees were then fed with 3 μ l of 1.5 M sucrose solution and kept in an incubator at
158 28°C for 150 min.

159 We conducted three different experiments. In the **first experiment**, we asked if ingestion
160 of 20 μ l of a 10 mM quinine solution induces an increase in mortality within few hours after
161 ingestion, consistent with the toxicosis that surviving rodents experience as a malaise state (Garcia,
162 Hankins, and Rusiniak, 1974; Rzóska, 1953). In a **second experiment**, we studied if CTA learning
163 following appetitive olfactory conditioning decreases not only US responses but also conditioned
164 CS (odor) responses acquired in the first phase of appetitive conditioning. In the **third experiment**,
165 we followed the same protocol as in the second experiment, but injected in addition antagonists of
166 dopamine (DA) and serotonin (5-HT) receptors into the bee brain to identify the neural
167 underpinning of the behavioral responses characterized in the second experiment.

168

169 2.2. *First experiment: survival analyses*

170 We first fed harnessed bees with 3 μ l of 1.5 M sucrose solution and kept them under rest
171 for two and half hours in an incubator. Afterwards, we split them in two groups, one of which was
172 fed with distilled water (n = 44), and the other with 10 mM quinine solution (n = 60). Quinine
173 hydrochloride dehydrate (Sigma Aldrich, France, CAS Number 6119-47-7) was used to prepare the
174 10 mM quinine solution. Previous results showed that bees can ingest up to 20 μ l of both distilled
175 water and other aversive substances when their proboscis is gently extended and the solutions are
176 delivered directly to it (Ayestarán et al., 2010). Each harnessed bee received in this way 20 μ l (4
177 times 5 μ l; i.e. one third of their full crop load) (Núñez, 1966) of its respective solution using a
178 graded micropipette that allowed verifying the volume ingested. We quantified the number of
179 dead bees every 30 min, from the end of the ingestion (feeding of the last bee in either group) until
180 240 min later (Fig. 1a).

181

182 2.3. *Second experiment: degradation of an appetitive odor memory via US devaluation by CTA*

183 2.3.1. *First phase: olfactory PER conditioning*

184 In the **first phase** (first day), bees were conditioned during four spaced trials to associate
185 the odorant 1-nonanol (Sigma Aldrich, France, CAS Number 143-08-8) as the CS with 1.66 M
186 fructose solution as the US (Sigma Aldrich, France, CAS Number 57-48-7). We chose fructose
187 solution as it can mediate associative learning despite being a weaker (Ayestarán et al., 2010) and
188 less preferred US (Wykes, 1952) than sucrose solution. Moreover, fructose can be devaluated via

189 its pairing with substances inducing toxicosis in CTA while sucrose is resistant to devaluation under
190 the same conditions due to its highly appetitive value (Ayestarán et al., 2010). Prior to conditioning,
191 harnessed bees were checked for PER to the fructose solution. To this end, we touched the
192 antennae of the bees with the solution and recorded if they extended the proboscis. Extension of
193 the proboscis beyond the virtual line separating the tip of the mandibles was counted as PER;
194 partial or incomplete responses were not considered.

195 We used four conditioning trials spaced by an intertrial interval of 15 min as conditioning
196 with multiple spaced trials induces the formation of long-term olfactory memories retrievable from
197 24 h on (Menzel, 1999). Each trial started when the bee was placed in front of the odor delivery
198 setup (Raiser, Galizia, and Szyszka, 2016), which was controlled by a microcomputer (Arduino Uno).
199 The apparatus released a continuous flow of clean air (3300 ml/min) pointed towards the bee
200 head. Twelve seconds after the placement in front of the odor delivery setup, the airflow was
201 diverged through the vial containing 1-nonanol during 6 s. The fructose solution was delivered 3 s
202 later by means of a toothpick contacting the antennae and proboscis during 4 s. Thus, the CS lasted
203 6 s, the US lasted 4 s and they had an overlap of 3 s (Fig. 2). The bee was left in front of the clean
204 airflow for further 12 s, so that the training trial lasted 30 s in total. An air extractor was placed
205 behind the bee to prevent odorant accumulation. The entire phase lasted 50 min, including the
206 time needed to place the bees in front of the odor delivery setup.

207 We quantified the percentage of bees that exhibited conditioned PER to 1-nonanol during
208 the four trials. At the end of conditioning, we kept only those bees that responded to 1-nonanol in
209 the last conditioning trials to ensure that only learners participated in the next phases of the
210 experiment.

211 2.3.2. *Second phase: CTA learning*

212 In the **second phase** (first day), bees that learned the association were subjected to a
213 conditioned taste aversion protocol in which antennal stimulation with fructose was followed by
214 ingestion of a 10 mM quinine solution. This phase started 40 min after the end of the previous
215 conditioning phase (Fig. 2). The bees were assigned to five groups. The *Paired-Quinine group* (PQ; n
216 = 41) experienced four stimulations of fructose delivered to the antennae, each one followed by 5
217 μ l of 10 mM quinine delivered to the proboscis. The *Unpaired-Quinine group* (UQ; n = 36)
218 experienced the same fructose and quinine stimulations but in a non-contingent way. Thus, both
219 groups consumed 20 μ l of 10 mM quinine solution, which is sufficient to induce a malaise-like state
220 in the next 4 h following ingestion (see first experiment), but only the PQ group was subjected to
221 an associative conditioned food aversion based on the contingency between fructose and the
222 aversive effect induced by quinine ingestion. The *Paired-Water group* (PW; n = 42) received four

223 stimulations with fructose delivered to the antennae, each one contingent to 5 μ l of distilled water
224 delivered to the proboscis. The *Unpaired-Water group* (UW; n = 39) experienced the same fructose
225 and distilled water stimulations but in a non-contingent way. Finally, an *Unhandled Group* (UG; n =
226 46) was left untreated during the same amount of time (55 min) spent by the other four groups in
227 this second phase.

228 In all groups, except the UG group, bees experienced eight trials with 7 min intertrial
229 intervals. Paired groups (PQ and PW) experienced two types of trials: four paired trials during
230 which the taste of fructose solution on the antennae was paired with delivery of the corresponding
231 tastant (quinine or water) to the proboscis, and four placement trials during which bees were
232 placed in the experimental site for the same duration as for a paired trial but without receiving any
233 stimulation. Unpaired groups (UQ and UW) experienced also two types of trials: four trials during
234 which only fructose contacted the antennae and four other trials during which the corresponding
235 tastant (quinine or water) was delivered directly to the proboscis. For all four groups (PQ, PW, UQ
236 and UW) the sequence of the two types of trials was pseudorandomized (i.e. ABABBABA).

237 Each trial lasted 30 s. It started when the bee was placed in front of the experimenter for
238 stimulation. In paired trials (groups PQ and PW), 5 s after that placement, the bee was stimulated
239 with 1.66 M fructose on the antennae during 15 s; 2 s after the onset of this stimulation, and
240 coinciding with PER, the corresponding tastant of each group (quinine or water) was delivered to
241 the proboscis for further 15 s (Fig. 2). The interstimulus interval was thus 2 s. After the offset of
242 tastant delivery to the proboscis, the bee was left in the experimental site for 8 s to complete the
243 30 s. In unpaired trials, either the fructose solution was applied to the antennae without further
244 consequences, or the corresponding tastant (quinine or water) was directly delivered to the
245 proboscis. The timing of these stimulations followed the corresponding timing as in paired trials. In
246 placement trials, the bee was placed in the experimental site for 30 s without experiencing any
247 stimulation. The entire phase lasted 55 min, including the time needed to replace bees at the
248 experimental site.

249 After the end of this phase, bees were placed in an incubator at 28°C for 90 min. This time
250 was chosen based on the results of the first experiment (survival analysis; see above) and
251 corresponded to the time necessary to detect differences in survival between bees having ingested
252 distilled water or quinine solution. Afterwards, bees were fed with 20 μ l of 1.5 M sucrose solution
253 to ensure survival and placed again in the incubator for 17 h 30 min until the next day (see Fig. 2).

254 *2.3.3. Third phase: test of US and CS responses*

255 In the **third phase** (second day), bees were tested for US and CS responses. They were
256 taken from the incubator and fed with 3 μ l of 1.5 M sucrose solution to ensure survival during the
257 subsequent resting time (150 min), which they had to spend in the incubator. After this period,
258 they were expected to have exhausted the small amount of resources ingested and to be highly
259 responsive to sugars. After this resting period, the responses of bees to the CS and to the US were
260 tested. The tests took place 23 h after the end of the first phase and 21 h 30 min after the end of
261 the second phase. Bees were tested with their original CS, 1-nonanol, in the absence of reward and
262 with a novel odorant (Nod), which was not used during conditioning and which was delivered
263 without reward. The Nod was 1-hexanol (Sigma Aldrich, France, CAS Number 111-27-3), which is
264 well differentiated from 1-nonanol (Guerrieri, Schubert, Sandoz, and Giurfa, 2005). Odorants were
265 given in a random sequence, which varied from bee to bee. The two odorant presentations were
266 spaced by 15 min. Fifteen min after the olfactory tests, bees were tested with their original US by
267 touching the antennae with the 1.66 M fructose solution.

