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RESEARCH ARTICLE

# Aversive learning of odor–heat associations in ants

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## ABSTRACT

Ants have recently emerged as useful models for the study of olfactory learning. In this framework, the development of a protocol for the appetitive conditioning of the maxilla–labium extension response (MaLER) provided the possibility of studying Pavlovian odor–food learning in a controlled environment. Here we extend these studies by introducing the first Pavlovian aversive learning protocol for harnessed ants in the laboratory. We worked with carpenter ants *Camponotus aethiops* and first determined the capacity of different temperatures applied to the body surface to elicit the typical aversive mandible opening response (MOR). We determined that 75°C is the optimal temperature to induce MOR and chose the hind legs as the stimulated body region because of their high sensitivity. We then studied the ability of ants to learn and remember odor–heat associations using 75°C as the unconditioned stimulus. We studied learning and short-term retention after absolute (one odor paired with heat) and differential conditioning (a punished odor versus an unpunished odor). Our results show that ants successfully learn the odor–heat association under a differential-conditioning regime and thus exhibit a conditioned MOR to the punished odor. Yet, their performance under an absolute-conditioning regime is poor. These results demonstrate that ants are capable of aversive learning and confirm previous findings about the different attentional resources solicited by differential and absolute conditioning in general.

**KEY WORDS: Aversive conditioning, *Camponotus*, Mandible opening response, Thermal stimulation**

## INTRODUCTION

Learning is a widespread ability among animals that allows them to establish predictive relationships in their environment. One of the most studied learning forms is Pavlovian (or classical) conditioning (Pavlov, 1927). In this paradigm, an individual learns to associate an initially neutral stimulus (conditioned stimulus, CS) with a stimulus having an innate positive or negative value (unconditioned stimulus, US), which elicits an unconditioned, stereotyped response (unconditioned response). In this framework, learning consists of acquiring the capacity to respond to the CS (conditioned response) following its forward-pairing with the US.

Pavlovian learning has been extensively studied in both vertebrates (Farris, 1967; Davey, 1992) and invertebrates (Bitterman et al., 1983; Watanabe et al., 2003). Among invertebrates, insects have played a major role in improving our understanding of the behavioral, neural and molecular mechanisms of Pavlovian learning and memory (Giurfa, 2007). This is due both to the fact that several Pavlovian protocols have been developed for different species, which exhibit excellent learning performances in the laboratory, and to the tractability of their relatively simple nervous systems (e.g. Giurfa, 2003, 2012; Mizunami et al., 2004; Davis, 2005). Among insects, the honey bee, *Apis mellifera*, has been one of the traditionally favored models in learning and memory studies (Menzel, 1985; Giurfa, 2007; Sandoz, 2011; Giurfa and Sandoz, 2012). Pavlovian protocols have been established in both appetitive and aversive contexts, which allowed the study of the mechanisms underlying these learning forms. For instance, the olfactory conditioning of the proboscis extension response (PER; Takeda, 1961; Bitterman et al., 1983; Giurfa and Sandoz, 2012; Matsumoto et al., 2012) constitutes an appetitive case of Pavlovian learning. In this case, harnessed honey bees learn to associate an odor (CS) with a sucrose solution (US), a protocol that aims to recreate the learning of natural odor–nectar relationships that occurs while foraging on flowers. In this context, the odor acquires an appetitive valence as it acquires the capacity to predict the food reward. Alternatively, the olfactory conditioning of the sting extension response (SER; Carcaud et al., 2009; Giurfa et al., 2009; Vergoz et al., 2007; Junca et al., 2014) constitutes an aversive case of Pavlovian learning as, in this case, harnessed bees learn the association between an odor (CS) and a mild electric shock or heat (US). The odor thus acquires the value of being a predictor for punishment.

Beside bees, other social insects such as ants have attracted the attention of scholars interested in various aspects of the biology of learning. Ants are a major and diverse group of social insects with highly plastic behaviors at the collective level (Gordon, 2010). Yet, it is only recently that their individual learning abilities have been characterized. While some studies characterized olfactory learning in free-walking ants trained to collect food in an arena or a Y-maze (Dupuy et al., 2006; Josens et al., 2009; Bos et al., 2012), other studies relied on a protocol for harnessed *Camponotus* ants, which allows a more precise quantification of olfactory learning and memory in an appetitive context (Guerrieri and d'Ettorre, 2010). This protocol was inspired by the PER conditioning method developed for bees (see above) and uses the extension of mouthparts (maxilla–labium extension response, MaLER) as the appetitive response that is conditioned by associating an odor (CS) with a food reward (sucrose solution, US). The MaLER could be successfully conditioned in several ant species (e.g. van Wilgenburg et al., 2011; Perez et al., 2013; Udino et al., 2016). In this way, comparative studies on appetitive learning can be performed using ants and bees as models. However, the absence of an aversive learning protocol for ants precludes the development of these studies in an aversive modality. Here we aimed to achieve two goals: (1) developing the first Pavlovian, aversive-conditioning protocol for ants; and (2) comparing the learning and short-memory performances induced by absolute and differential conditioning, i.e. between conditioning with a single reinforced CS (absolute) or with two CSs, one reinforced and the other not (differential).

