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

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ABSTRACT

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

1. Introduction

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

A well-established form of Pavlovian learning is taste aversion learning (or conditioned taste aversion, CTA), which was established by Garcia, Kimeldorf, and Koelling (1955). This form of aversive learning relies on associating a preferred food or its taste with a gastric malaise usually produced by intraperitoneal injections of lithium chloride (LiCl), or by irradiations delivered immediately after food ingestion. In this way, animals associate food taste as a CS with gastric illness as a US, and avoid in consequence consuming the previously preferred food. This learning is extremely robust and can induce long-lasting aversive memories after just one forward pairing of food with gastric malaise

Abbreviations: CTA, conditioned taste aversion; CS, conditioned stimulus; US, unconditioned stimulus; SER, sting extension response; UQ, unpaired quinine group; UW, unpaired water group; DA, dopamine; PER, proboscis extension response; PQ, paired quinine group; PW, paired water group; 5-HT, serotonin

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(Reilly & Schachtman, 2008). It is a ubiquitous phenomenon, which has been observed in many species, from invertebrates to humans (Reilly & Schachtman, 2008), and which has a clear ecological relevance (Garcia, Ervin, & Koelling, 1966) as it mediates avoidance of substances that are potentially toxic and may result in an individual's death.

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

An interesting animal model to study this problem is the honey bee *Apis mellifera.* Honey bees have been widely studied to understand behavioral and neural principles governing Pavlovian conditioning based on the existence of a laboratory Pavlovian protocol, which allows the study of olfactory appetitive learning (Bitterman, Menzel, Fietz, & Schäfer, 1983; Giurfa & Sandoz, 2012; Giurfa, 2007). In this protocol, termed the olfactory conditioning of the proboscis extension response (PER), harnessed bees are exposed to an odorant (CS) followed by a reward of sucrose solution (US), which is delivered to their antennae and then to the proboscis. The antennal stimulation with sucrose elicits PER in hungry bees. After successful learning, the odorant acquires the capacity to elicit PER *per se*. Olfactory PER conditioning leads to robust long-term memories that are stabilized in time (Menzel, 1999, 2001; Muller, 2012), even after a single trial conditioning (Villar, Marchal, Viola, & Giurfa, 2020), thanks to the process of protein synthesis.

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

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

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

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

2. Materials and methods

2.1. Insects

Experiments were performed on honeybee workers (*Apis mellifera* L.) obtained from the experimental apiary located at the University Paul Sabatier. Bees were captured every morning, enclosed in glass vials, and cooled down on ice until they stopped moving. They were then harnessed in individual plastic tubes using tape strips and low-temperature melting wax applied to the back of the head. In this way, they could only move their antennae and mouthparts, including the proboscis. Bees were then fed with 3 µl of 1.5 M sucrose solution and kept in an incubator at 28°C for 150 min.

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

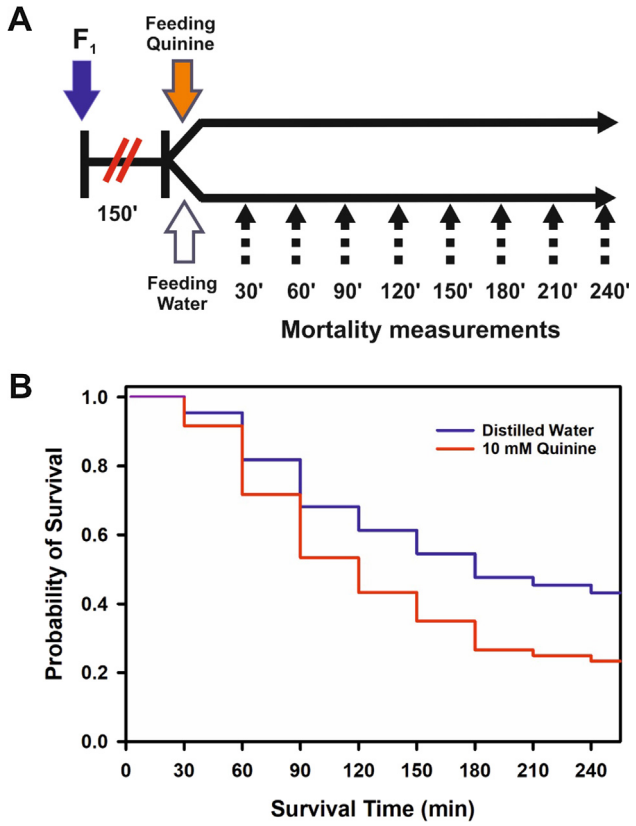

Fig. 1. First experiment. (A) Experimental schedule. The experiment started when the bees were captured and harnessed in individual tubes. They were then fed with 3 µl of 1.5 M sucrose solution (F_1) and kept in an incubator for 150 min. Bees were split in two groups, one fed with 10 mM quinine solution 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 honeybees having ingested a 10 mM quinine solution (n = 44) exhibited a significant decrease in survival compared to the group fed with distilled water (n = 60) during the 4 h post-ingestion period.

2.2. First experiment: Survival analyses

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

2.3. Second experiment: Degradation of an appetitive odor memory via US devaluation by CTA

2.3.1. First phase: Olfactory PER conditioning

In the **first phase** (first day), bees were conditioned during four spaced trials to associate the odorant 1-nonanol (Sigma Aldrich, France, CAS Number 143-08-8) as the CS with 1.66 M fructose solution as the US (Sigma Aldrich, France, CAS Number 57-48-7). We chose fructose solution as it can mediate associative learning despite being a weaker (Ayestarán et al., 2010) and less preferred US (Wykes, 1952) than sucrose solution. Moreover, fructose can be devaluated via its pairing with substances inducing toxicosis in CTA while sucrose is resistant to devaluation under the same conditions due to its highly appetitive value (Ayestarán et al., 2010). Prior to conditioning, harnessed bees were checked for PER to the fructose solution. To this end, we touched the antennae of the bees with the solution and recorded if they extended the proboscis. Extension of the proboscis beyond the virtual line separating the tip of the mandibles was counted as PER; partial or incomplete responses were not considered.