268

269 2.4. *Third experiment: the neural bases of CS-memory degradation via CTA*

270 The experiment followed the same schedule as the second experiment with the difference
271 that in the second phase bees were assigned to five PQ (*paired quinine*) groups and one PW (*paired*
272 *water*) group. All groups had a sample size of 30 bees. The PQ treatment was chosen, as it was the
273 one inducing US devaluation and the degradation of the CS-specific memory in the previous
274 experiment. As in the previous experiment, the PQ groups experienced four stimulations of
275 fructose delivered to the antennae, each one followed by 5 μ l of 10 mM quinine solution delivered
276 to the proboscis. The PW group was subjected to four stimulations of fructose delivered to the
277 antennae, each one contingent to 5 μ l of distilled water delivered to the proboscis.

278 After the end of this phase, bees received intraocellar injections via a small hole pricked
279 into the cornea of the median ocellus, which allowed inserting a 10 μ l-syringe (World Precision
280 Instrument). Drugs were injected into the brain of immobilized bees along the median ocellar
281 nerve. The ocellar nerve consists of a thick fiber bundle, approximately 40 μ m in diameter, which
282 runs medially and caudally from the dorsal margin of the head capsule into a depth of 300 μ m into
283 the protocerebrum. Drugs migrate through the ocellar tract into the bee brain where they
284 distribute in a fast (less than 5 min) and homogenous way (Menzel, Heyne, Kinzel, Gerber, and
285 Fiala, 1999).

286 Drugs injected differed between groups. The PW group was injected with phosphate
287 buffered saline (PBS) as a control. One of the PQ groups was also injected with PBS in order to

288 reproduce the US devaluation and the degradation of the CS-specific memory induced by
289 conditioned food aversion. From the four remaining PQ groups, two were injected with the DA-
290 receptor antagonist cis-(Z)-flupentixol dihydrochloride (henceforth flupentixol; Sigma Aldrich,
291 France, CAS Number 2413-38-9; see Blenau, Erber, and Baumann, 1998) and the other two with
292 the 5-HT receptor antagonist methiothepin mesylate (henceforth methiothepin; Sigma Aldrich,
293 France, CAS Number 74611-28-2; see Blenau and Thamm, 2011). Flupentixol is a potent blocker of
294 invertebrate dopamine receptors, which in the case of the honey bee antagonizes two of the three
295 dopamine receptors (*Amdop1* and *Amdop2*) (Beggs et al., 2011; Mustard et al., 2003).
296 Methiothepin acts as a competitive inhibitor in the presence of 5-HT and antagonizes in a non-
297 specific way all four serotonin receptors known for the honey bee ($Am5-HT_{1A}$, $Am5-HT_7$, $Am5-HT_{2\alpha}$
298 and $Am5-HT_{2\beta}$), although to a lesser degree $Am5-HT_{2\beta}$ (Schlenstedt, Balfanz, Baumann, and Blenau,
299 2006; Thamm, Balfanz, Scheiner, Baumann, and Blenau, 2010; Thamm, Rolke, Jordan, Balfanz,
300 Schiffer, Baumann, and Blenau, 2013). In either case, two doses were employed: one of 0.2 μ M and
301 another of 2 mM. These doses proved to be effective both in the case of flupentixol and
302 methiothepin as they affect significantly aversive responsiveness to a series of electric shock of
303 increasing voltage (Tedjakumala et al., 2014). Both drugs were dissolved in PBS. After injection,
304 bees were handled as in the previous experiment before the CS and US tests on the next day.

305

306 2.5. Statistics

307 In the first experiment, survival rates were analyzed using as censored observations the
308 individuals that survived at the end of the measuring period (Bewick, Cheek, and Ball, 2004). For
309 each treatment, we computed the cumulative proportion of surviving bees and established Kaplan-
310 Meier's survival functions defined as the probability of surviving at least to time t . A log rank test
311 was used to compare the two groups (water-fed and quinine-fed). This test computes a Z score
312 referred to a standard normal (chi square) distribution in the case of a two-sample comparison.

313 In the second and the third experiment, olfactory learning was evaluated by quantifying the
314 number of bees extending the proboscis (PER) to the conditioned odorant 1-nonanol. The change
315 in conditioned responses during trials was analyzed using a Cochran test for repeated
316 measurements performed on binomial variables (PER: 1, no PER: 0).

317 Test responses in the last phase of the second and the third experiment were quantified by
318 recording number of individuals exhibiting PER to fructose solution (US responses) and to the
319 successive presentation of 1-nonanol (CS responses) and 1-hexanol (novel odorant or Nod). To
320 determine whether US responses varied according to the treatment experienced, we analyzed data

321 according to a $N \times 2^2$ table, which segregated the bees that responded from those that did not
322 respond to the US, according to the N groups of each experiment (5 in the first experiment and 6 in
323 the third experiment). We tested the null hypothesis of US responses being independent of the
324 treatment applied in the 2nd phase. To detect the group(s) introducing significant rejection of the
325 null hypothesis, we subdivided the analysis following standard procedures for contingency tables
326 (Zar, 1999; p. 502).

327 CS and Nod responses were analyzed by means of a repeated-measurement ANOVA with
328 factors 'group' and 'odorant'. ANOVA procedures are applicable in the case of binary response
329 variables despite their lack of normality if comparisons imply equal cell frequencies and at least 40
330 degrees of freedom of the error term (d'Agostino, 1971; Lunney, 1970; Matsumoto, Menzel,
331 Sandoz, and Giurfa, 2012), conditions that were met by our experiments. As this analysis confounds
332 different bee categories (bees responding to the CS and not to the Nod, bees responding to both
333 odorants, bees responding to none, and bees responding only to the Nod) and may hide important
334 features of memory retention (Pamir, Chakroborty, Stollhoff, Gehring, Antemann, Morgenstern,
335 Felsenberg, Eisenhardt, Menzel, and Nawrot, 2011; Pamir, Szyszka, Scheiner, and Nawrot, 2014;
336 Villar et al., 2020), we focused on a more robust proxy of memory retention, which is the bees that
337 showed CS-specific memory (i.e. that responded to the CS and not to the Nod)(Matsumoto et al.,
338 2012; Villar et al., 2020). To determine if CS-specific responses varied between groups, we analyzed
339 data according to a $N \times 2^2$ table, which segregated the bees with specific memory from those
340 lacking such specificity, according to the N groups of each experiment. Again, subdividing the
341 analysis (Zar, 1999; p. 502) allowed identifying the group(s) that introduced significant rejection of
342 the null hypothesis stating that CS-specific responses were independent of the treatment applied in
343 the 2nd phase.

344

345 **3. Results**

346 *3.1. First experiment: survival analyses following ingestion of quinine solution or distilled water*

347 Figure 1b shows the Kaplan-Meier survival curves (Bewick et al., 2004) obtained through
348 assessment of mortality every 30 min during 4 h for the group fed with distilled water (n = 44) and
349 for the group fed with 10 mM quinine solution (n = 60). Survival differed significantly between
350 groups (log-rank test: $Z = 2.22$, $df:1$, $P = 0.027$). Bees that ingested quinine solution exhibited
351 higher mortality than bees fed with distilled water, even if in both cases survival decreased
352 dramatically during the 4-h period considered (80% decrease in the quinine group and 60%
353 decrease in the water group). In the case of bees fed with water, mortality resulted probably from
354 the scarce energetic resources available to them (i.e. they did not receive any sugar solution during

355 the 4-h evaluation period and the previous 2 ½ h). These results confirm the harmful nature of
356 quinine ingestion and the fact that this substance induces toxicosis in bees (Ayestarán et al., 2010).
357 Concentrations of quinine solution at least 10 times lower than the one used in this experiment
358 were shown to induce behavioral responses typical of malaise in honey bees (Hurst et al., 2014).

359

360 3.2. *Second experiment: degradation of an appetitive odor memory via US devaluation by CTA*

361 This experiment consisted of three phases performed over two consecutive days (Fig. 2).
362 On the first day, bees were conditioned to associate the odorant 1-nonanol as a CS with a 1.66 M
363 fructose solution as a US during four trials. Bees (n = 249) learned to respond to 1-nonanol which
364 anticipated the food reward and attained a level of 83% correct responses in the last conditioning
365 trial (Cochran test: $Q = 424.72$, $df : 3$, $P < 0.0001$). Yet, for assessing the impact of US devaluation on
366 the olfactory memory established upon this conditioning, it is necessary to ascertain that all the
367 bees subjected to the devaluation procedure had indeed learned efficiently the odor-fructose
368 association. We thus kept for the remaining phases of the experiment only those bees that
369 responded with PER to the odorant in the last conditioning trial (n = 204). All these bees had
370 responded with PER to fructose stimulation on their antennae prior to conditioning and had
371 increased significantly their conditioned responses during the conditioning procedure (Fig. 3a; $Q =$
372 420.60 , $df: 3$, $P < 0.0001$).