To reach these goals, we took inspiration from an aversive conditioning protocol recently developed for honey bees, which

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uses heat as an aversive US and odorants as CSs (Junca et al., 2014; Junca and Sandoz, 2015). While the SER was the behavioral readout for odor–heat learning in bees, here we made use of the mandible opening response (MOR; Fig. 1A). The MOR is a stereotyped aggressive behavior typically emitted by ants in response to threatening stimuli, such as the odor of an enemy, which has been formally characterized as a binary variable in harnessed *Camponotus* ants (Guerrieri and d'Ettorre, 2008). Differently from the SER in bees, which involves the use of abdominal body parts and corresponding neural pathways clearly segregated from a feeding context, the MOR in ants involves the mandibles, which are also opened in an appetitive context to extend the maxilla–labium (MaLER) and acquire food. However, in response to aversive stimulations, the MOR always occurs without the maxilla–labium extension, and in a context that is not associated with feeding but with potential biting of a threatening stimulus. Moreover, in the MOR, the mandibles are wide open, which is not the case of MaLER. The MOR therefore constitutes an appropriate readout for behavioral aversion in ants (Guerrieri and d'Ettorre, 2008).

## MATERIALS AND METHODS

### Ant housing and preparation

Ants used in the experiments belonged to five colonies of carpenter ants *Camponotus aethiops* (Latreille 1798) (colony size 250 individuals on average) collected in Pompertuzat (Midi-Pyrénées, France, 43.5°N, 1.5167°E). Colonies were kept under standardized laboratory conditions (25±1°C, photoperiod=12 h, 50% humidity) in artificial nests composed of two plastic boxes (26×19×10 cm) connected by a plastic hose. One box paved with plaster served as a nest and was kept in the dark by means of opaque walls; the second box was exposed to light and served as foraging area. The inner faces of the two boxes were coated with Fluon® (AGC Chemicals Europe, Thornton Cleveleys, Lancashire, UK) to prevent ants from escaping. Ants were fed three times per week with a sucrose solution (33% w/w) and proteins (crickets and mealworms). Water was provided *ad libitum*.

On the day of the experiment, medium-sized worker ants (foragers) were collected in the foraging area of at least three different colonies, anesthetized after remaining on crushed ice for a few minutes, and then harnessed in the conditioning holder. This consisted of an individual support made of a foam strip (1.3×2 cm) on which the ant was attached vertically by two strings while keeping its abdomen oriented forward (Fig. 1B). The first string, set at the junction of the head and the thorax, restricted the movements of the head without hindering the mandible opening, while the second string, set at the thorax between the first and second pair of legs, allowed the flexion of the abdomen (gaster). We chose to leave the abdomen free to move because the faster flexing behavior, which is typically accompanied by the release of formic acid, constitutes a typical aggressive response often complementing the MOR in forlicine ants such as *C. aethiops*. Restraining the ants in an enclosed holder similar to that used for the MaLER (i.e. an Eppendorf tube with the apex cut off; Guerrieri and d'Ettorre, 2010) could damage the ant following prolonged exposure to released formic acid. Moreover, limiting the expression of the gaster flexion could increase stress and affect the expression of the MOR.

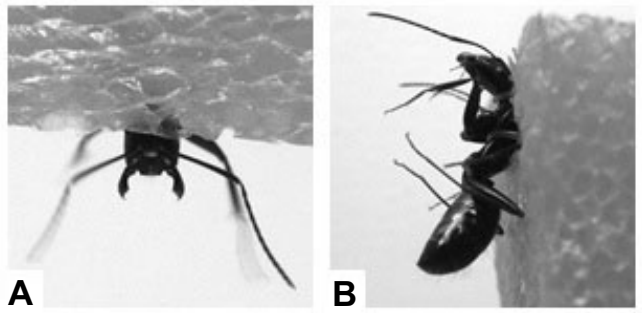


**Fig. 1. Experimental set-up.** (A) Mandible opening response (MOR) of harnessed *Camponotus aethiops* ants. (B) Lateral view showing the position of the ant, attached by two strings to the experimental holder made of a foam strip. The ant is fixed vertically, with its abdomen oriented forward. Photo credit: Paul Devienne.

After being placed in the holder, each ant received water *ad libitum* to avoid dehydration and standardize water level. The ants were then kept in a dark and humid (50%) box for 3 h to let them recover from the anesthesia and habituate to the harness. To observe the behavioral response (MOR), the ants were placed one by one under a stereomicroscope (Carl Zeiss Stemi 2000-C Stereo Microscope, ocular ×10, zoom 7.7:1, Marly le Roi, France).

### Effect of temperature on the MOR

We first studied the capacity of different temperatures to elicit the aggressive response (MOR) upon stimulation of different parts of the ants' body. In this way, we could determine both the best aversive US and the most sensitive body region for eliciting the unconditioned response. Thermal stimulations were applied for 1 s on the ventral abdomen (sternite segments 4 and 5), the dorsal abdomen (tergite segments 3–5) or the hind legs (tarsus and tibia) depending of the experimental group ($n$=31 per group). The head was not stimulated to avoid interfering with the MOR. Thermal stimulation was applied through a metal probe (Toolcraft MST-01, widest diameter=3 mm, tip diameter=1 mm, Georgensgmünd, Germany) inserted at the end of a micro soldering iron (Toolcraft MS-7512) whose temperature was adjusted via a laboratory power supply (Velleman HQ-power, PS1503, Gavere, Belgium). The temperature of the probe was measured with a contact thermometer (Voltcraft VC-150-1, Hirschau, Germany) at the beginning and end of each series of ants tested. This setup was similar to that used by Junca et al. (2014).

Each ant received a series of seven increasing thermal stimulations, from 25°C (ambient temperature) to 85°C, in consecutive steps of 10°C. The thermal stimulations were alternated with tactile stimulations (control) applied on the same body part with a second metal probe maintained at room temperature (25°C). To this end, an individual ant was placed under the stereomicroscope and, after 20 s, it received either a thermal or a tactile stimulation lasting 1 s; it was left in place for an additional 20 s (to avoid contextual learning) and was then removed to be replaced by the next ant. As groups of 12 ants were tested in a series, the inter-stimulus interval was 10 min.

