

## 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 due to their high sensitivity. We then studied the ability of ants to learn and remember odor-heat associations using 75°C as 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 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.

## INTRODUCTION

Learning is a widespread ability among animals, which allows them establishing predictive relationships in their environment. One of the most studied learning forms is Pavlovian (or classical) conditioning (Pavlov and Anrep, 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 in acquiring the capacity of responding 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 for understanding 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 a traditionally privileged model in learning and memory studies (Menzel, 1985; Giurfa, 2007; Sandoz, 2011; Giurfa and Sandoz, 2012). Both in appetitive and aversive contexts, Pavlovian protocols have been established, which allowed studying 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 at recreating 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 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 bee, 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. On the contrary, the absence of an aversive learning protocol for ants precludes the development of these studies in an aversive modality. Here we aimed at achieving two goals: 1) developing the first Pavlovian, aversive-conditioning protocol for ants; 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 uses heat as an aversive US and odorants as CSs (Junca et al., 2014; Junca and Sandoz, 2015). While SER (sting extension response) was the behavioral readout for odor-heat learning in bees, here we made use of the mandible opening response (MOR, Fig. 1A). 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 binary variable in harnessed *Camponotus* ants (Guerrieri and d'Ettorre, 2008). Differently from SER in bees, which involves the use of abdominal body parts and corresponding neural pathways clearly segregated from a feeding context, MOR in ants involves the mandibles, which are also opened in an appetitive context as to extend the *maxilla-labium* (MaLER) and acquire food. However, in response to aversive stimulations, MOR occurs always 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

MOR the mandibles are wide opened, which is not the case of MaLER. 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* (colony size 250 individuals on average) collected in Pompertuzat (Midi-Pyrénées, France, latitude 43.5, longitude 1.5167). Colonies were kept under standardized laboratory conditions ( $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ , photoperiod = 12h, 50% humidity) in artificial nests composed of two plastic boxes ( $26 \times 19 \times 10$  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® 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 given *ad libitum*.

On the day of experiment, medium-size worker ants (foragers) were collected in the foraging area of at least three different colonies, anesthetized after remaining a few minutes on crushed ice and harnessed in the conditioning holder. This consisted of an individual support made of a foam strip ( $1.3 \times 2$  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 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 foricine ants such as *Camponotus 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.

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 hours to let them recover from the anesthesia and habituate to the harness. To observe the behavioral response (mandible opening response, MOR), the ants were placed one by one under a stereoscopic microscope (Microscope Zeiss Stemi 2000-C, ocular  $\times 10$ , zoom 7.7:1).

### **Temperature effect on the mandible opening response**

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 aimed at choosing both the best aversive US and the most sensitive body region for eliciting the unconditioned response. Thermal stimulations were applied for one second on the ventral abdomen (sternite segments 4-5), 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) 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). The temperature of the probe was measured with a contact thermometer (Votcraft VC-150-1) 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 seconds, it received either a thermal or a tactile stimulation lasting one second; it was left in place for additional 20 seconds (to avoid contextual learning) and then removed to be replaced by the next ant. As groups of twelve ants were tested in a series, the inter-stimulus interval was 10 minutes.

The presence/absence of MOR was noted during 6 seconds following the stimulus (tactile and thermal). Six seconds is the average latency for mandible opening following a high thermal stimulation ( $>55^{\circ}\text{C}$ , see supplementary material). The MOR response was noted as 1 when the mandibles were wide opened (see, Fig. 1A) and 0 when the mandibles were closed or only slightly opened.

### Aversive conditioning of the mandible opening response

The conditioned stimulus (CS) was either octanal or 1-hexanol (floral odors, Sigma-Aldrich, France, purity > 99%). Half of ants received octanal paired with heat and the other half 1-hexanol paired with heat. These odors have been successfully used to train *Camponotus 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 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 unconditioned stimulus (US) was a thermal stimulation of 75°C applied to the hind legs. The intensity of the temperature used as 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 mandible opening response.

We used two conditioning procedures, differential and absolute conditioning. In *differential conditioning*, ants had to learn two odors as conditioned stimuli, one of which (CS+) was associated with the thermal stimulation, while 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 six times the odor (CS) and six times the heat (US) in 12 trials.