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

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

2.3.2. Second phase: CTA learning

In the **second phase** (first day), bees that learned the association were subjected to a conditioned taste aversion protocol in which antennal stimulation with fructose was followed by ingestion of a 10 mM quinine solution. This phase started 40 min after the end of the previous conditioning phase (Fig. 2). The bees were assigned to five groups. The *Paired-Quinine group* (PQ; n = 41) experienced four stimulations of fructose delivered to the antennae, each one followed by 5 µl of 10 mM quinine delivered to the proboscis. The *Unpaired-Quinine group* (UQ; n = 36) experienced the same fructose and quinine stimulations but in a non-contingent way. Thus, both groups consumed 20 µl of 10 mM quinine solution, which is sufficient to induce a malaise-like state in the next 4 h following ingestion (see first experiment), but only the PQ group was subjected to an associative conditioned food aversion based on the contingency between fructose and the aversive effect induced by quinine ingestion. The *Paired-Water group* (PW; n = 42) received four stimulations with fructose delivered to the antennae, each one contingent to 5 µl of distilled water delivered to the proboscis. The *Unpaired-Water group* (UW; n = 39) experienced the same fructose and distilled water stimulations but in a non-contingent way. Finally, an *Unhandled Group* (UG; n = 46) was left untreated during the same amount of time (55 min) spent by the other four groups in this second phase.

In all groups, except the UG group, bees experienced eight trials with 7 min intertrial intervals. Paired groups (PQ and PW) experienced two types of trials: four paired trials during which the taste of fructose solution on the antennae was paired with delivery of the corresponding tastant (quinine or water) to the proboscis, and four placement trials during which bees were placed in the experimental site for the same duration as for a paired trial but without receiving any stimulation. Unpaired groups (UQ and UW) experienced also two types of trials: four