373 In the second phase, which started 40 min after the end of conditioning (Fig. 2), the bees
374 were assigned to five groups: the *Paired-Quinine group* (PQ; n = 41), the *Unpaired-Quinine group*
375 (UQ; n = 36), the *Paired-Water group* (PW; n = 42), the *Unpaired-Water group* (UW; n = 39) and the
376 *Unhandled Group* (UG; n = 46). Each group received a different treatment during a period of 60
377 min. After this phase and 90 additional min of rest, all bees were fed with 20 µl of 1.5 M sucrose
378 solution to ensure survival until the next day (see Figure 2). All bees were kept in an incubator for
379 17 h 30 min, recovered on the next morning to be fed with 3 µl of 1.5 M sucrose solution and
380 placed again in the incubator for 150 min.

381 In the third phase, performed on the second day after the 150 min resting period, the five
382 groups were tested for responses to the 1st-phase US fructose and for their CS memory. In testing
383 olfactory retention, we presented the CS and a novel odor ('Nod': 1-hexanol) in a random order to
384 check for the specificity of the olfactory memory. Figure 3b shows the 1st-phase US responses of
385 the five groups of bees in terms of the percentage of bees responding with PER to antennal
386 fructose stimulation. The PQ group exhibited the lowest level of responses to fructose (56.09%)
387 while the UG group had the highest level of responses (91.30%). The other groups (UQ, PW and
388 UW) had comparable levels of responses, which varied between 74.36% and 83.33%. We tested

389 the null hypothesis of responses to the 1st-phase US being independent of the treatment applied in
 390 the 2nd phase by means of a 5 x 2 contingency table, which segregated the bees that responded
 391 from those that did not respond to the fructose US, according to the five groups. This analysis
 392 showed that responses to the fructose US varied significantly according to the treatment group (χ^2
 393 = 17.77, df:4, $P < 0.005$). Moreover, subdividing the analysis (Zar, 1999; p. 502) revealed that the
 394 only group introducing a significant variation was the PQ group; in its absence, responses did not
 395 vary between the remaining four groups (χ^2 = 4.39, df:3, NS). This result thus demonstrates an
 396 associative devaluation of the fructose US by the contingent experience of quinine ingestion (the
 397 2nd-phase US), as the response of the PQ group differed from that of the UQ group. No other
 398 reduction of responses to the fructose US was found, thus showing that neither water nor unpaired
 399 quinine had a negative effect on these responses.

400 Figure 3c shows the response of the five groups of bees to the odorants (CS and Nod) in the
 401 retention test of the third phase. A repeated-measurement ANOVA with factors 'group' and
 402 'odorant' (repeated measurement) revealed significant effects for the factor 'odorant' ($F_{1,199} =$
 403 218.35 , $P < 0.0001$) but not for 'group' ($F_{4,199} = 1.47$, $P = 0.21$). Thus, bees generally responded
 404 more to the CS than to the Nod and showed a similar average level of responses. The interaction
 405 between both factors was, however, significant ($F_{4,199} = 3.62$, $P < 0.01$), showing that differentiation
 406 between CS and Nod was not the same in all groups. In order to appreciate in more details possible
 407 effects of 2nd phase treatments on CS memory, we focused on the proportion of bees that showed
 408 specific memory (i.e. that responded to the CS and not to the Nod)(Matsumoto et al., 2012; Villar
 409 et al., 2020). Figure 3d shows the percentage of bees showing specific memory within each group.
 410 All the other bees correspond to individuals responding to both odorants, to none of them or only
 411 to the Nod (very rare) and were considered as lacking specific retention. The PQ group, which
 412 exhibited devaluation of the fructose US as the result of the contingent experience of fructose and
 413 quinine (2nd-phase US) showed the lowest level of specific memory (26.8%). The UG group
 414 presented the highest level of CS specific memory (65.2%) while the other three groups varied
 415 between 52.4% and 56.4%. To determine if CS responses varied between the five groups, we
 416 analyzed data according to a 5 x 2 χ^2 table, which segregated the bees with specific memory from
 417 those lacking such specificity, according to the five groups. Levels of CS-specific memory varied
 418 significantly across the groups (χ^2 = 13.84, df:4, $P < 0.01$). As for responses to the fructose US,
 419 subdividing the analysis showed that only the PQ group exhibited a degraded CS memory:
 420 excluding this group from a subdivided analysis yielded no significant differences between the
 421 remaining four groups (χ^2 = 1.90, df:3, NS). Thus, devaluation of the fructose US induced a long-
 422 term degradation of the CS memory that was specific for the group that experienced contingent
 423 fructose and quinine.

424

425 3.3. *The neural bases of CS-memory degradation via CTA*

426 We next aimed at studying the neural bases of CS-memory degradation induced by the CTA phase
427 using a neuropharmacological approach that targeted DA and 5-HT receptors, given their
428 importance in different forms of aversive learning and responsiveness in bees. We thus injected
429 antagonists flupentixol (DA-receptor antagonist; Beggs et al., 2011; Mustard et al., 2003) and
430 methiothepin (5-HT-receptor antagonist; Blenau and Thamm, 2011; Tedjakumala et al., 2014) into
431 the bee brain via the ocellar tract, and determined their impact on CS-specific memory and US
432 responses following the experimental schedule used in the prior experiment. Injections were
433 performed immediately after the end of the second phase, i.e. after CTA (see Fig. 2). Control bees
434 were injected with PBS (phosphate buffered saline).

435 In the first phase, bees ($n = 234$) were again trained with four pairings of 1-nonanol and
436 1.66 M fructose. Bees learned the association and at the end of conditioning, a level of 77% of
437 correct responses was attained (Cochran test: $Q = 360.21$, $df:3$, $P < 0.0001$). As in the previous
438 experiment, we kept for the rest of the experiment only those bees that learned efficiently the
439 odor-fructose association and responded with PER to the odorant in the last conditioning trial ($n =$
440 180). Figure 4a shows that the increase of conditioned responses was also highly significant in this
441 group ($Q = 375.1$, $df:3$, $P < 0.0001$).

442 In the second phase, the bees were assigned to six groups ($n = 30$ each): one PW (paired-
443 water) group as a control and five PQ (paired-quinine) groups. Focus was set on the PQ treatment
444 because it was the one inducing devaluation of the fructose US and the degradation of the CS-
445 specific memory in the previous experiment. Immediately after the end of this phase, each bee
446 received an intraocellar injection, which differed between groups. The PW group was injected with
447 PBS. One of the PQ groups was also injected with PBS in order to reproduce the devaluation of the
448 fructose US and the degradation of the CS-specific memory induced by conditioned food aversion.
449 From the four remaining PQ groups, two were injected with flupentixol, one with a lower
450 concentration (PQ-fl, 0.2 μ M) and the other with a higher concentration (PQ-fh, 2 mM). Similarly,
451 two groups were injected with methiothepin, one with a lower concentration (PQ-ml, 0.2 μ M) and
452 the other with a higher concentration (PQ-mh, 2 mM).

453 Twenty-three hours after the end of the first phase (olfactory conditioning), the six groups
454 were tested for responses to the fructose US and for responses to the CS 1-nonanol and to the
455 Nod. Figure 4b shows the responses of the six groups of bees upon antennal stimulation with the
456 fructose US. No significant variation of PER was observed in this case ($\chi^2 = 3.25$, $df:5$, NS) despite
457 the fact that the PQ group injected with PBS exhibited the lowest level of responses to fructose

458 (66.67%). Figure 4c shows the bees' responses in the test for CS memory. As in the previous
 459 experiment, responses to the CS and to the Nod varied between odorants (repeated measurement
 460 ANOVA, $F_{1,174} = 91.5$, $P < 0.0001$) but not between groups ($F_{5,174} = 1.66$, $P = 0.15$). However, the
 461 interaction between both factors was significant ($F_{5,174} = 3.70$, $P < 0.01$), suggesting differences
 462 among groups in the way they differentiated between CS and NOd.

463 A focus on CS-specific responses confirmed significant differences among groups (Fig. 4d:
 464 $\chi^2 = 18.1$, $df:5$, $P < 0.001$). Subdividing the analysis (Zar, 1999; p. 502) showed that the two groups
 465 with higher levels of CS-specific memory, the PW-PBS and the PQ group injected with the highest
 466 concentration of methiothepin (PQ-mh), did not differ from each other ($\chi^2 = 1.07$, $df:1$, NS).
 467 Similarly, the four remaining PQ groups (injected with PBS, with the two doses of flupentixol and
 468 with the lowest dose of methiothepin) showed equivalent impairment of CS-specific memory ($\chi^2 =$
 469 3.58 , $df:1$, NS). On the contrary, a comparison between the pooled data of the PW-PBS and the PQ-
 470 mh groups vs. the pooled data of the other four remaining PQ groups yielded highly significant
 471 differences ($\chi^2 = 92.11$, $df:1$, $P < 0.0001$). Thus, pairing fructose with quinine induced again a long-
 472 term degradation of the CS memory, which was rescued by injection of the highest dose of
 473 methiothepin. Serotonergic signaling underlies therefore the malaise effect induced by quinine,
 474 which affects the stability of the olfactory memory.