In both conditioning procedures, each conditioning trial lasted 50 seconds according to a predefined sequence. The ant was placed under the binocular microscope and left undisturbed during 23 seconds to allow familiarization with the experimental context. Then, the CS was delivered for four seconds. After three seconds (inter-stimulus interval), the thermal stimulation (US) was delivered for one seconds, thus overlapping with the end of the CS presentation. The ant was then left in the device for further 23 seconds to prevent the

establishment of predictive associations between the context and the thermal stimulation. A group of 12 ants was tested in a series so that the inter-trial interval (ITI) was 10 minutes.

The presence/absence of MOR was noted during the three seconds in which the odor (CS+ or CS-) was presented alone (conditioned response), as well as during the six seconds 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:  $\approx 10\%$  of 94 ants; absolute conditioning:  $\approx 14.5\%$  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 new 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 MOR was still elicited by the aversive US. Individuals which did not respond to this last thermal stimulation were excluded from the analyses because the absence of response could reflect a lack of motivation or poor physical condition (differential conditioning:  $\approx 6\%$  of 85 ants; absolute conditioning:  $\approx 10.5\%$  of 153 ants).

### **Statistical analysis**

Data were analyzed with R software, version 3.3.2 (R Development Core Team, 2009). The significance level was set at 5%. The requirements for using each statistical test were verified.

*Temperature effect on the mandible opening response (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., 2014). The response (0 or 1 for each stimulation) was used as 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 significant model with the highest explanatory power (i.e. the lowest AIC value). In the selected model, the interaction (*region\*stimulus\*trial*) 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 generalized linear mixed model (GLMM) for binomial data, with a logit link function (*lme4* package). When necessary, models were optimized with the iterative algorithm BOBYQA (Powell, 2009). The MOR (0 or 1 for each trial) was used as response variable. The stimulus (for differential conditioning, CS+ or CS-), the group (for absolute conditioning, paired or unpaired) and the nature of the stimulus (octanal or 1-hexanol) were included as fixed factors. Trials were included as covariate. 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 significant 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 minutes 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

### Temperature effect on the mandible opening response (MOR)

This experiment aimed at determining 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 MOR. Thermal stimulations were applied on 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,  $region*stimulus*trial$ :  $\chi^2 = 25.507$ ,  $df = 3$ ,  $p < 0.0001$ , Fig. 2). Stimulating with heat any of the three body parts resulted in a higher percentage of ants exhibiting MOR compared to the effect of the tactile 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, MOR increased over successive stimulations of increasing temperature, but remained constant and low for tactile controls (Fig. 2).

While 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 differed neither between the ventral abdomen and the hind legs ( $p = 0.15$ ) nor between the ventral abdomen and the dorsal abdomen ( $p = 0.97$ ). On the other hand, 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 vs ventral abdomen*:  $p = 0.37$ ; *dorsal abdomen vs hind legs*:  $p = 0.75$ ; *ventral abdomen vs hind legs*:  $p = 0.98$ ).

Thus, the regions that are more sensitive to thermal stimulation are the hind legs and, to a lower extent, the ventral abdomen. This experiment also shows that a high and comparable level of MOR is attained for both regions from 75°C on. 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 conditioning 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 would be observable if ants exhibit 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:  $\chi^2 = 0.6649$ ,  $df = 1$ ,  $p = 0.41$ ). Moreover, retention levels 10 min after conditioning were also unaffected by the reinforcement contingency both for CS+ ( $\chi^2 = 2.8422$ ,  $df = 1$ ,  $p = 0.091$ ) and CS- responses ( $\chi^2 = 0.50598$ ,  $df = 1$ ,  $p = 0.48$ ). This allowed pooling the results of both subgroups and presenting them in terms of a CS+ vs. CS- discrimination learning (Fig. 3).