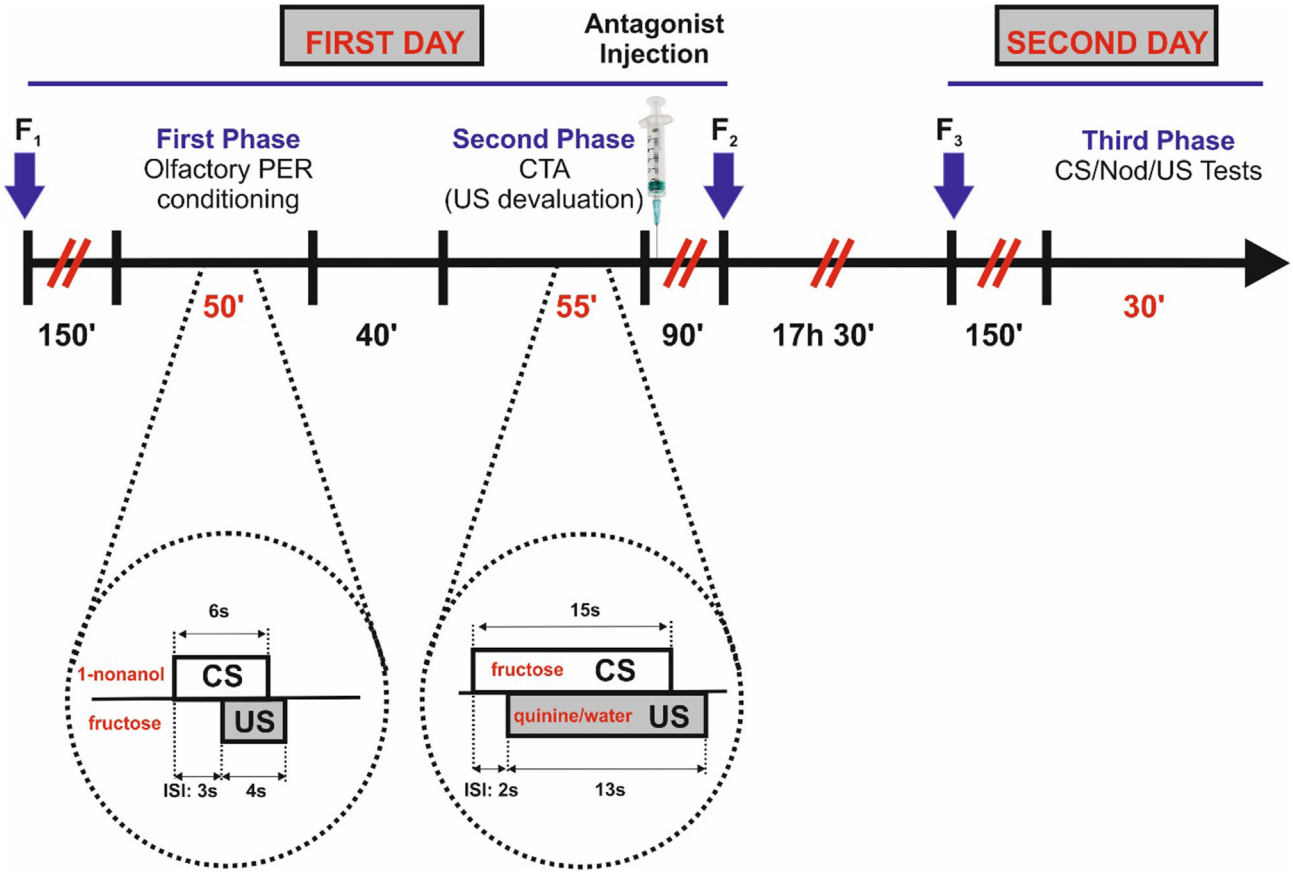

Fig. 2. Experimental schedule of the second and third experiments performed to study US devaluation by quinine ingestion and the subsequent degradation of an appetitive CS memory. The experiment started when the bees were captured and harnessed in individual tubes. It included (a) a first phase of olfactory conditioning (50 min) during which 1-nonanol (CS) was paired with 1.66 M fructose solution (US), (b) a second phase of conditioned food aversion (55 min) during which the fructose solution was paired or not with a 10 mM quinine solution (control groups received distilled water in place of quinine solution), and c) a third phase (30 min) performed 23 h min after the first phase (and 21 h 30 min after the second phase) during which the responses to the US (1.66 M fructose), the CS (1-nonanol) and a novel odorant (Nod: 1-hexanol) were evaluated. F_1, F_2 and F_3 indicate feeding episodes performed to ensure survival. F_1: feeding of 3 µl of 1.5 M sucrose solution; F_2: feeding of 20 µl of 1.5 M sucrose solution; F_3: feeding of 3 µl of 1.5 M sucrose solution. The duration of each experimental phase is indicated in red (min). In a second experiment, the same general protocol was used to determine the neural underpinnings of the variations in responses observed in this experiment. In this second experiment, antagonists of the dopaminergic system (flupentixol) and of the serotonergic system (methiothepin) were injected immediately after the end of the second phase into the bee brain ('Antagonist Injection').

trials during which only fructose contacted the antennae and four other trials during which the corresponding tastant (quinine or water) was delivered directly to the proboscis. For all four groups (PQ, PW, UQ and UW) the sequence of the two types of trials was pseudorandomized (i.e. ABABBABA).

Each trial lasted 30 s. It started when the bee was placed in front of the experimenter for stimulation. In paired trials (groups PQ and PW), 5 s after that placement, the bee was stimulated with 1. 66 M fructose on the antennae during 15 s; 2 s after the onset of this stimulation, and coinciding with PER, the corresponding tastant of each group (quinine or water) was delivered to the proboscis for further 15 s (Fig. 2). The interstimulus interval was thus 2 s. After the offset of tastant delivery to the proboscis, the bee was left in the experimental site for 8 s to complete the 30 s. In unpaired trials, either the fructose solution was applied to the antennae without further consequences, or the corresponding tastant (quinine or water) was directly delivered to the proboscis. The timing of these stimulations followed the corresponding timing as in paired trials. In placement trials, the bee was placed in the experimental site for 30 s without experiencing any stimulation. The entire phase lasted 55 min, including the time needed to replace bees at the experimental site.

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

2.3.3. Third phase: Test of US and CS responses

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

tests, bees were tested with their original US by touching the antennae with the 1.66 M fructose solution.

2.4. Third experiment: The neural bases of CS-memory degradation via CTA

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

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

Drugs injected differed between groups. The PW group was injected with phosphate buffered saline (PBS) as a control. One of the PQ groups was also injected with PBS in order to reproduce the US devaluation and the degradation of the CS-specific memory induced by conditioned food aversion. From the four remaining PQ groups, two were injected with the DA-receptor antagonist cis-(Z)-flupentixol dihydrochloride (henceforth flupentixol; Sigma Aldrich, France, CAS Number 2413-38-9; see Blenau, Erber, & Baumann, 1998) and the other two with the 5-HT receptor antagonist methiothepin mesylate (henceforth methiothepin; Sigma Aldrich, France, CAS Number 74611-28-2; see Blenau & Thamm, 2011). Flupentixol is a potent blocker of invertebrate dopamine receptors, which in the case of the honey bee antagonizes two of the three dopamine receptors (*Amdop1* and *Amdop2*) (Beggs et al., 2011; Mustard et al., 2003). Methiothepin acts as a competitive inhibitor in the presence of 5-HT and antagonizes in a non-specific way all four serotonin receptors known for the honey bee (Am5-HT_{1A}, Am5-HT_{7}, Am5-$HT_{2\alpha}$ and Am5-$HT_{2\beta}$), although to a lesser degree Am5-$HT_{2\beta}$ (Schlenstedt, Balfanz, Baumann, & Blenau, 2006; Thamm et al., 2013; Thamm, Balfanz, Scheiner, Baumann, & Blenau, 2010). In either case, two doses were employed: one of 0.2 μM and another of 2 mM. These doses proved to be effective both in the case of flupentixol and methiothepin as they affect significantly aversive responsiveness to a series of electric shock of increasing voltage (Tedjakumala et al., 2014). Both drugs were dissolved in PBS. After injection, bees were handled as in the previous experiment before the CS and US tests on the next day.

2.5. Statistics

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

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

Test responses in the last phase of the second and the third experiment were quantified by recording number of individuals exhibiting PER to fructose solution (US responses) and to the successive presentation of 1-nonanol (CS responses) and 1-hexanol (novel odorant or Nod). To determine whether US responses varied according to the treatment experienced, we analyzed data according to a N × 2 χ^2 table, which segregated the bees that responded from those that did not respond to the US, according to the N groups of each experiment (5 in the first experiment and 6 in the third experiment). We tested the null hypothesis of US responses being independent of the treatment applied in the 2nd phase. To detect the group(s) introducing significant rejection of the null hypothesis, we subdivided the analysis following standard procedures for contingency tables (Zar, 1999; p. 502).