475

476 4. Discussion

477 4.1. Degradation of appetitive CS memory after US devaluation via CTA learning

478 Our results provide the first clear evidence of a post-conditioning degradation of a CS
 479 memory by post-ingestive US devaluation in an insect. The reduction in CS and US responses in the
 480 third phase of our second experiment was due to an effect induced by the quinine solution during
 481 CTA learning as the *Paired-Water* (PW) and the *Unpaired-Water* (UW) groups did not show any
 482 response variation. It could be argued that the devaluation of the fructose US by quinine during the
 483 CTA was not due to a post-ingestive effect but was of pre-ingestive nature. If quinine taste were
 484 distasteful to bees, pairing fructose with this aversive stimulus would induce fructose devaluation
 485 in the *Paired-Quinine Group* (PQ) but not in the *Unpaired-Quinine Group* (UQ). Yet, no evidence for
 486 the existence of 'bitter-tuned' receptors such as those existing in other insects (e.g. fruit flies) has
 487 been found in the honey bee (Robertson and Wanner, 2006). Moreover, cumulative evidence -
 488 behavioral, electrophysiological and molecular - indicates that honey bees have a reduced
 489 sensitivity to bitter tastes (de Brito Sanchez, 2011; de Brito Sanchez, Lorenzo, Su, Fanglin, Zhan, and
 490 Giurfa, 2014; Guiraud, Hotier, Giurfa, and de Brito Sanchez, 2018). The pre-ingestive interpretation
 491 could be nevertheless maintained in this scenario of reduced bitter sensitivity if it is assumed that

492 the PQ group would experience fructose followed by a non-sweet, aqueous solution. In this case,
493 the mismatch between the appetitive expectation induced by fructose and the solution received at
494 the level of the proboscis could be an aversive event. Yet, in this scenario, a similar fructose
495 devaluation and degradation of the prior olfactory memory should have been observed in the
496 *Paired-Water Group* (PW), and this was never the case. These considerations lead us to favor a
497 post-ingestive interpretation of the fructose devaluation resulting from CTA, which we attribute to
498 the induction of a malaise-like state following quinine ingestion.

499 After the CTA phase, bees were subjected to a resting period of 90 min, which was
500 sufficient to detect differences in mortality between bees having ingested distilled water and bees
501 having ingested quinine solution in the survival experiment (see Fig. 1b). The feeding episodes
502 following this period (F_2 and F_3 in Fig. 2) and the prolonged spacing between the end of the CTA
503 phase and the tests of CS and US responsiveness (21 h 30 min) ensured that the illness state had
504 been already overcome when bees were tested with the CS and US. The decrease in CS and US
505 responses following CTA occurred only in the *Paired-Quinine* group (PQ) but not the *Unpaired-*
506 *Quinine* group (UQ) (see Fig. 3), thus demonstrating the associative nature of this phenomenon.
507 This difference is interesting as in principle both groups ingested the same quantity of quinine
508 solution, and were thus subjected to a malaise that developed during and after CTA learning (i.e.
509 during the 90 min rest introduced at the end of the CTA phase). In both groups, CTA conditioning
510 included eight trials spaced by 7 min, and in four of them quinine was delivered (the PQ group had
511 four placement trials besides the fructose-quinine trials, and the UQ group had non-contingent
512 quinine and fructose trials). Thus at the end of the 55 min required by CTA conditioning and the 90
513 min rest, the malaise state was presumably present in both groups (see Fig. 1b). The fact that only
514 the PQ group reduced its responses to fructose and to the odorant previously paired with it
515 indicates that for this group it was possible to associate the contingent stimulation of fructose and
516 quinine with the development of the malaise, even if this state appeared delayed in time. Delayed
517 associations in the form of trace conditioning – a conditioning form in which an interval is imposed
518 between the presentations of the CS and the US – have been shown in the honey bee (Szyzka,
519 Demmler, Oemisch, Sommer, Biergans, Birnbach, Silbering, and Galizia, 2011). In this perspective,
520 the double stimulation of fructose and quinine could have acted as an unambiguous CS associated
521 with the delayed malaise US. For the UQ group, the impact of CTA would be reduced if the
522 associative strength supported by the malaise had to be shared between two separate CSs (i.e.
523 presented in separate trials), quinine and fructose, which would create ambiguity in terms of the
524 origin of the illness state. In consequence, the decrease of CS responses observed in the PQ group
525 was mediated by a long-term recall of the devaluated fructose. By evoking the CS in the testing
526 phase, PQ bees retrieved the memory of the devaluated US and its consequences in terms of

527 malaise, thus showing the nature of the associations established throughout the experimental
528 phases.

529 Theories on Pavlovian conditioning differ in their interpretation of the elements connected
530 by the associations established in this learning form. On the one hand, Pavlovian conditioning was
531 said to rely on the formation of a stimulus-response (S - R) link, so that the CS (S) becomes capable
532 of activating the motor program (R) directly through learning (Hull, 1943; Spence, 1956). On the
533 other hand, an alternative view (Bolles, 1972) proposed that during Pavlovian conditioning subjects
534 form a stimulus - stimulus (S - S*) association binding the CS (S) with the US (S*) with which it was
535 paired. The latter model suggests that Pavlovian conditioned responses are elicited by the cognitive
536 expectation of the predicted US. Our results clearly support the S - S* model as they show the
537 flexibility of the CS responses, which diminish upon recall of the devaluated US. This finding is
538 consistent with a neural model proposed to account for second-order conditioning in crickets
539 ('Mizunami Unoki Model'; Mizunami and Matsumoto, 2010), in which an odor (CS1) is paired with
540 water or sodium chloride solution and a visual pattern (CS2) is paired with the odor (CS1). The
541 model shows that connections from neurons representing the conditioned stimulus (CS) to
542 aminergic neurons, which in insects provide instructive appetitive or aversive valence information
543 (Giurfa, 2006) (i.e. S - S* connections) account for the learning observed experimentally,
544 consistently with the S - S* interpretation.

545 A basic principle of Pavlovian conditioning is stimulus substitution, the fact that the CS
546 acquires the value of the original US as a result of conditioning (García-Hoz, 2014). This notion is
547 well captured by the Rescorla and Wagner model proposed for Pavlovian learning (Rescorla and
548 Wagner, 1972), which states that conditioning, i.e. the associative strength binding the CS and the
549 US, progresses along trials towards a limit (λ) set by the US. In other words, full conditioning
550 (maximal associative strength) is attained when that limit is reached and the CS activates the
551 internal representation of the US in a way comparable to that produced by the US itself. The
552 odorant used in the first conditioning phase reached maximal associative strength in the bees that
553 were selected for the subsequent phases of the experiment because conditioned responses were
554 at their maximal possible level in the last conditioning trial (100%), which corresponds to the level
555 elicited by pure fructose stimulation before conditioning (see white bars in Figures 3a and 4a). The
556 devaluation of fructose induced by the explicit pairing with quinine affected the US representation
557 and strength, and translated into the odor-fructose contingency established in the first phase. The
558 decrease in CS responses observed in the last experimental phase thus reflects the expectation of
559 an aversive outcome associated *a posteriori* with fructose. Similar results have been obtained in
560 vertebrates, including fish (Nordgreen, Janczak, Hovland, Ranheim, and Horsberg, 2010), rodents
561 (Colwill and Motzkin, 1994; Holland, 1981; Holland and Straub, 1979; Kraemer, Hoffmann, Randall,

562 and Spear, 1992; Sage and Knowlton, 2000; Yin and Knowlton, 2002), and humans (Bray, Rangel,
563 Shimojo, Balleine, and O'Doherty, 2008; Gottfried, O'Doherty, and Dolan, 2003), where different
564 procedures implemented to devalue the US resulted in degraded CS memories. Our findings in an
565 invertebrate extend the universality of this phenomenon and provide further evidence that
566 encoding of a CS-US association underlies Pavlovian learning.

567

568 4.2. CTA in honey bees and other insect species

569 An essential component of the three-phase experiment we performed is the aversive CTA
570 induced by pairing the antennal fructose stimulation with the ingestion of quinine solution. This
571 pairing induced the devaluation of fructose that affected the appetitive olfactory memory
572 established prior to the devaluation procedure. CTA learning has been shown in various insect
573 species, which learn to avoid food based on the negative consequences associated with their
574 ingestion. For instance, mantids *Tenodera ardifolia* learn to avoid a preferred prey, the milkweed
575 bug *Oncopeltus fasciatus*, when the latter was raised on a diet of plants containing secondary toxic
576 compounds (Berenbaum and Miliczky, 1984). Grasshoppers *Schistocerca americana* also learn to
577 avoid spinach and broccoli leaves when their ingestion is associated with an abdominal injection of
578 nicotine hydrogen tartrate (NHT), quinine solution or lithium chloride (Bernays and Lee, 1988; Lee
579 and Bernays, 1990). A more detailed analysis of this effect was achieved in the desert locust
580 (*Schistocerca gregaria*), in which the experience of a preferred odor followed by food
581 supplemented with NHT results in an aversion for that odor, which is expressed 1 h later and is still
582 observable 24 h later (Simoes, Ott, and Niven, 2012). When food ingestion was uncoupled from
583 malaise by pairing the preferred odor with toxin-free food on the one hand, and delivering an
584 injection of NHT into the body on the other hand, aversion towards the preferred odor was also
585 observed 4 h later, but only if the injection was simultaneous to the odor-food experience, or
586 occurred up to 30 minutes after that experience. If the preferred odor was directly paired with the
587 NHT injection (without food delivery), aversion memory was only observed in a test performed 4 h
588 later for the case in which NHT injection occurred simultaneously with odor stimulation. These
589 results indicate the presence of two different effects: 1) one depending on ingestion that forms
590 aversive memories even if the toxic effect of NHT is delayed from the odor up to 30 minutes, and 2)
591 another that is independent on ingestion and that forms aversive memories only if the toxic effect
592 of NHT is simultaneous to the odor (Simoes et al., 2012).