Figure 3 shows that ants trained under a differential conditioning regime responded differently to the punished and the unpunished odor along the successive trials (GLMM, significant *stimulus\*trial* interaction:  $\chi^2 = 20.037$ ,  $df = 1$ ,  $p < 0.0001$ , Fig. 3A). Indeed, ants responded differently to the CS+ and to the CS- over the course of the acquisition phase (GLMM: stimulus:  $\chi^2 = 8.34$ ,  $df = 1$ ,  $p = 0.0039$ , Fig. 3A). Precisely, the responses to the CS+ and the CS- differed only in the last trial (GLMM, Tukey's post hoc test, CS+ vs 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 ( $\chi^2 = 22.5$ ,  $df = 1$ ,  $p < 0.0001$ , 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+:  $\chi^2 = 1.81$ ,  $df = 1$ ,  $p = 0.18$ ; CS-:  $\chi^2 = 0.49$ ,  $df = 1$ ,  $p = 0.48$ ) 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*,  $\chi^2 = 1.94$ ,  $df = 1$ ,  $p = 0.16$ ). A significant interaction between trial and treatment (i.e. paired and unpaired groups) was found, thus indicating that experiencing associations between odor and heat determined a different response compared to experiencing the same sensory stimulation in a non-associative way (GLMM: *group\*trial*,  $\chi^2 = 4.76$ ,  $df = 1$ ,  $p = 0.03$ ). Although the odorant chosen as CS did not influence acquisition, it affected retention performances 10 min after

conditioning. The performance varied according to the odor conditioned (unpaired group:  $\chi^2 = 4.08021$ ,  $df = 1$ ,  $p = 0.028$ , paired group,  $\chi^2 = 0.21$ ,  $df = 1$ ,  $p = 0.64$ ). This result precludes pooling performances and favors representing and analyzing them separately, according to the nature of CS (octanal or 1-hexanol).

The performance of ants trained with octanal as CS was compared to 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 responding to odors between the paired and the unpaired group along the successive trials (GLMM:  $\chi^2 = 8.0560$ ,  $df = 1$ ,  $p = 0.0045$ ). Indeed, ants of the paired group increased MOR during trials (from ca. 30% to ca. 60%;  $\chi^2 = 11.289$ ,  $df = 1$ ,  $p = 0.00078$ ) whereas ants of the unpaired group remained at a constant level of responses that oscillated around 30% ( $\chi^2 = 0.5142$ ,  $df = 1$ ,  $p = 0.47$ ). 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 ( $\chi^2 = 0.06$ ,  $df = 1$ ,  $p = 0.8$ ). 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 ten minutes after the last conditioning trial (Fig. 4B) ants experiencing octanal in the paired and the unpaired group did not differ in their response to octanal ( $\chi^2 = 1.1996$ ,  $df = 1$ ,  $p = 0.27$ ). 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 ( $\chi^2 = 0.1387$ ,  $df = 1$ ,  $p = 0.70$ ). Furthermore, their level of response to the novel odor was not different from that to the CS (paired group:  $\chi^2 = 1$ ,  $df = 1$ ,  $p = 0.32$ ; unpaired group:  $\chi^2 = 0.8181$ ,  $df = 1$ ,  $p = 0.36$ ). 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 CS as no significant difference was found between the paired and the unpaired groups during the conditioning phase (GLMM: *group*:  $\chi^2 = 0.134$ ,  $df = 1$ ,  $p = 0.71$ , *group\*trial*:  $\chi^2 = 0.038$ ,  $df = 1$ ,  $p = 0.84$ , Fig. 4C). In this case, the response to the punished odor remained stable in the paired group despite of its association with heat ( $\chi^2 = 0$ ,  $df = 1$ ,  $p = 1$ ). The proportion of ants responding to the CS+ in the last acquisition trial and in the retention test (see below) were not statistically different ( $\chi^2 = 1.58$ ,  $df = 1$ ,  $p = 0.21$ ), thus showing that additional trials would

not necessarily improve learning in this case. As expected, the unpaired group also showed no evidence of learning ( $\chi^2 = 0.0823$ ,  $df = 1$ ,  $p = 0.7742$ ).

The results of the retention test following 1-hexanol conditioning were somehow surprising as ants of the paired group responded more to the CS+ than ants in the unpaired group ( $\chi^2 = 7.7829$ ,  $df = 1$ ,  $p = 0.0052$ , Fig. 4D) despite not showing significant acquisition. Ants of the paired and unpaired groups did not respond differently to the novel odor ( $\chi^2 = 0.1602$ ,  $df = 1$ ,  $p = 0.68$ ) and this level of responses was similar to that of the paired group for the CS (paired group:  $\chi^2 = 0$ ,  $df = 1$ ,  $p = 1$ ; unpaired group:  $\chi^2 = 10.889$ ,  $df = 1$ ,  $p = 0.001$ ). 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.

## DISCUSSION

Our study shows that heat applied on the body surface of carpenter ants *Camponotus aethiops* elicits the typical aversive mandible opening response (MOR) and that 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 stimulation region due 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 to absolute conditioning.