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

3. Results

3.1. First experiment: Survival analyses following ingestion of quinine solution or distilled water

Fig. 1b shows the Kaplan-Meier survival curves (Bewick et al., 2004) obtained through assessment of mortality every 30 min during 4 h for the group fed with distilled water (n = 44) and for the group fed with 10 mM quinine solution (n = 60). Survival differed significantly between groups (log-rank test: Z = 2.22, df:1, P = 0.027). Bees that ingested quinine solution exhibited higher mortality than bees fed with distilled water, even if in both cases survival decreased dramatically during the 4-h period considered (80% decrease in the quinine group and 60% decrease in the water group). In the case of bees fed with water, mortality resulted probably from the scarce energetic resources available to them (i.e. they did not receive any sugar solution during the 4-h evaluation period and the previous 2 ½ h). These results confirm the harmful nature of quinine ingestion and the fact that this substance induces toxicosis in bees (Ayestarán et al., 2010). Concentrations of quinine solution at least 10 times lower than the one used in this experiment were shown to induce behavioral responses typical of malaise in honey bees (Hurst et al., 2014).

3.2. Second experiment: Degradation of an appetitive odor memory via US devaluation by CTA

This experiment consisted of three phases performed over two

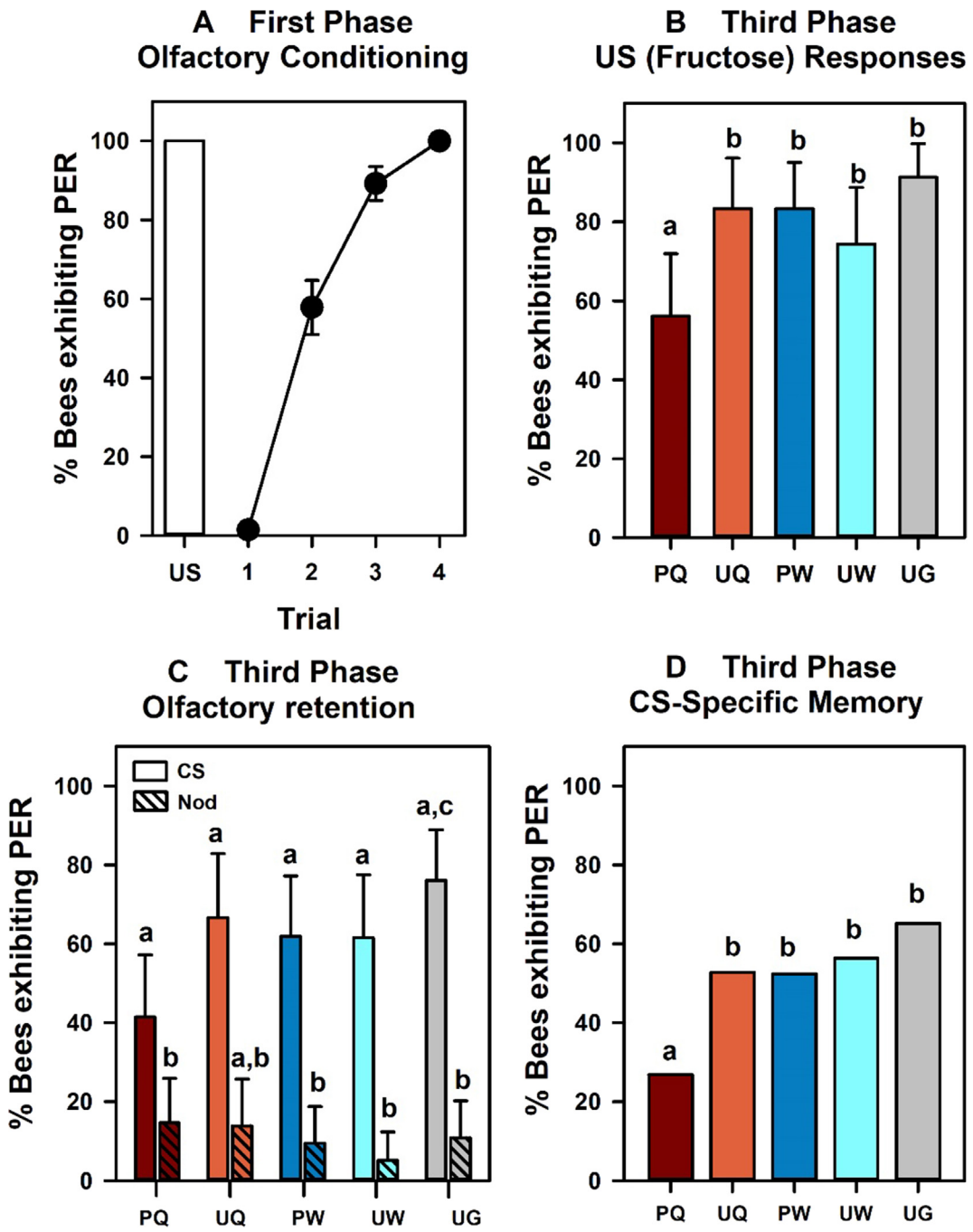

Fig. 3. Results of the second experiment. (A) First Phase: Acquisition performances of learner bees (i.e. bees responding correctly at the last conditioning trial) in an absolute conditioning associating 1-nonanol (CS) and a 1.66 M fructose solution (US). The white bar indicates the US-response level (100%) of these bees prior to conditioning. The percentage of learners responding in each trial is shown ± 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 phase: US responses (% of bees responding to 1.66 M fructose solution + 95% confidence interval) of the five groups of bees established in the second phase, 23 h after the first phase (olfactory conditioning) and 21 h 30 min after the second phase (CTA). (C) Third phase: CS and Nod (novel odor) responses of the five groups of bees in a retention test performed 23 h after the first phase (olfactory conditioning) and 21 h 30 min after the second phase (CTA). The bars show the percentage of bees responding to the odorants + 95% confidence interval. (D) Third phase: Levels of CS-specific memory (% bees responding only to the odorant conditioned and not to a novel odorant) of the five groups of bees established in the second phase 23 h after the first phase (olfactory conditioning) and 21 h 30 min after the second phase (CTA). In the three third-phase panels (B–D), different letters above the bars indicate significant differences ($P < 0.05$; B, D: contingency 5 × 2 χ^2 analysis; C: ANOVA for repeated measurements & Tukey post hoc comparisons).

consecutive days (Fig. 2). On the first day, bees were conditioned to associate the odorant 1-nonanol as a CS with a 1.66 M fructose solution as a US during four trials. Bees (n = 249) learned to respond to 1-nonanol which anticipated the food reward and attained a level of 83% correct responses in the last conditioning trial (Cochran test: $Q = 424.72$, df : 3, $P < 0.0001$). Yet, for assessing the impact of US devaluation on the olfactory memory established upon this conditioning, it is necessary to ascertain that all the bees subjected to the

devaluation procedure had indeed learned efficiently the odor-fructose association. We thus kept for the remaining phases of the experiment only those bees that responded with PER to the odorant in the last conditioning trial (n = 204). All these bees had responded with PER to fructose stimulation on their antennae prior to conditioning and had increased significantly their conditioned responses during the conditioning procedure (Fig. 3a; Q = 420.60, df: 3, P < 0.0001).