The presence/absence of the MOR was noted during 6 s following the stimulus (tactile and thermal). Six seconds is the average latency for mandible opening following a high thermal stimulation (>55°C, see Appendix and Fig. S1). The MOR response was noted as 1 when the mandibles were wide open (see Fig. 1A) and 0 when the mandibles were closed or only slightly opened.

### Aversive conditioning of the MOR

The CS was either octanal or 1-hexanol (floral odors, Sigma-Aldrich, France, purity >99%). Half of the ants received octanal paired with heat and the other half 1-hexanol paired with heat. These odors have been successfully used to train *C. aethiops* ants in appetitive differential conditioning (Guerrieri and d'Ettorre, 2010; Perez et al., 2013). The odors were presented to the antennae using a 20 ml syringe containing a piece of filter paper (1×1.5 cm) soaked



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with 5 µl of pure odorant (Urlacher et al., 2010; Perez et al., 2015). An air extractor was placed behind the ant during conditioning in order to prevent the accumulation of odors. The US was a thermal stimulation of 75°C applied to the hind legs. The intensity of the temperature used as the US and the stimulated area were chosen based on the results obtained in the experiments described in the previous section, where a stimulation of the hind legs at 75°C induced a high rate of MOR.

We used two conditioning procedures: differential and absolute conditioning. In differential conditioning, ants had to learn two odors as conditioned stimuli; one (CS+) was associated with the thermal stimulation, and the other (CS−) was presented without reinforcement. Training consisted of 12 trials (six reinforced and six non-reinforced) during which the two CSs were presented in a pseudo-random sequence (e.g. ABABBABAABAB). The same stimulus was never presented more than twice consecutively. In absolute conditioning, two experimental groups, paired and unpaired, were conditioned in parallel. In the paired group, six presentations of the odor–heat association were alternated with six blank trials in which the ants were placed under the stereomicroscope without any stimulation. In the unpaired group, ants received the CS and the US in separate trials following a pseudo-random sequence. Thus, ants in the unpaired group experienced the odor (CS) six times and the heat (US) six times in 12 trials.

In both conditioning procedures, each conditioning trial lasted 50 s according to a predefined sequence. Each ant was placed under the stereomicroscope and left undisturbed for 23 s to allow familiarization with the experimental context. Then, the CS was delivered for 4 s. After 3 s (inter-stimulus interval), the thermal stimulation (US) was delivered for 1 s, thus overlapping with the end of the CS presentation. The ant was then left in the device for a further 23 s to prevent the establishment of predictive associations between the context and the thermal stimulation. A group of 12 ants was tested in series so that the inter-trial interval (ITI) was 10 min.

The presence/absence of the MOR was noted during the 3 s in which the odor (CS+ or CS−) was presented alone (conditioned response), as well as during the 6 s following thermal stimulation. Ants that did not respond to half of the thermal stimulations (three out of six trials) were excluded from the analyses (Guerrieri and d'Ettorre, 2010; Junca et al., 2014) as they were considered unresponsive to thermal stimulation (differential conditioning: ∼10% of 94 ants; absolute conditioning: ∼14.5% of 179 ants).

Memory retention in the short-term range was evaluated 10 min after conditioning. To this end, conditioned ants were presented with two odors without heat reinforcement: in the case of ants trained under differential conditioning, the CS+ and the CS− were delivered; in the case of ants trained under absolute conditioning, the CS and a novel odor (NOd, either octanal or 1-hexanol, depending on the CS) were delivered. The order of presentation of the two odors was randomized between ants.

After the retention test, the thermal stimulation was presented again to each ant to verify whether the MOR was still elicited by the aversive US. Individuals that did not respond to this last thermal stimulation were excluded from the analyses because the absence of a response could reflect a lack of motivation or poor physical condition (differential conditioning: ∼6% of 85 ants; absolute conditioning: ∼10.5% of 153 ants).

### Statistical analysis

Data were analyzed with R software, version 3.3.2 (http://www.R-project.org). The significance level was set at 5%. The requirements for using each statistical test were verified.

#### Effect of temperature on the MOR

The sensitivity curves to stimuli (thermal or tactile) were analyzed using a generalized linear mixed model (GLMM; Bolker et al., 2009) with a binomial error structure and a logit link function (lme4 packages; Bates et al., 2015). The response (0 or 1 for each stimulation) was used as the dependent variable. The stimulated body region (ventral abdomen, dorsal abdomen or hind legs) and the type of stimulus (thermal or tactile) were entered in the model as fixed factors. Trials (successive stimulations) were used as a covariate. The individuals' identity and the colony of origin were set as random factors to account for repeated measures and for within-colony similarities. Interactions between fixed factors and covariates were included in the models to detect differences in response slopes between trials for each stimulus. We retained the best model with the highest explanatory power [i.e. the lowest Akaike's information criterion (AIC) value]. In the selected model, the region×stimulus×trial interaction was significant. We used Tukey's *post hoc* tests to detect differences both between stimuli and between regions (glht function from R package multcomp; Bretz et al., 2011).