593 In the honey bee, contradictory evidence has been reported concerning their capacity to
594 develop CTA. Yet, this discrepancy seems to rely on procedural methods rather than on a true
595 biological capacity (or incapacity) for learning to avoid toxic food. Ethanol (EtOH) was one of the

596 substances used to induce conditioned food aversion (Varnon, Dinges, Black, Wells, and Abramson,
597 2018). Bees were fed a 2M sucrose solution scented with an odorant and containing EtOH, which
598 was used to induce an aversion towards the odorant present in the sucrose solution. To visualize
599 this possible aversion, bees were trained 30 min later to associate the same odorant with 2M pure
600 sucrose solution using the olfactory PER conditioning protocol. In theory, here, CTA should result in
601 a lower initial response to the odorant and a deficient learning performance. This prediction was
602 not verified. On the contrary, bees exhibited high levels of appetitive spontaneous responses to the
603 odorant already in the first conditioning trial. It was thus concluded that bees do not develop CTA
604 after EtOH consumption. Yet, this lack of effect may have been due to the highly concentrated
605 sucrose solution used in these experiments, which may have provided enough energetic resources
606 to counteract the noxious effect of EtOH (Varnon et al., 2018).

607 In a different approach, PER conditioning was performed by stimulating the antennae with
608 pure 1M sucrose solution and delivering a mixture of that solution with amygdaline to the
609 proboscis (Wright et al., 2010). Bees ingested the mixture of sucrose and amygdaline and rapidly
610 learned the odorant in the first three to four conditioning trials, but then showed a pronounced
611 decay in the conditioned responses to the odorant in subsequent trials. This decay was explained
612 as the result of a post-ingestive malaise induced by the mixture of sucrose solution and amygdalin,
613 which would become important after the first conditioning trials (Wright et al., 2010). Although this
614 interpretation is attractive, an alternative explanation could focus on the contrast occurring
615 repeatedly along trials as a consequence of receiving a strong 1 M sucrose solution on the
616 antennae and then a lower-value (less sweet) sucrose solution upon proboscis extension. This
617 decrease in value is clearly visible at the gustatory-receptor level as the presence of bitter
618 substances such as quinine in sucrose solution inhibits the response of sucrose receptor cells in the
619 honey bee (de Brito Sanchez et al., 2005). In other words, the contrast between the US expectation
620 and the US actually received could have decreased the appetitive motivation and the interest for
621 the conditioned odorant. In this experiment, mortality curves spanning the same period as the
622 conditioning experiment were absent (only mortality 24h after ingestion was reported) so that it is
623 difficult to determine if bees were suffering from a real malaise. In particular because, as in the
624 case of the EtOH-treated bees (Varnon et al., 2018; see above), mixing amygdalin with sucrose
625 solution may counteract the noxious effect of the toxin. Mortality curves for a mixture of sucrose
626 and amygdalin were established in a different work (Ayestarán et al., 2010) and no significant
627 mortality could be detected during a period of 1 h, which corresponds to the period during which
628 the decay of conditioned responses was observed (Wright et al., 2010). It thus seem that mixing
629 sucrose solution with toxins decreases the perceptual value of sucrose and that the presence of

630 sucrose in the mixture provides energetic resources capable of counteracting the illness induced by
631 the toxin.

632 On the contrary, when bees ingest pure solutions of quinine or amygdalin, significant
633 mortality is detected already 1 or 2 h after ingestion (Ayestarán et al., 2010). Surviving bees thus
634 experienced an illness-like state during this period. Therefore, pairing a weak sugar solution (e.g.
635 1.66 M fructose or 1.66 M glucose) with the ingestion of these pure toxins resulted in a significant
636 reduction of appetitive responses (PER) to these sugars (Ayestarán et al., 2010). This reduction was
637 not observed if a strong sugar solution (1 M sucrose) was used (Ayestarán et al., 2010). Taken
638 together, these results indicate that bees can indeed develop CTA, in particular when the ingestion
639 of a weak appetitive food is followed by ingestion/injection of an illness-inducing toxin not mixed
640 with sucrose solution. The use of sucrose solution mixed with toxins does not guarantee the
641 development of CTA as the mixture may not be harmful enough to generate illness and aversion.

642

643 4.3. *Hedonic value of tastants and their use for appetitive olfactory and aversive food* 644 *conditioning*

645 Three tastants were used in our experiments based on their different reinforcing
646 properties: 1) a 1.66 M fructose solution, an appetitive US that can induce significant olfactory
647 learning but has a weak hedonic value (Ayestarán et al., 2010; see above); 2) a pure quinine
648 solution (10 mM), which induces a malaise-like state a few hours after ingestion (Ayestarán et al.,
649 2010; see above), and 3) distilled water, a neutral tastant. As mentioned above, fructose solution
650 was chosen instead of sucrose because the latter has a high intrinsic appetitive value, which
651 renders difficult its subsequent devaluation. On the contrary, fructose, even at the same
652 concentration as sucrose solution (30% w/w), can be devaluated by ingestion of quinine solution,
653 thus showing that identity-specific features of a US render it susceptible or not to devaluation.
654 Quinine solution, on the other hand, induces a malaise-like state after ingestion because it results
655 in higher mortality than distilled water, which in the absence of supplementary energetic
656 resources, also induces mortality (see Fig. 2 and Ayestaran et al., 2010). Bees that were injected
657 with quinine solution at an even lower concentration than the one used in our work (1 mM)
658 diminish their walking activity and increase grooming behavior, consistently with a malaise-like
659 state (Hurst et al., 2014). Importantly, we determined the devaluating effect of quinine 23 h after
660 conditioning, i.e. 21 h 30 min after the end of the pairing of fructose and quinine. Only bees that
661 survived the quinine ingestion were available on the next day for testing US and CS responses.
662 These bees therefore experienced the malaise, survived and were subjected to the US and CS tests
663 after an interval long enough to ensure that they were no longer under the effect of the malaise.

664 Figure 3b,c confirms this conclusion by showing that only the PQ group exhibited a decrease in US
665 and CS responses. The UQ group, which ingested the same amount of quinine, did not exhibit this
666 reduction. This result is different from the one obtained after testing CS and US responses only 90
667 min after ingestion (Ayestarán et al., 2010): in this case, both the PQ and the UQ groups exhibited
668 reduced CS and US responsiveness, probably as a consequence of a generalized malaise state and
669 not as a result of an associative US devaluation and degradation of a CS memory. In the present
670 study, the decrease of US and CS responses was of associative nature as it was due to the
671 association between the contingent fructose-quinine stimulation and the subsequent malaise-like
672 state induced by quinine ingestion. Distilled water was used as a control for the effects induced by
673 quinine ingestion; it neither induced US devaluation nor CS-memory degradation, as shown by the
674 performances of the PW group. Yet, the mismatch between an antennal stimulation with fructose
675 and the subsequent ingestion of water could have, in some circumstances, a negative effect *per se*
676 (see above). In our case, this effect proved to be negligible and it did not affect the performances
677 recorded.

678

679 4.4. *The neural bases of appetitive memory degradation via aversive US devaluation*

680 The demonstration that 5-HT mediates an aversive, malaise-dependent degradation of
681 memory adds new evidence on the role of this biogenic amine in the processing of aversive
682 stimulations in the insect brain, yet in a context different from those previously known. In a natural
683 situation, bees exposed to aversive or potentially nociceptive stimuli release an alarm pheromone
684 carried by their stinger and whose main component is isoamyl acetate (IAA) (Boch, Shearer, and
685 stone, 1962). This pheromone alerts and recruits more defenders to organize a collective attack
686 (Boch et al., 1962; Collins and Blum, 1983; Nouvian, Reinhard, and Giurfa, 2016). Exposure to IAA
687 upregulates brain levels of 5-HT, and to a lesser degree of DA, thereby increasing the likelihood of
688 an individual bee to attack and sting (Nouvian, Mandal, Jamme, Claudianos, d'Ettorre, Reinhard,
689 Barron, and Giurfa, 2018). Pharmacological enhancement of the levels of both amines induces
690 higher defensive responsiveness, while pharmacological blockade of their corresponding receptors
691 decreases stinging. In the laboratory, aversive responsiveness is evaluated *via* the quantification of
692 the sting extension response (SER), a reflexive response that is elicited in a harnessed bee *via*
693 delivery of mild electric or thermal shocks (Junca, Carcaud, Moulin, Garnery, and Sandoz, 2014;
694 Junca, Garnery, and Sandoz, 2019; Núñez, Almeida, Balderrama, and Giurfa, 1997; Núñez,
695 Maldonado, Miralto, and Balderrama, 1983; Roussel, Carcaud, Sandoz, and Giurfa, 2009;
696 Tedjakumala et al., 2014). In these experiments, bees are subjected to a series of increasing
697 voltages or contact temperatures and their SER to these stimuli is evaluated. A pharmacological
698 analysis of this behavior showed that injection of DA- and 5-HT-receptor antagonists into the bee

699 brain induces an increase in sting responsiveness to shocks of intermediate voltage (Tedjakumala
700 et al., 2014). The effect was particularly evident in the case of 5-HT antagonists, one them being
701 methiothepin, the antagonist used in our work. Thus, the response to the succession of aversive
702 shocks experienced by the bee was an enhanced 5-HT signaling, which diminished responsiveness
703 (i.e. made animals more tolerant) to the shocks. Blocking such signaling rendered the bees more
704 responsive to the shocks.