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

In the third phase, performed on the second day after the 150 min resting period, the five groups were tested for responses to the 1st-phase US fructose and for their CS memory. In testing olfactory retention, we presented the CS and a novel odor ('Nod': 1-hexanol) in a random order to check for the specificity of the olfactory memory. Fig. 3b shows the 1st-phase US responses of the five groups of bees in terms of the percentage of bees responding with PER to antennal fructose stimulation. The PQ group exhibited the lowest level of responses to fructose (56.09%) while the UG group had the highest level of responses (91.30%). The other groups (UQ, PW and UW) had comparable levels of responses, which varied between 74.36% and 83.33%. We tested the null hypothesis of responses to the 1st-phase US being independent of the treatment applied in the 2nd phase by means of a 5 × 2 contingency table, which segregated the bees that responded from those that did not respond to the fructose US, according to the five groups. This analysis showed that responses to the fructose US varied significantly according to the treatment group (χ^2 = 17.77, df:4, P < 0.005). Moreover, subdividing the analysis (Zar, 1999; p. 502) revealed that the only group introducing a significant variation was the PQ group; in its absence, responses did not vary between the remaining four groups (χ^2 = 4.39, df:3, NS). This result thus demonstrates an associative devaluation of the fructose US by the contingent experience of quinine ingestion (the 2nd-phase US), as the response of the PQ group differed from that of the UQ group. No other reduction of responses to the fructose US was found, thus showing that neither water nor unpaired quinine had a negative effect on these responses.

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

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

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

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

In the second phase, the bees were assigned to six groups (n = 30 each): one PW (paired-water) group as a control and five PQ (paired-quinine) groups. Focus was set on the PQ treatment because it was the one inducing devaluation of the fructose US and the degradation of the CS-specific memory in the previous experiment. Immediately after the end of this phase, each bee received an intraocellar injection, which differed between groups. The PW group was injected with PBS. One of the PQ groups was also injected with PBS in order to reproduce the devaluation of the fructose US and the degradation of the CS-specific memory induced by conditioned food aversion. From the four remaining PQ groups, two were injected with flupentixol, one with a lower concentration (PQ-fl, 0.2 µM) and the other with a higher concentration (PQ-fh, 2 mM). Similarly, two groups were injected with methiothepin, one with a lower concentration (PQ-ml, 0. 2 µM) and the other with a higher concentration (PQ-mh, 2 mM).

Twenty-three hours after the end of the first phase (olfactory conditioning), the six groups were tested for responses to the fructose US and for responses to the CS 1-nonanol and to the Nod. Fig. 4b shows the responses of the six groups of bees upon antennal stimulation with the fructose US. No significant variation of PER was observed in this case (χ^2 = 3.25, df:5, NS) despite the fact that the PQ group injected with PBS exhibited the lowest level of responses to fructose (66.67%). Fig. 4c shows the bees' responses in the test for CS memory. As in the previous experiment, responses to the CS and to the Nod varied between odorants (repeated measurement ANOVA, $F_{1,174}$ = 91.5, P < 0.0001) but not between groups ($F_{5,174}$ = 1.66, P = 0.15). However, the interaction between both factors was significant ($F_{5,174}$ = 3.70, P < 0.01), suggesting differences among groups in the way they differentiated between CS and NOd.