### Heat effect 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 unconditioned stimuli (US) 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 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

*Camponotus aethiops*, in which it induces MOR reproducibly and in an intensity-dependent manner. Applying heat to the body surface triggers 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 MOR at significantly lower and constant levels, showing that heat was a specific trigger of 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 to the dorsal abdomen, similarly to previous observations in bees (Junca et al., 2014). While information about thermal receptors in these areas are still missing, this result suggests that they are indeed present thereon. In natural conditions, high thermal sensitivity to heat in the legs and ventral abdomen could help preventing prolonged contact with particularly hot surfaces, which could happen when ants forage during warm days in their natural southern-Europe 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 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 never been explored up to now. Our results show that MOR can be conditioned efficiently using odors as conditioned stimuli predicting heat as an aversive unconditioned stimulus, 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 determinant to promote a broad spectrum of studies comparing the different circuits and neurotransmitters that mediate both learning forms (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 high level of spontaneous MOR responses along conditioning both for an unpunished CS in differential conditioning and for a CS not contingent with heat in an 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 sec to the hind legs), we obtained substantial rates of conditioned responses at the end of a 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. Not only could 1-hexanol be learned under differential but not under absolute conditioning (compare Figs. 3B and 4B) but also the short-term memory induced by absolute conditioning was weak and non-specific while that induced by differential conditioning was more specific and was even better for 1-hexanol. 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 performances and inconsistent short-term memory retention.

The fact that differential conditioning induces better performances 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 modelling 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 by the CS- odors (Perez et al., 2016). In free-flying bees trained to associate color stimuli with sucrose reward, colors that appeared to be non-discernible 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 that have therefore an associated risk of erroneous non-rewarded or penalized choices, improve significantly 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, promotes 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 to increase the reinforcement contrast.

Our new protocol renders possible studying memory formation and retrieval by testing the ants' response to the CS at different periods post-conditioning, including long-term ones (e.g. 24 and 72h). 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).



## **Aknowledgements**

We are grateful to Jean-Christophe Sandoz for his help regarding the device for thermal stimulation. Many thanks to Paul Devienne for technical assistance and to Margot Perez for advice. This work was supported by the French National Agency grant PheroMod (ANR-14-CE18-0003-03). Martin Giurfa thanks the Institut Universitaire de France for support, the CNRS and the University Paul Sabatier of Toulouse.

## **Author contributions**

PdE, MG and JMD conceived the experiments. LD performed the experiments. DB and LD analyzed the data. All authors wrote the paper and approved the final manuscript.