#### Aversive conditioning of the MOR

Acquisition curves were analyzed using a GLMM for binomial data, with a logit link function (lme4 package). When necessary, models where optimized with the iterative algorithm BOBYQA (Powell, 2009). The MOR (0 or 1 for each trial) was used as the response variable. The stimulus (for differential conditioning, CS+ or CS−), group (for absolute conditioning, paired or unpaired) and nature of the stimulus (octanal or 1-hexanol) were included as fixed factors. Trials were included as covariates. The identity of individuals and the colony of origin were entered as random factors. Interactions between the stimulus or the group (according to the conditioning procedure), the nature of the stimulus and the trial were included in the model to detect slope differences along the trials between the two stimuli (CS+ or CS−) or the two groups (paired or unpaired) and the possible influence of the nature of CS+ (octanal or 1-hexanol). We retained the significant model with the highest explanatory power (i.e. the lowest AIC value). To analyze separately the ant responses according to the odor used as CS+, the same GLMM models were applied, excluding the factor 'nature of the stimulus'. The best model for each odor was selected based on its explanatory power according to AIC values (i.e. the lowest AIC value).

To evaluate memory retention 10 min after the last conditioning trial, a McNemar's test was conducted to compare the proportion of responses of the two odors (CS+/CS− or CS/NOd), and a $\chi^2$ test was applied to compare the response of paired and unpaired groups.

## RESULTS

### Effect of temperature on the MOR

This experiment aimed to determine both the ants' sensitivity to a range of increasing temperatures and the most sensitive body region for eliciting the MOR upon thermal stimulation. We increased thermal stimulations from 25°C (ambient temperature) to 85°C in 10°C steps and determined the occurrence of the MOR. Thermal stimulations were applied to three body parts: the ventral abdomen, the dorsal abdomen and the hind legs. A significant interaction between stimulus type, trial and the stimulated body part revealed that responses to thermal and tactile stimulation differed over successive trials in different body parts (GLMM, region×stimulus×trial: $\chi^2$=25.507, d.f.=3, $P$<0.0001, Fig. 2). Stimulating with heat any of the three body parts resulted in a higher percentage of ants exhibiting MOR compared with the effect of the tactile

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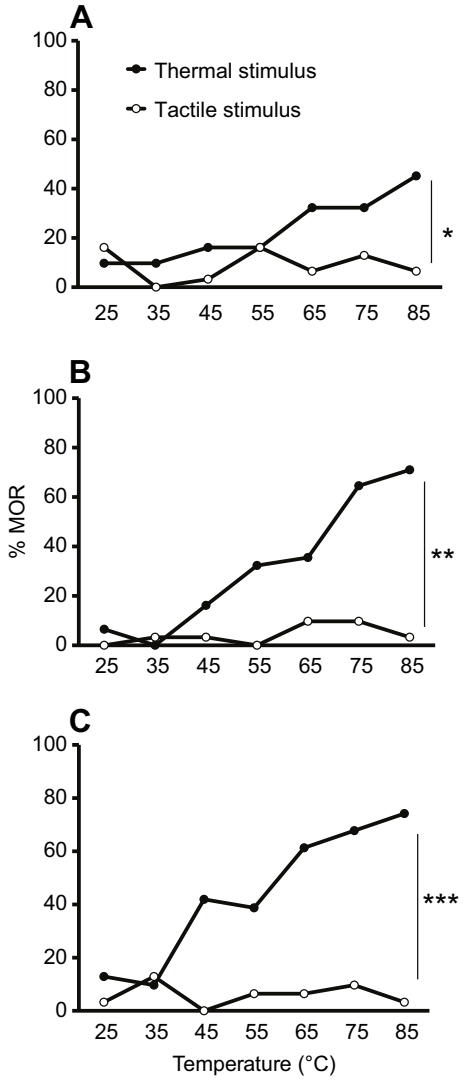


**Fig. 2. Unconditioned MOR: dependence on temperature intensity and localization of the thermal stimulation.** Percentage of ants displaying unconditioned MOR to successive thermal (black circles) and tactile (white circles) stimulations. Stimulations were applied to (A) the dorsal abdomen ($n$=31), (B) the ventral abdomen ($n$=31) and (C) the hind legs ($n$=31). For all the three body parts studied, ants responded more to thermal stimuli than to tactile controls (GLMM, *$P$<0.01; **$P$<0.0001; ***$P$<0.00001).

stimulation in the same body regions (GLMM, Tukey's *post hoc* test, dorsal abdomen: $P$=0.0068; ventral abdomen: $P$<0.0001; hind legs: $P$<0.00001). Indeed, for all three body parts, the MOR increased over successive stimulations of increasing temperature, but remained constant and low for tactile controls (Fig. 2).

Although the sensitivity to temperature was significantly different between the hind legs and the dorsal abdomen (GLMM, Tukey's *post hoc* test, $P$=0.011), it did not differ between the ventral abdomen and the hind legs ($P$=0.15) or between the ventral abdomen and the dorsal abdomen ($P$=0.97). In contrast, the percentage of ants exhibiting MOR was equally low and constant along trials in the case of tactile stimulation (GLMM, Tukey's *post hoc* test, dorsal abdomen versus ventral abdomen: $P$=0.37; dorsal abdomen versus hind legs: $P$=0.75; ventral abdomen versus hind legs: $P$=0.98).

Thus, the regions that are more sensitive to thermal stimulation are the hind legs and, to a lesser extent, the ventral abdomen. This experiment also shows that a high and comparable level of MOR is attained for both regions from 75°C upwards. This temperature was therefore chosen as US for conditioning experiments and the hind legs as the region for US stimulation.

## Learning of odor–heat associations

We studied the capacity of ants to learn odor–heat associations under a differential and an absolute conditioning regime. To this end, we paired odor stimulations with a thermal stimulation of 75°C applied to the hind legs. Learning was observable if ants exhibited the MOR to the odor associated with the heat punishment.