A focus on CS-specific responses confirmed significant differences among groups (Fig. 4d: χ^2 = 18.1, df:5, P < 0.001). Subdividing the

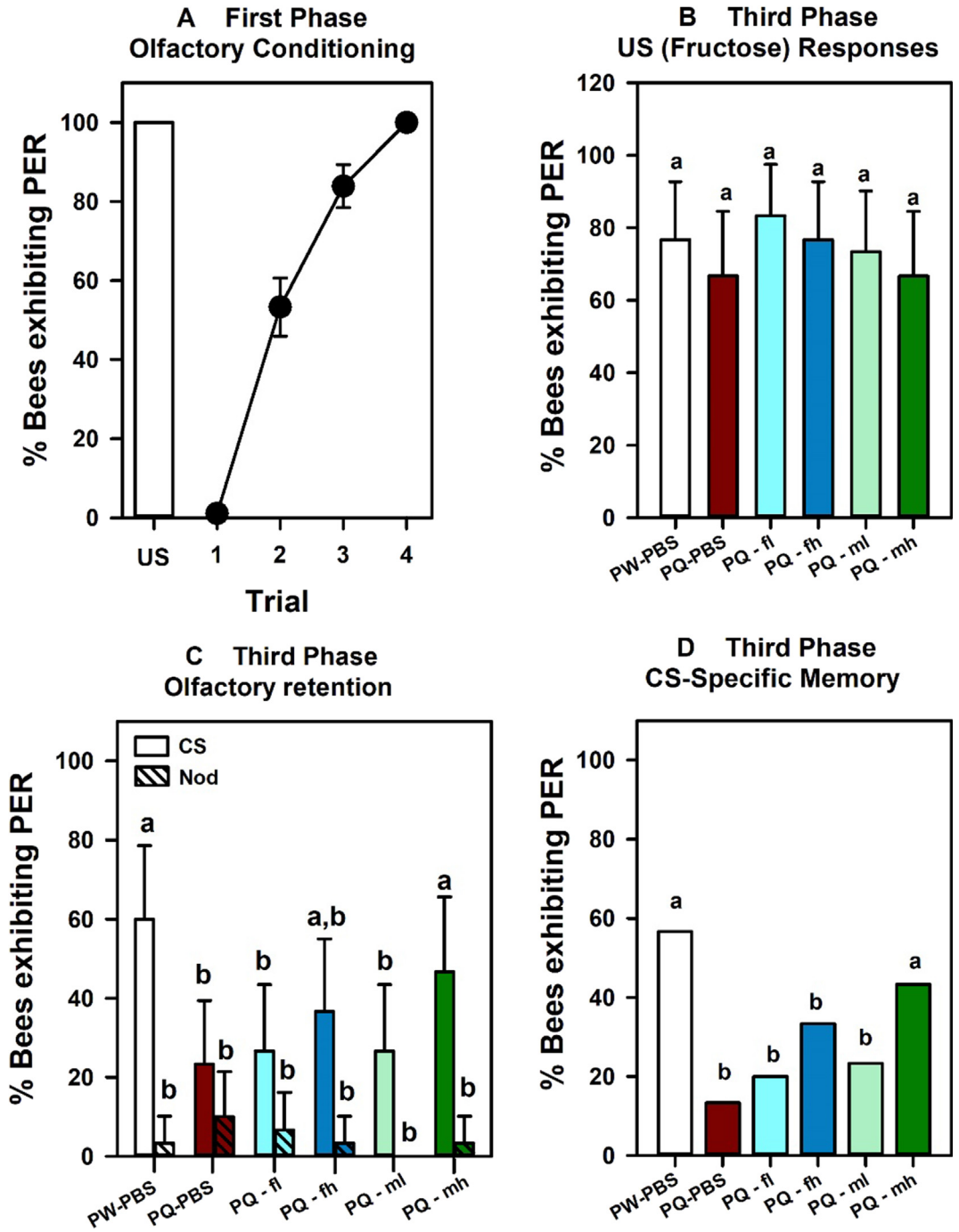

Fig. 4. Results of the third experiment. (A) First Phase: Acquisition performances of learner bees (i.e. bees responding correctly at the last conditioning trial) in an absolute conditioning associating 1-nonanol (CS) and a 1.66 M fructose solution. The white bar indicates the US-response level of these bees prior to conditioning. The percentage of learners responding at each trial is shown ± 95% confidence interval. In a second phase, bees were split in six groups. Five of them were fed with 10 mM quinine solution upon contingent (PQ: *Paired Quinine*) antennal stimulation with 1.66 M fructose solution; the remaining group was fed with distilled water upon contingent (PW: *Paired Water*) antennal stimulation with 1.66 M fructose solution. At the end of this second phase, two groups were injected with PBS (PW-PBS and PQ-PBS), two PQ groups with two concentrations of flupentixol, low and high (PQ-fl and PQ-fh), and two PQ groups with two concentrations of methiothepin, low and high (PQ-ml and PQ-mh). All groups had n = 30. (B) Third phase: US responses (% of bees responding to 1.66 M fructose solution + 95% confidence interval) of the six groups of bees established in the second phase, 23 h after the first phase (olfactory conditioning) and 21 h 30 min after the second phase (CTA). (C) Third phase: CS and Nod (novel odor) responses of the six groups of bees in a retention test performed 23 h after the first phase (olfactory conditioning) and 21 h 30 min after the second phase (CTA). The bars show the percentage of bees responding to the odorants + 95% confidence interval. (D) Third phase: Levels of CS-specific memory (% bees responding only to the odorant conditioned and not to a novel odorant) of the six groups of bees established in the second phase, 23 h after the first phase (olfactory conditioning) and 21 h 30 min after the second phase (CTA). In the three third-phase panels (B–D), different letters above the bars indicate significant differences ($P < 0.05$; B, D: contingency 5 × 2 χ^2 analysis; C: ANOVA for repeated measurements & Tukey post hoc comparisons).

analysis (Zar, 1999; p. 502) showed that the two groups with higher levels of CS-specific memory, the PW-PBS and the PQ group injected with the highest concentration of methiothepin (PQ-mh), did not differ from each other (χ^2 = 1.07, df:1, NS). Similarly, the four remaining PQ groups (injected with PBS, with the two doses of flupentixol and with the lowest dose of methiothepin) showed equivalent impairment of CS-specific memory (χ^2 = 3.58, df:1, NS). On the contrary, a comparison between the pooled data of the PW-PBS and the PQ-mh groups vs. the pooled data of the other four remaining PQ groups yielded highly significant differences (χ^2 = 92.11, df:1, P < 0.0001). Thus, pairing fructose with quinine induced again a long-term degradation of the CS memory, which was rescued by injection of the highest dose of methiothepin. Serotonergic signaling underlies therefore the malaise effect induced by quinine, which affects the stability of the olfactory memory.