705 The two previous neural responses (to alarm pheromone and to electric shocks) have in
706 common the coincident activation of both 5-HT and DA signaling, even if in both cases, the effects
707 related to 5-HT were more important. In another work discussed above (olfactory PER conditioning
708 with an odorant followed by a compound US made of antennal stimulation with 1 M sucrose
709 solution and a mixture of the same solution with amygdalin delivered to the proboscis; Wright et
710 al., 2010), DA signaling was irrelevant for the decrease in PER to the conditioned odorant observed
711 along trials, and interpreted as the consequence of a developing malaise (see above for an
712 alternative interpretation of this effect). On the contrary, blockade of 5-HT receptors using a
713 mixture of methiothepin and ketanserin rescued PER to the conditioned odorant, thus showing
714 that the decay of responses, be it for malaise or for a decrease in appetitive motivation, was due to
715 5-HT signaling (Wright et al., 2010). However, the use of another serotonergic blocker, mianserin
716 (Tierney, 2018), which was used mistakenly as an octopaminergic blocker, did not modify the decay
717 in PER responses. Irrespective of this inconsistency, several lines of evidence underline the
718 important role of 5-HT in response to a broad spectrum of aversive events.

719 In our work, only the higher dose of the 5-HT-receptor antagonist methiothepin was able to
720 rescue the CS-specific memory (Fig. 4d). This result is consistent with the role attributed to 5-HT
721 signaling in aversive situations, and shows that the degradation of memory based on CTA was
722 mediated by 5-HT signaling. Although, we did not find a clear effect of the DA-receptor antagonist
723 flupentixol, a tendency to memory improvement was also visible for the higher concentration (Fig.
724 4 c,d, dark blue bar); yet, this increase did not reach significance.

725 We were not able to see the expected US devaluation in the PQ group injected with PBS
726 (Figure 4b), which would be the equivalent of the PQ group of the behavioral experiment (Figure
727 3b). Despite the lack of evident US devaluation in this group, the aversive, associative effect of the
728 malaise induced by quinine ingestion occurred as this group exhibited the lowest degraded CS-
729 specific memory (Fig. 4d). In fact, all PQ groups, except the one injected with the highest
730 concentration of methiothepin (see above), had an impaired CS-specific memory when compared
731 with the PW group injected with PBS. This comparison shows that CTA took place in the PQ groups,
732 even if responses to fructose in the final phase of the experiment did not always reveal it.

733

734 4.5. *Mechanisms of 5-HT signaling in relation to post-ingestive malaise in the bee nervous*
735 *system*

736 5-HT regulates feeding and feeding-related processes such as hunger, gut motility and
737 dieresis in numerous insect species such as crickets (Cooper and He, 1994), migratory locusts
738 (Huddart and Oldfield, 1982), fall armyworms (Howarth, Prince, Dyker, Losel, Seinsche, and
739 Osborne, 2002), cabbage worms (Walker and Bloomquist, 1999), blow flies (Haselton, Downer,
740 Zylstra, and Stoffolano, 2009), kissing bugs (Orchard, 2009) and stick insects (Luffy and Dorn, 1992),
741 among others. In another social insect, the carpenter ant *Camponotus mus*, an increase in 5-HT
742 levels *via* oral administration impairs ingestive behavior (Falibene, Rossler, and Josens, 2012),
743 which is consistent with the findings of our work: if a feeding aversive event is signaled by an
744 increase in 5-HT, reducing feeding behavior would be an adaptive response.

745 The relationship between feeding, malaise and 5-HT signaling was studied in the honey
746 bee, using a combination of behavioral methods, immunostaining of 5-HT processes, gene-
747 expression analysis and quantification of 5-HT levels after ingestions of pure sucrose solution or
748 sucrose solution spiked with amygdalin (French, Simcock, Rolke, Gartside, Blenau, and Wright,
749 2014). A rich innervation by 5-HT neural processes was found at the level of the esophagus, crop,
750 proventriculus and midgut and in the first fused thoracic and abdominal ganglia of the ventral
751 nerve chord. Moreover, high levels of expression of the four 5-HT receptors characterized in the
752 honey bee (*Am5-ht1*, *Am5-ht2*, *Am5-ht2*, *Am5-ht7*) were found in the crop. These results
753 indicate a tight control of ingestion processes by the serotonergic system. However, the functional
754 measurements did not always yield consistent results. For instance, based on prior evidence, an
755 increase in 5-HT levels was expected after ingestion of a mixture of sucrose solution and amygdalin
756 because this increase would reflect the malaise that was attributed to the ingestion of this mixture
757 (Wright et al., 2010). Yet, contrary to this expectation, 5-HT levels in the hemolymph were higher
758 after feeding bees with pure sucrose solution compared with feeding with the mixture of sucrose
759 and amygdalin. Moreover, these levels did not vary in time, contrary to the decay in PER responses
760 observed during olfactory conditioning (Wright et al., 2010). Injection of 5-HT into the brain
761 suppressed feeding of pure sucrose solution and of sucrose supplemented with amino acids, but
762 did not change the ingestion levels of sucrose supplemented with amygdalin, which remained low
763 and comparable to those observed in the absence of injection or after injection of water into the
764 brain (French et al., 2014). It was thus concluded that the hypothesis of 5-HT mediating a malaise
765 signal released by the gut in response to sucrose mixed with toxins was not tenable and that 5-HT
766 levels in the brain might instead control the motor program responsible for PER (French et al.,
767 2014). This reinterpretation thus indicates that if bees ceased responding to an odor after receiving

768 sucrose mixed with amygdalin during successive conditioning trials, it was because of an effect on
769 PER and thus on the amount of food ingested rather than because of a malaise state. This
770 argument is compatible with the motivational hypothesis proposed above and rises again the
771 potential problem of using a sucrose mixed with amygdalin as a potential inductor of malaise. It
772 would be therefore interesting to consider performing these experiments again, yet using pure
773 toxins instead of mixing them with sucrose solution.

774

775 **5. Conclusions**

776 The present work shows that in the honey bee conditioned food aversion not only reduces
777 responses to a US paired with negative consequences, but also degrades prior appetitive memories
778 engaging a representation of that US. In an ecological context, bees experiencing nectars spiked
779 with toxins may ingest them or decide to abandon them based on their less attractive hedonic
780 value. In a situation in which a high floral diversity is available, the individual and collective
781 response would be to switch to another floral species offering nectar that is more valuable. Yet, in
782 agricultural landscapes dominated by monocultures, this possibility would not be granted. This
783 could have dramatic consequences for the health and survival of bees: although foragers tend to
784 reject noxious food if they can choose between alternatives varying in toxicity, they consume it
785 when choice is no longer available (Desmedt, Hotier, Giurfa, Velarde, and de Brito Sanchez, 2016).
786 This would result in decreased foraging efficiency and probable higher mortality.

787

788 **CRedit authorship contribution statement**

789 Y.L. performed all experiments. E.D. performed preliminary experiments, which gave origin to this
790 work. M.G. de B.S. conceived the work. M.G. de B.S., S.S. and M.G. supervised the work. M.G.
791 provided the funding and the materials for the experiments. Y.L. and M.G. analyzed the data. M.G.
792 and L.Y. wrote the manuscript. All authors (Y.L., J.-C.S., S.S. and M.G. de B.S. and M.G.) discussed
793 the results and edited the manuscript.

794

795 **Competing Interests**

796 The author(s) declare no potential conflicts of interest with respect to the research, authorship,
797 and/or publication of this article.