### Differential aversive olfactory conditioning

Octanal and 1-hexanol were used as conditioned stimuli. Their role as CS+ and CS− was balanced between two groups of conditioned ants (octanal+/1-hexanol−, $n$=36; 1-hexanol+/octanal−, $n$=42). The learning performance of both groups during the acquisition phase was the same irrespective of the reinforcement contingency (octanal +/1-hexanol− or 1-hexanol+/octanal−, GLMM: odor: $\chi^2$=0.6649, d.f.=1, $P$=0.41). Moreover, retention levels 10 min after conditioning were also unaffected by the reinforcement contingency both for CS+ ($\chi^2$=2.8422, d.f.=1, $P$=0.091) and CS− responses ($\chi^2$=0.50598, d.f.=1, $P$=0.48). This allowed us to pool the results of both subgroups and present them in terms of a CS+ versus CS− discrimination learning (Fig. 3).

Fig. 3 shows that ants trained under a differential conditioning regime responded differently to the punished and the unpunished odor throughout the successive trials (GLMM, significant stimulus×trial interaction: $\chi^2$=20.037, d.f.=1, $P$<0.0001; Fig. 3A). Indeed, ants responded differently to the CS+ and to the CS− over the course of the acquisition phase (GLMM: stimulus: $\chi^2$=8.34, d.f. =1, $P$=0.0039; Fig. 3A). Precisely, the responses to the CS+ and the CS− differed only in the last trial (GLMM, Tukey's *post hoc* test, CS+ versus CS−, $P$=0.0009; Fig. 3A).

Ten minutes after the end of conditioning, ants responded more to the odor previously paired with heat than to the odor that was previously unpunished ($\chi^2$=22.5, d.f.=1, $P$<0.0001; Fig. 3B). This result shows effective retrieval of a specific short-term memory of the aversive odor–heat association. The fact that the CS+/CS− performance in the retention test was not significantly different from the CS+/CS− performance in the last acquisition trial (CS+: $\chi^2$=1.81, d.f.=1, $P$=0.18; CS−: $\chi^2$=0.49, d.f.=1, $P$=0.48) indicates that six reinforced trials were sufficient to reach the learning plateau. Taken together, these results show that ants learned efficiently to discriminate the heated from the unheated odor.

### Absolute aversive olfactory conditioning

As in the previous experiment, octanal and 1-hexanol were used as conditioned stimuli. Two groups of ants were trained, one with octanal paired with heat ($n$=34) and the other with 1-hexanol paired with heat ($n$=32). Each of these groups had an unpaired group as a control (unpaired, octanal: $n$=33, 1-hexanol: $n$=38). Overall, the performance during the acquisition phase was independent of the reinforced odor (GLMM: odor×trial, $\chi^2$=1.94, d.f.=1, $P$=0.16). A significant interaction between trial and treatment (i.e. paired and unpaired groups) was found, thus indicating that experiencing associations between odor and heat resulted in a different response compared with experiencing the same sensory stimulation in a non-associative way (GLMM: group×trial, $\chi^2$=4.76, d.f.=1, $P$=0.03). Although the odorant chosen as CS did not influence acquisition, it affected retention performance 10 min after conditioning. The performance varied according to the odor conditioned (unpaired

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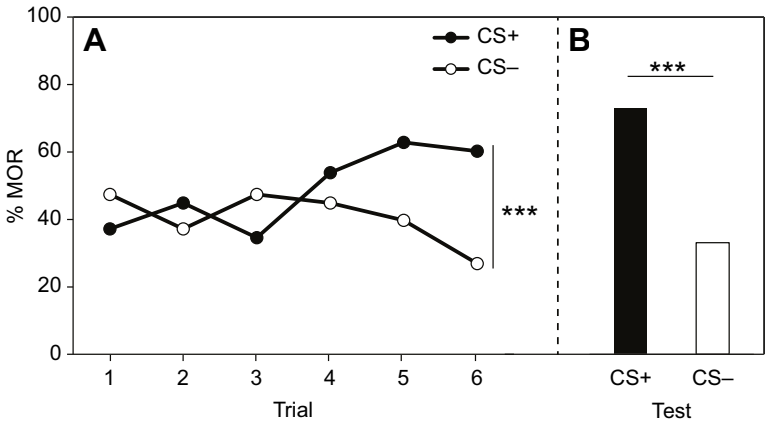


**Fig. 3. Olfactory differential conditioning of the MOR and short-term retention.** Percentage of ants ($n$=78) showing MOR to the odor (octanal, $n$=36; 1-hexanol, $n$=42, pooled) reinforced with thermal stimulation of 75°C (CS+, black circles/bars) and to the unreinforced odor (CS–, white circles/bars). (A) During the 12-trial learning phase, ants learned to respond more to the reinforced odor than to the non-reinforced odor (GLMM: stimulus×trials interaction: ***$P$<0.0001). (B) This difference was also visible in the retention test performed 10 min after the end of acquisition (McNemar $\chi^2$ test, ***$P$<0.0001).

group: $\chi^2$=4.08021, d.f.=1, $P$=0.028, paired group, $\chi^2$=0.21, d.f.=1, $P$=0.64). This result precludes pooling performances and favors representing and analyzing them separately, according to the nature of the CS (octanal or 1-hexanol).