4. Discussion

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

Our results provide the first clear evidence of a post-conditioning degradation of a CS memory by post-ingestive US devaluation in an insect. The reduction in CS and US responses in the third phase of our second experiment was due to an effect induced by the quinine solution during CTA learning as the *Paired-Water* (PW) and the *Unpaired-Water* (UW) groups did not show any response variation. It could be argued that the devaluation of the fructose US by quinine during the CTA was not due to a post-ingestive effect but was of pre-ingestive nature. If quinine taste were distasteful to bees, pairing fructose with this aversive stimulus would induce fructose devaluation in the *Paired-Quinine Group* (PQ) but not in the *Unpaired-Quinine Group* (UQ). Yet, no evidence for the existence of 'bitter-tuned' receptors such as those existing in other insects (e.g. fruit flies) has been found in the honey bee (Robertson & Wanner, 2006). Moreover, cumulative evidence - behavioral, electrophysiological and molecular – indicates that honey bees have a reduced sensitivity to bitter tastes (de Brito Sanchez et al., 2014; de Brito Sanchez, 2011; Guiraud, Hotier, Giurfa, & de Brito Sanchez, 2018). The pre-ingestive interpretation could be nevertheless maintained in this scenario of reduced bitter sensitivity if it is assumed that the PQ group would experience fructose followed by a non-sweet, aqueous solution. In this case, the mismatch between the appetitive expectation induced by fructose and the solution received at the level of the proboscis could be an aversive event. Yet, in this scenario, a similar fructose devaluation and degradation of the prior olfactory memory should have been observed in the *Paired-Water Group* (PW), and this was never the case. These considerations lead us to favor a post-ingestive interpretation of the fructose devaluation resulting from CTA, which we attribute to the induction of a malaise-like state following quinine ingestion.

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

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

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

2010), rodents (Colwill & Motzkin, 1994; Holland & Straub, 1979; Holland, 1981; Kraemer, Hoffmann, Randall, & Spear, 1992; Sage & Knowlton, 2000; Yin & Knowlton, 2002), and humans (Bray, Rangel, Shimojo, Balleine, & O'Doherty, 2008; Gottfried, O'Doherty, & Dolan, 2003), where different procedures implemented to devaluate the US resulted in degraded CS memories. Our findings in an invertebrate extend the universality of this phenomenon and provide further evidence that encoding of a CS-US association underlies Pavlovian learning.

4.2. CTA in honey bees and other insect species

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

In the honey bee, contradictory evidence has been reported concerning their capacity to develop CTA. Yet, this discrepancy seems to rely on procedural methods rather than on a true biological capacity (or incapacity) for learning to avoid toxic food. Ethanol (EtOH) was one of the substances used to induce conditioned food aversion (Varnon, Dinges, Black, Wells, & Abramson, 2018). Bees were fed a 2 M sucrose solution scented with an odorant and containing EtOH, which was used to induce an aversion towards the odorant present in the sucrose solution. To visualize this possible aversion, bees were trained 30 min later to associate the same odorant with 2 M pure sucrose solution using the olfactory PER conditioning protocol. In theory, here, CTA should result in a lower initial response to the odorant and a deficient learning performance. This prediction was not verified. On the contrary, bees exhibited high levels of appetitive spontaneous responses to the odorant already in the first conditioning trial. It was thus concluded that bees do not develop CTA after EtOH consumption. Yet, this lack of effect may have been due to the highly concentrated sucrose solution used in these experiments, which may have provided enough energetic resources to counteract the noxious effect of EtOH (Varnon et al., 2018).

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

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

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

Three tastants were used in our experiments based on their different reinforcing properties: 1) a 1.66 M fructose solution, an appetitive US that can induce significant olfactory learning but has a weak hedonic value (Ayestarán et al., 2010; see above); 2) a pure quinine solution (10 mM), which induces a malaise-like state a few hours after ingestion (Ayestarán et al., 2010; see above), and 3) distilled water, a neutral tastant. As mentioned above, fructose solution was chosen instead of sucrose because the latter has a high intrinsic appetitive value, which renders difficult its subsequent devaluation. On the contrary, fructose, even at the same concentration as sucrose solution (30% w/w), can be devaluated by ingestion of quinine solution, thus showing that identity-specific features of a US render it susceptible or not to devaluation. Quinine solution, on the other hand, induces a malaise-like state after ingestion because it results in higher mortality than distilled water, which in the absence of supplementary energetic resources, also induces mortality (see Fig. 2 and Ayestaran et al., 2010). Bees that were injected with quinine solution at an even lower concentration than the one used in our work (1 mM) diminish their walking activity and