798

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804

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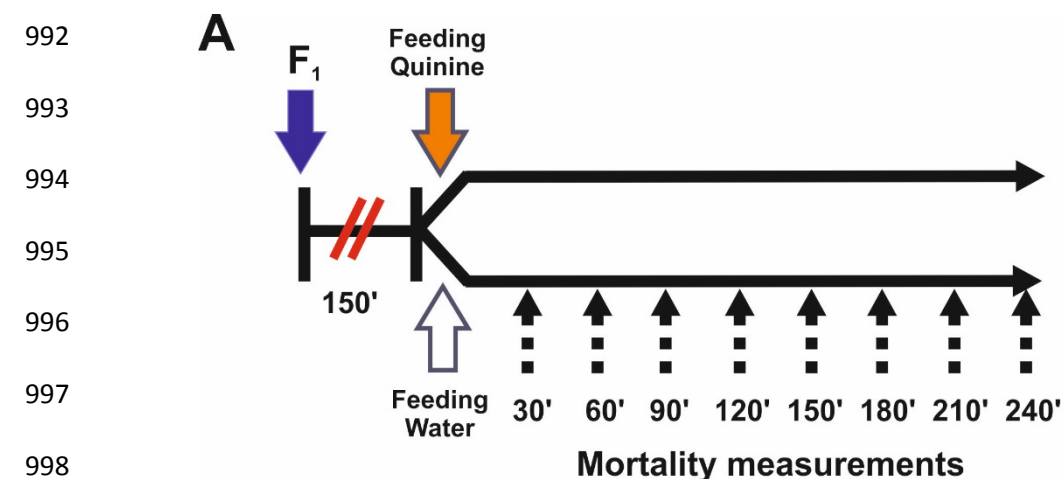
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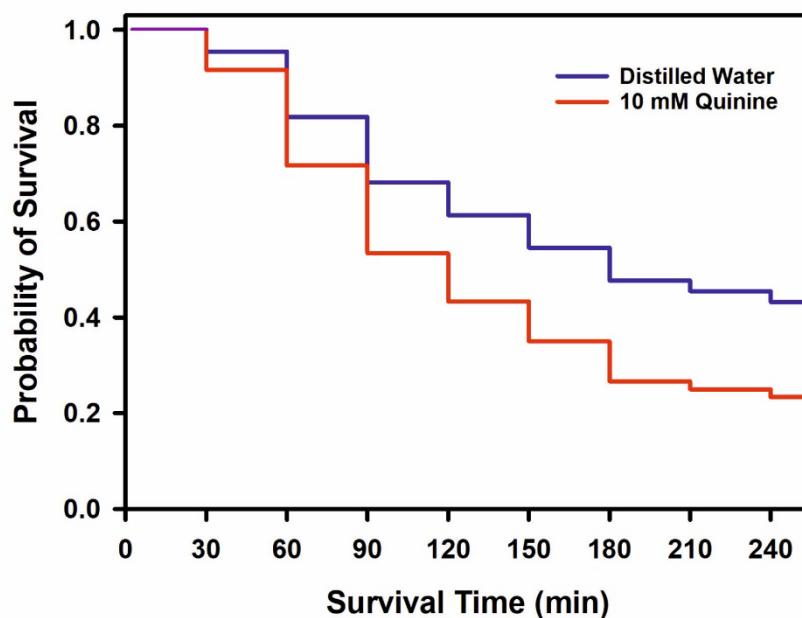
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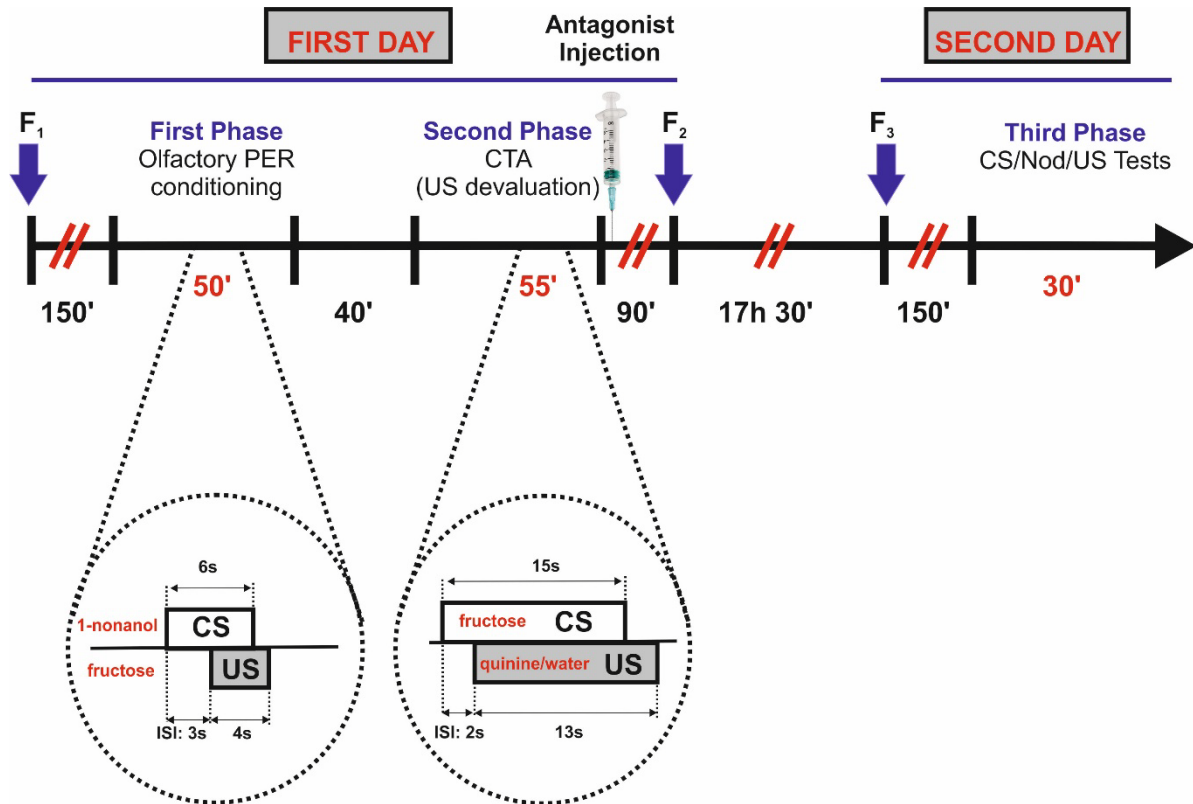
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Fig.1. First experiment. (A) Experimental schedule. The experiment started when the bees were captured and harnessed in individual tubes. They were then fed with 3 μ l of 1.5 M sucrose solution (F_1) and kept in an incubator for 150 min. Bees were split in two groups, one fed with 10 mM quinine solution and another fed with distilled water. Mortality was quantified every 30 min (dashed arrows) during a 4 h post-ingestion period. **(B) Kaplan–Meier curves of survival for harnessed honeybees fed with distilled water and 10 mM quinine solution.** The group of

1019 honeybees having ingested a 10 mM quinine solution (n=44) exhibited a significant decrease in
 1020 survival compared to the group fed with distilled water (n=60) during the 4 h post-ingestion period.

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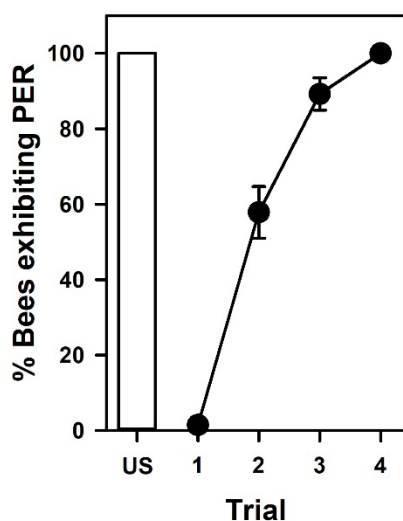
1023 **Fig. 2. Experimental schedule of the second and third experiments performed to study US**
 1024 **devaluation by quinine ingestion and the subsequent degradation of an appetitive CS memory.**

1025 The experiment started when the bees were captured and harnessed in individual tubes. It
 1026 included a) a **first phase** of olfactory conditioning (50 min) during which 1-nonanol (CS) was paired
 1027 with 1.66 M fructose solution (US), b) a **second phase** of conditioned food aversion (55 min) during
 1028 which the fructose solution was paired or not with a 10 mM quinine solution (control groups
 1029 received distilled water in place of quinine solution), and c) a **third phase** (30 min) performed 23 h
 1030 min after the first phase (and 21 h 30 min after the second phase) during which the responses to
 1031 the US (1.66 M fructose), the CS (1-nonanol) and a novel odorant (Nod: 1-hexanol) were evaluated.
 1032 F₁, F₂ and F₃ indicate feeding episodes performed to ensure survival. F₁: feeding of 3μl of 1.5 M
 1033 sucrose solution; F₂: feeding of 20 μl of 1.5 M sucrose solution; F₃: feeding of 3μl of 1.5 M sucrose
 1034 solution. The duration of each experimental phase is indicated in red (min). In a second
 1035 experiment, the same general protocol was used to determine the neural underpinnings of the
 1036 variations in responses observed in this experiment. In this second experiment, antagonists of the
 1037 dopaminergic system (flupentixol) and of the serotonergic system (methiothepin) were injected

1038 immediately after the end of the second phase into the bee brain ('Antagonist Injection').