The performance of ants trained with octanal as the CS was compared with that of ants in the corresponding unpaired group, which experienced octanal and heat but in a non-contingent manner (Fig. 4A). A significant group×trial interaction revealed different responses to odors between the paired and the unpaired groups throughout the successive trials (GLMM: $\chi^2$=8.0560, d.f.=1, $P$=0.0045). Indeed, ants of the paired group increased the MOR during trials (from ca. 30% to ca. 60%; $\chi^2$=11.289, d.f.=1, $P$=0.00078) whereas ants of the unpaired group remained at a constant level of responses that oscillated around 30% ($\chi^2$=0.5142, d.f.=1, $P$=0.47). Six paired conditioning trials were sufficient for the ants to reach a learning plateau as the response to the CS in the retention test (see below) was not significantly different from that in the last acquisition trial ($\chi^2$=0.06, d.f.=1, $P$=0.8). These results thus show that ants of the paired group learned the association between octanol and heat.

Further analysis of the retention performance shows that 10 min after the last conditioning trial (Fig. 4B) ants experiencing octanal in the paired and unpaired groups did not differ in their response to octanal ($\chi^2$=1.1996, d.f.=1, $P$=0.27). This result indicates that short-term retention was inconsistent, a conclusion that was confirmed by the high and similar level of responses to the novel odor (generalization) exhibited by the paired and unpaired groups ($\chi^2$=0.1387, d.f.=1, $P$=0.70). Furthermore, their level of response to the novel odor was not different from that to the CS (paired group: $\chi^2$=1, d.f.=1, $P$=0.32; unpaired group: $\chi^2$=0.8181, d.f.=1, $P$=0.36). Thus, pairing octanal with aversive heat induced significant learning but the resulting short-term memory (in the range of 10 min) was weak in terms of its associative nature and specificity.

The situation was different for the group trained with 1-hexanol as the CS, as no significant difference was found between the paired and unpaired groups during the conditioning phase (GLMM: group: $\chi^2$=0.134, d.f.=1, $P$=0.71; group×trial: $\chi^2$=0.038, d.f.=1, $P$=0.84; Fig. 4C). In this case, the response to the punished odor remained stable in the paired group despite its association with heat ($\chi^2$=0, d.f.=1, $P$=1). The proportions of ants responding to the CS+ in the last acquisition trial and in the retention test (see below) were not statistically different ($\chi^2$=1.58, d.f.=1, $P$=0.21), thus showing that additional trials would not necessarily improve learning in this case. As expected, the unpaired group also showed no evidence of learning ($\chi^2$=0.0823, d.f.=1, $P$=0.7742).

The results of the retention test following 1-hexanol conditioning were surprising, as ants of the paired group responded more to the CS+ than ants in the unpaired group ($\chi^2$=7.7829, d.f.=1, $P$=0.0052; Fig. 4D) despite not showing significant acquisition. Ants in the paired and unpaired groups did not respond differently to the novel odor ($\chi^2$=0.1602, d.f.=1, $P$=0.68), and this level of response was similar to that of the paired group for the CS (paired group: $\chi^2$=0, d.f.=1, $P$=1; unpaired group: $\chi^2$=10.889, d.f.=1, $P$=0.001). As for the group trained with punished octanal, we conclude that training with punished 1-hexanol induces a non-specific response, at least in the short range of 10 min.

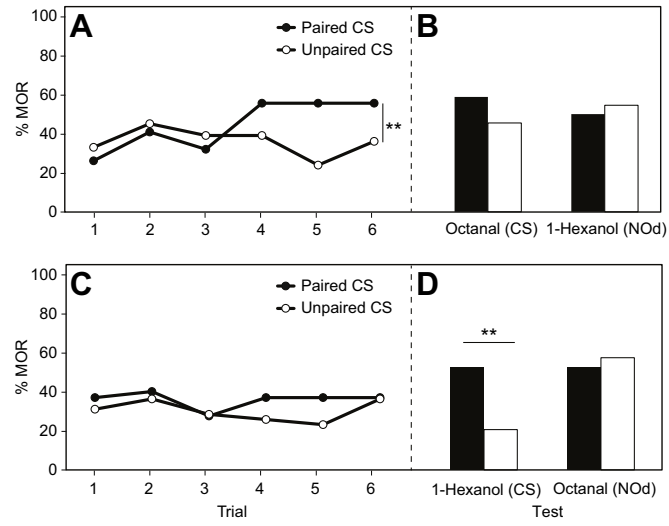


**Fig. 4. Olfactory conditioning of the MOR response: absolute conditioning.** Percentage of ants showing MOR to octanal (A,B) or 1-hexanol (C,D) reinforced (CS) with a thermal stimulation of 75°C. Paired group: black circles/bars; unpaired group, white circles/bars. During the 12-trial learning phase (A,C), the frequency of conditioned responses did not vary if odor and heat were unpaired, but it increased when octanal was paired with heat (GLMM group×trial: **$P$<0.01). (B,D) Memory retention test: 10 min after acquisition, ants responded more to 1-hexanol than to the novel odor (NOd) if it was previously paired than if it was not ($\chi^2$ test, **$P$<0.01). Octanal/paired: $n$=34, octanal/unpaired: $n$=33, 1-hexanol/paired: $n$=32, 1-hexanol/unpaired: $n$=38.

## DISCUSSION

Our study shows that heat applied on the body surface of carpenter ants *C. aethiops* elicits the typical aversive MOR, and the probability of this response increases with the temperature used to stimulate the ants and varies according to the body region to which the stimulation is applied. Here we chose the hind legs as the

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stimulation region owing to their high sensibility to heat. We also show that ants can successfully learn odor–heat associations and thus exhibit MOR to the punished odor, in particular under a differential-conditioning regime, which improves learning and retention performances compared with absolute conditioning.