increase grooming behavior, consistently with a malaise-like state (Hurst et al., 2014). Importantly, we determined the devaluating effect of quinine 23 h after conditioning, i.e. 21 h 30 min after the end of the pairing of fructose and quinine. Only bees that survived the quinine ingestion were available on the next day for testing US and CS responses. These bees therefore experienced the malaise, survived and were subjected to the US and CS tests after an interval long enough to ensure that they were no longer under the effect of the malaise. Fig. 3b,c confirms this conclusion by showing that only the PQ group exhibited a decrease in US and CS responses. The UQ group, which ingested the same amount of quinine, did not exhibit this reduction. This result is different from the one obtained after testing CS and US responses only 90 min after ingestion (Ayestarán et al., 2010): in this case, both the PQ and the UQ groups exhibited reduced CS and US responsiveness, probably as a consequence of a generalized malaise state and not as a result of an associative US devaluation and degradation of a CS memory. In the present study, the decrease of US and CS responses was of associative nature as it was due to the association between the contingent fructose-quinine stimulation and the subsequent malaise-like state induced by quinine ingestion. Distilled water was used as a control for the effects induced by quinine ingestion; it neither induced US devaluation nor CS-memory degradation, as shown by the performances of the PW group. Yet, the mismatch between an antennal stimulation with fructose and the subsequent ingestion of water could have, in some circumstances, a negative effect *per se* (see above). In our case, this effect proved to be negligible and it did not affect the performances recorded.

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

The demonstration that 5-HT mediates an aversive, malaise-dependent degradation of memory adds new evidence on the role of this biogenic amine in the processing of aversive stimulations in the insect brain, yet in a context different from those previously known. In a natural situation, bees exposed to aversive or potentially nociceptive stimuli release an alarm pheromone carried by their stinger and whose main component is isoamyl acetate (IAA) (Boch, Shearer, & Stone, 1962). This pheromone alerts and recruits more defenders to organize a collective attack (Boch et al., 1962; Collins & Blum, 1983; Nouvian, Reinhard, & Giurfa, 2016). Exposure to IAA upregulates brain levels of 5-HT, and to a lesser degree of DA, thereby increasing the likelihood of an individual bee to attack and sting (Nouvian et al., 2018). Pharmacological enhancement of the levels of both amines induces higher defensive responsiveness, while pharmacological blockade of their corresponding receptors decreases stinging. In the laboratory, aversive responsiveness is evaluated *via* the quantification of the sting extension response (SER), a reflexive response that is elicited in a harnessed bee *via* delivery of mild electric or thermal shocks (Junca, Carcaud, Moulin, Garnery, & Sandoz, 2014; Junca, Garnery, & Sandoz, 2019; Núñez, Almeida, Balderrama, & Giurfa, 1997; Núñez, Maldonado, Miralto, & Balderrama, 1983; Roussel, Carcaud, Sandoz, & Giurfa, 2009; Tedjakumala et al., 2014). In these experiments, bees are subjected to a series of increasing voltages or contact temperatures and their SER to these stimuli is evaluated. A pharmacological analysis of this behavior showed that injection of DA- and 5-HT-receptor antagonists into the bee brain induces an increase in sting responsiveness to shocks of intermediate voltage (Tedjakumala et al., 2014). The effect was particularly evident in the case of 5-HT antagonists, one them being methiothepin, the antagonist used in our work. Thus, the response to the succession of aversive shocks experienced by the bee was an enhanced 5-HT signaling, which diminished responsiveness (i.e. made animals more tolerant) to the shocks. Blocking such signaling rendered the bees more responsive to the shocks.

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

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

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

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

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

The relationship between feeding, malaise and 5-HT signaling was studied in the honey bee, using a combination of behavioral methods, immunostaining of 5-HT processes, gene-expression analysis and quantification of 5-HT levels after ingestions of pure sucrose solution or sucrose solution spiked with amygdalin (French et al., 2014). A rich innervation by 5-HT neural processes was found at the level of the esophagus, crop, proventriculus and midgut and in the first fused thoracic and abdominal ganglia of the ventral nerve chord. Moreover, high levels of expression of the four 5-HT receptors characterized in the honey bee (*Am5-ht1, Am5-ht2α, Am5-ht2β, Am5-ht7*) were found in the crop. These results indicate a tight control of ingestion processes by the serotonergic system. However, the functional measurements did not always yield consistent results. For instance, based on prior evidence,

an increase in 5-HT levels was expected after ingestion of a mixture of sucrose solution and amygdalin because this increase would reflect the malaise that was attributed to the ingestion of this mixture (Wright et al., 2010). Yet, contrary to this expectation, 5-HT levels in the hemolymph were higher after feeding bees with pure sucrose solution compared with feeding with the mixture of sucrose and amygdalin. Moreover, these levels did not vary in time, contrary to the decay in PER responses observed during olfactory conditioning (Wright et al., 2010). Injection of 5-HT into the brain suppressed feeding of pure sucrose solution and of sucrose supplemented with amino acids, but did not change the ingestion levels of sucrose supplemented with amygdalin, which remained low and comparable to those observed in the absence of injection or after injection of water into the brain (French et al., 2014). It was thus concluded that the hypothesis of 5-HT mediating a malaise signal released by the gut in response to sucrose mixed with toxins was not tenable and that 5-HT levels in the brain might instead control the motor program responsible for PER (French et al., 2014). This reinterpretation thus indicates that if bees ceased responding to an odor after receiving sucrose mixed with amygdalin during successive conditioning trials, it was because of an effect on PER and thus on the amount of food ingested rather than because of a malaise state. This argument is compatible with the motivational hypothesis proposed above and rises again the potential problem of using a sucrose mixed with amygdalin as a potential inductor of malaise. It would be therefore interesting to consider performing these experiments again, yet using pure toxins instead of mixing them with sucrose solution.

5. Conclusions

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

Declaration of Competing Interest

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

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