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A First Phase Olfactory Conditioning



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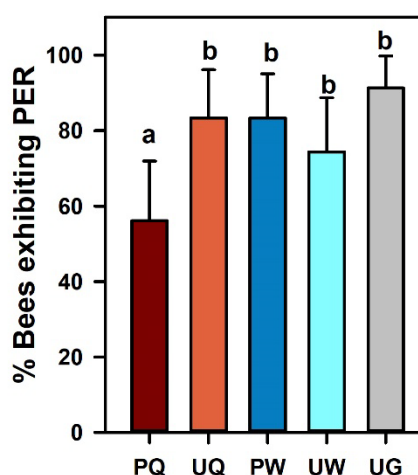
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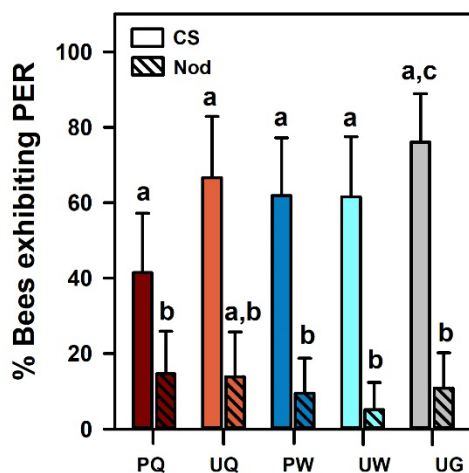
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B Third Phase US (Fructose) Responses



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C Third Phase Olfactory retention



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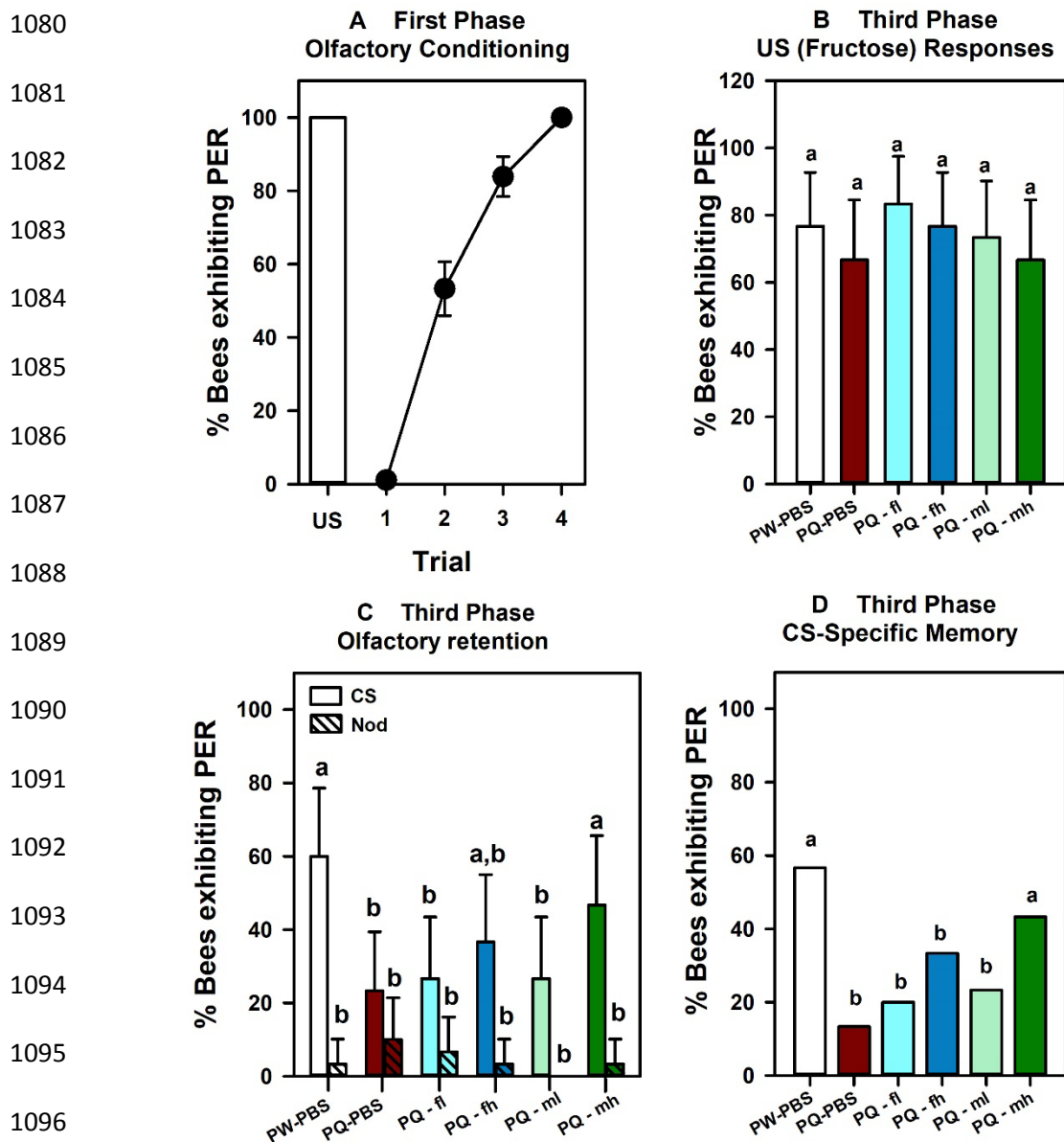
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Fig. 3. Results of the second experiment. (A) First Phase: Acquisition performances of learner bees (i.e. bees responding correctly at the last conditioning trial) in an absolute conditioning associating 1-nonanol (CS) and a 1.66 M fructose solution (US). The white bar indicates the US-response level (100 %) of these bees prior to conditioning. The percentage of learners responding in each trial is shown \pm 95% confidence interval. In a **second phase**, bees were split in five groups. Two of them were fed with 10 mM quinine solution upon contingent (PQ: *Paired Quinine*, $n = 41$) or non-contingent (UQ: *Unpaired Quinine*, $n = 36$) antennal stimulation with 1.66 M fructose solution; two other groups were stimulated on the antennae with 1.66 M fructose solution and fed with contingent (PW: *Paired Water*, $n = 42$) or non-contingent (UW: *Unpaired Water*, $n = 39$) distilled water. The final group (UG: *Untreated Group*, $n = 46$) did not experience any handling. (B) **Third**

1067 **phase:** US responses (% of bees responding to 1.66 M fructose solution + 95% confidence interval)
1068 of the five groups of bees established in the second phase, 23 h after the first phase (olfactory
1069 conditioning) and 21 h 30 min after the second phase (CTA). **(C) Third phase:** CS and Nod (novel
1070 odor) responses of the five groups of bees in a retention test performed 23 h after the first phase
1071 (olfactory conditioning) and 21 h 30 min after the second phase (CTA). The bars show the
1072 percentage of bees responding to the odorants + 95% confidence interval. **(D) Third phase:** Levels
1073 of CS-specific memory (% bees responding only to the odorant conditioned and not to a novel
1074 odorant) of the five groups of bees established in the second phase 23 h after the first phase
1075 (olfactory conditioning) and 21 h 30 min after the second phase (CTA). In the three third-phase
1076 panels **(B, C, D)**, different letters above the bars indicate significant differences ($P < 0.05$; **B, D:**
1077 contingency 5×2 χ^2 analysis; **C:** ANOVA for repeated measurements & Tukey post hoc
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1101 **Fig. 4. Results of the third experiment. (A) First Phase:** Acquisition performances of learner bees
1102 (i.e. bees responding correctly at the last conditioning trial) in an absolute conditioning associating
1103 1-nanol (CS) and a 1.66 M fructose solution. The white bar indicates the US-response level of
1104 these bees prior to conditioning. The percentage of learners responding at each trial is shown \pm
1105 95% confidence interval. In a **second phase**, bees were split in six groups. Five of them were fed
1106 with 10 mM quinine solution upon contingent (PQ: *Paired Quinine*) antennal stimulation with 1.66
1107 M fructose solution; the remaining group was fed with distilled water upon contingent (PW: *Paired*
1108 *Water*) antennal stimulation with 1.66 M fructose solution. At the end of this second phase, two

1109 groups were injected with PBS (PW-PBS and PQ-PBS), two PQ groups with two concentrations of
1110 flupentixol, low and high (PQ-fl and PQ-fh), and two PQ groups with two concentrations of
1111 methiothepin, low and high (PQ-ml and PQ-mh). All groups had $n = 30$. **(B) Third phase:** US
1112 responses (% of bees responding to 1.66 M fructose solution + 95% confidence interval) of the six
1113 groups of bees established in the second phase, 23 h after the first phase (olfactory conditioning)
1114 and 21 h 30 min after the second phase (CTA). **(C) Third phase:** CS and Nod (novel odor) responses
1115 of the six groups of bees in a retention test performed 23 h after the first phase (olfactory
1116 conditioning) and 21 h 30 min after the second phase (CTA). The bars show the percentage of bees
1117 responding to the odorants + 95% confidence interval. **(D) Third phase:** Levels of CS-specific
1118 memory (% bees responding only to the odorant conditioned and not to a novel odorant) of the six
1119 groups of bees established in the second phase, 23 h after the first phase (olfactory conditioning)
1120 and 21 h 30 min after the second phase (CTA). In the three third-phase panels **(B, C, D)**, different
1121 letters above the bars indicate significant differences ($P < 0.05$; **B, D:** contingency 5×2 χ^2 analysis;
1122 **C:** ANOVA for repeated measurements & Tukey post hoc comparisons).