### Effect of heat on the mandible opening response

Traditionally, electric shocks have been used to elicit unconditioned responses in order to evaluate the individual response threshold or to study aversive learning and memory abilities (Busto et al., 2010; Kahsai and Zars, 2011). This principle applies both to vertebrates (e.g. fear conditioning protocols: Maren, 1996; Maren, 2001; Rosen, 2004) and invertebrates (e.g. classical conditioning in *Aplysia*: Hawkins, 1984; Abrams, 1985; Levy and Susswein, 1999). Electric shocks are used as USs in aversive olfactory conditioning of fruit flies (*Drosophila melanogaster*; e.g. Tully and Quinn, 1985; Davis, 2005) and honey bees (Vergoz et al., 2007; Roussel et al., 2009). More recently, a thermal stimulation was used as the US in olfactory aversive conditioning in bees (Junca et al., 2014; Junca and Sandoz, 2015). Our results show that heat also acts as an efficient aversive US for carpenter ants *C. aethiops*, in which it induces MOR reproducibly and in an intensity-dependent manner. Applying heat to the body surface triggers the MOR, in particular in the case of high temperatures, thus suggesting that these temperatures act as nociceptive stimuli for ants. This is confirmed by the fact that tactile stimulations induced the MOR at significantly lower and constant levels, showing that heat was a specific trigger of the MOR with low or no sensitization component.

We studied the sensitivity to heat of three body parts, which are all easily accessible to the experimenter: the dorsal abdomen, the ventral abdomen and the hind legs. A higher proportion of MOR was observed when thermal stimulations were applied to the ventral abdomen and hind legs, indicating a higher thermal sensitivity of these two body parts as compared with the dorsal abdomen, similarly to previous observations in bees (Junca et al., 2014). Although information about thermal receptors in these areas are still missing, this result suggests that they are indeed present. Under natural conditions, high thermal sensitivity to heat in the legs and ventral abdomen could help prevent prolonged contact with particularly hot surfaces, which could happen when ants forage during warm days in their natural southern European biotopes. Indeed, these ants adapt their foraging activity to the soil temperature (O'Neill and Kemp, 1990; van Oudenhove et al., 2012). The presence of thermosensitive sensilla, which has been demonstrated on the antennae of other ants' species (Ruchty et al., 2009; Nagel and Kleineidam, 2015), could mediate such behavioral plasticity.

### Aversive olfactory conditioning of the MOR

In ants, the MOR is a reliable indicator of inter-individual aggressive behavior (Guerrieri and d'Ettorre, 2008). Yet, the possibility of conditioning it via the pairing of a neutral odor with heat as a nociceptive stimulus had not been explored until now. Our results show that MOR can be conditioned efficiently using odors as CSs predicting heat as an aversive US, in a Pavlovian framework. In this way, we extended the range of controlled conditioning protocols available for ants and make possible comparative studies between aversive (via the present protocol) and appetitive learning and memory (via MaLER conditioning: Guerrieri and d'Ettorre, 2010; Guerrieri et al., 2011). In honey bees, the existence of both conditioning variants, appetitive (via PER conditioning: Bitterman et al., 1983; Giurfa and Sandoz, 2012) and aversive (via SER conditioning; Vergoz et al., 2007; Giurfa et al., 2009; Junca et al., 2014) has been a determining factor in promoting a broad spectrum of studies comparing the different circuits and neurotransmitters that mediate both learning forms (e.g. Giurfa, 2007; Tedjakumala and Giurfa, 2013). A comparable research agenda will now be possible in ants and will pave the way for future studies spanning behavioral, cellular and molecular levels.

Despite these positive aspects, we observed a high level of spontaneous MORs in both conditioning regimes both for an unpunished CS in differential conditioning and for a CS not contingent with heat in unpaired absolute conditioning (between 30 and 40%; see Figs 3 and 4). This spontaneous MOR indicates that ants were somehow aroused by the experimental situation and responded aggressively to non-relevant olfactory stimulation, even if they managed to learn a specific odor–heat association, particularly in the case of differential conditioning. In the case of the unpaired group in absolute conditioning, the ants experienced just one odor, which was never reinforced. In this case, the aversive experience in heat trials seems to generalize partially to olfactory trials, even if heat and odor were not contingent. This conclusion is confirmed by the high response levels to the novel odor in the short-term retention test. To reduce the ants' arousal and their tendency to generalize between heated and unheated events, one could conceive different holders, possibly providing contact with the substrate and thus reducing potential stress associated with immobilization.

### Absolute and differential conditioning induce different learning and retention performances

Using the conditions yielding the highest MOR probability (75°C applied for 1 s to the hind legs), we obtained substantial rates of conditioned responses at the end of differential conditioning, i.e. 60% in response to the US-paired CS, regardless of its chemical identity (octanal or 1-hexanol). Yet, this was not the case in absolute conditioning, as ants associated octanal with the US at the same level of 60% but did not exhibit learning for 1-hexanol during the acquisition phase. This was unexpected, as our choice of 1-hexanol as a relevant CS was based on its successful learning in appetitive conditioning (Bos et al., 2013; Perez et al., 2015). Generally, in thermal MOR conditioning, differential conditioning induced better learning and short-term retention performances than absolute conditioning. For example, 1-hexanol could be learned under differential but not absolute conditioning (compare Figs 3B and 4B); further, the short-term memory induced by absolute conditioning was weak and non-specific whereas that induced by differential conditioning was more specific and even better for this odor. The retention data reveal an additional feat: although performance during absolute conditioning of 1-hexanol showed no improvement during acquisition, there was a significant retention 10 min after conditioning, thus indicating effective learning of this odor in some ants. Taken together, these results indicate that absolute conditioning induces poor learning performance and inconsistent short-term memory retention.

The fact that differential conditioning induces better performance than absolute conditioning is a trend that is common to other species (e.g. flies; Barth et al., 2014) and learning paradigms (e.g. appetitive conditioning in ants; Perez et al., 2016). It can be explained by the fact that differential conditioning provides a CS− against which the relevant CS+/US association can be contrasted. A modeling approach showed that the enhanced olfactory discrimination after differential learning is a consequence of the interaction between excitatory and inhibitory generalization gradients mediated by the CS+ and CS− odors (Perez et al., 2016). In free-flying bees trained to associate color stimuli with sucrose reward, colors that appeared to be

non-discriminable after absolute conditioning became discriminable after differential conditioning, even if the same rewarded target was used in both conditioning forms (Dyer and Chittka, 2004; Giurfa, 2004). It was thus suggested that conditioning procedures that imply a comparison between rewarded and non-rewarded stimuli, and therefore that have an associated risk of erroneous non-rewarded or penalized choices, significantly improve color discrimination (Giurfa, 2004; Avarguès-Weber and Giurfa, 2014). These results, as well as the present data on heat conditioning in ants, support the notion that differential conditioning, in particular in the case of perceptually similar stimuli, induces more attention than absolute conditioning owing to a higher probability of erroneous choices; aversive reinforcements would increase even more this penalty, thus enhancing attention (Avarguès-Weber and Giurfa, 2014). In theory, aversive learning performance could be further improved by reinforcing the CS− with a positive US (e.g. sucrose) so as to increase the reinforcement contrast.

Our new protocol renders possible the study of memory formation and retrieval by testing the ants' response to the CS for different post-conditioning periods, including long-term ones (e.g. 24 and 72 h). This would allow comparisons with appetitive long-term memories (Guerrieri and d'Ettorre, 2010; Guerrieri et al., 2011) in terms of duration and stability. Future studies will also elucidate whether aversive learning in a Pavlovian context translates into effective odor rejection when the trained ants have the possibility to choose between odorants (Carcaud et al., 2009; de Brito Sanchez et al., 2015). Further explorations of aversive learning in ants will pave the way for comparisons between the dynamics and neural bases of appetitive and aversive memories, as achieved in other insect species (Mizunami et al., 2009; Perisse et al., 2013; Tedjakumala and Giurfa, 2013; Xie et al., 2013).

## APPENDIX

### Effect of temperature on the latency to perform MOR

#### Materials and methods

The ants were prepared as described in the main text (Fig. 1) and placed individually under a stereoscopic microscope (Leica M80, ocular ×10, zoom 8:1). An interval of 20 s was established before the presentation of the stimulus to avoid the contextual effects. The ant then received a series of seven increasing thermal stimulations with a metal probe, from 25°C (ambient temperature) to 85°C, in consecutive steps of 10°C. Thermal stimulations were alternated with tactile stimulations (control) applied on the same body part with a second metal probe maintained at room temperature (25°C). After the stimulation (tactile or thermal) of one of the three studied body parts (ventral abdomen, dorsal abdomen and hind legs), the MOR was recorded over a period of 20 s in three different groups of ants (one per each body part). This time period was considered sufficient to observe a response from all tested ants. The estimation of the average latency to perform MOR for the different temperatures tested and for each body part allowed quantification of MOR as a binary response.

#### Statistical analysis

Data were analyzed with R software version 3.3.0 (http://www.R-project.org). In order to compare MOR between the three body parts studied, Kruskal–Wallis tests were applied and Mann–Whitney *U*-tests were used for two-by-two comparisons.

#### Results

After the thermal stimulation, MOR latency was significantly different between the three body parts studied when the stimulation applied was 65°C (Kruskal–Wallis test: $H$=10.6, d.f.=2, $P$=0.005), 75°C ($H$=12.63, d.f.=2, $P$=0.002) and 85°C ($H$=9.16, d.f.=2, $P$=0.01). For these three temperatures, the thermal stimulation of the hind legs triggered the MOR within 6 s (Fig. S1A).

A thermal stimulation of 65°C applied to the hind legs triggered significantly more MOR than when the dorsal abdomen and the ventral abdomen were stimulated (Mann–Whitney *U*-test: $U$=702, $P$=0.002; $W$=637.5, $P$=0.026, respectively). When the hind legs were stimulated with 75°C or 85°C, the latency to perform MOR was significantly different than when the stimulation was applied to the dorsal abdomen (Mann–Whitney *U*-test: $U$=719, $P$=0.001; $W$=670.5, $P$=0.01, respectively). No significant differences were observed when the ventral abdomen was stimulated (Mann–Whitney *U*-test: $U$=524, $P$=0.54; $U$=529, $P$=0.49, respectively). Moreover, during a thermal stimulation at 75°C, the proportion of ants that responded with MOR in 6 s was 67.7% when stimulation was applied to the hind legs, compared with 64.5% and 32.3% when stimulation was applied to the ventral abdomen and dorsal abdomen, respectively.

MOR latency remained particularly long during successive tactile stimulations (Fig. S1B) and MOR latency was generally not influenced by the stimulated zone. These results indicate that the tactile stimulation is a reliable control.

We therefore chose to apply the thermal stimulation in the subsequent experiments to the hind legs, which show the shortest latency for the MOR at the three highest temperature tested. We used 75°C for the conditioning experiment.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: J.-M.D., M.G., P.d'E.; Methodology: L.D., D.B.; Formal analysis: L.D., D.B.; Investigation: L.D.; Writing - original draft: L.D.; Writing - review & editing: D.B., J.-M.D., M.G., P.d'E.; Supervision: M.G., P.d'E.; Project administration: P.d'E.; Funding acquisition: P.d'E.

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#### Supplementary information

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