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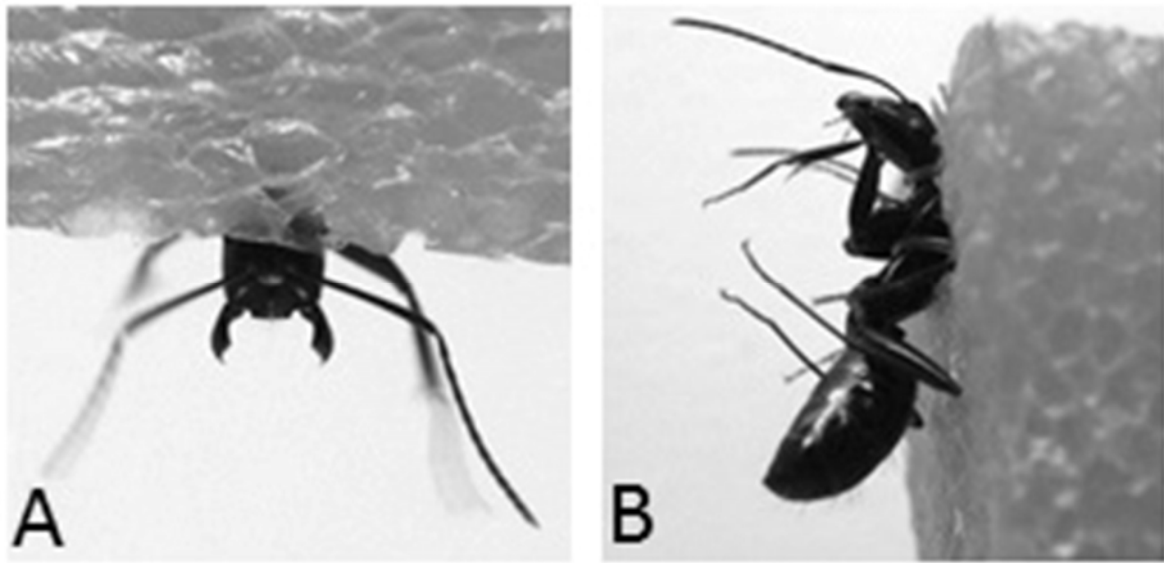
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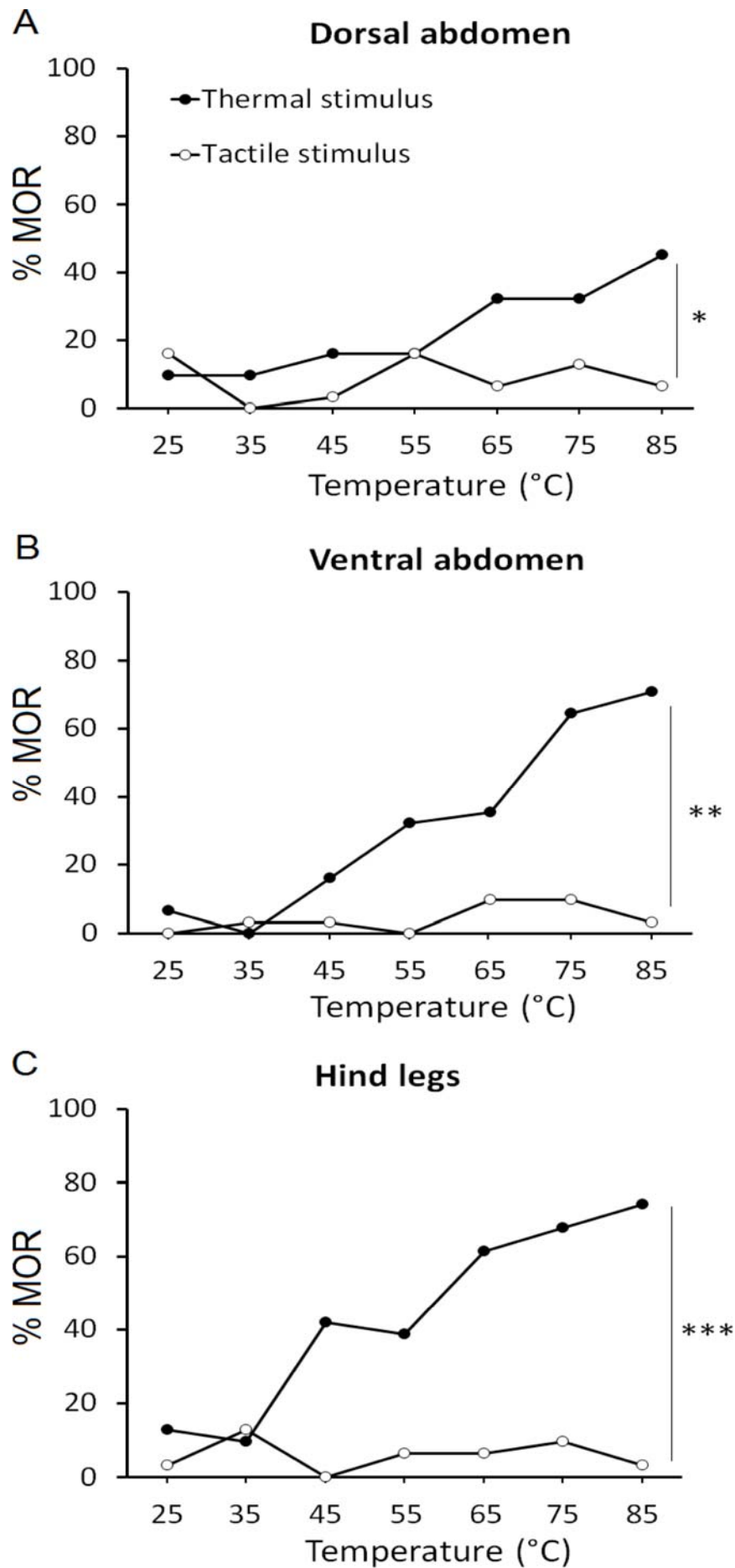
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## Figures

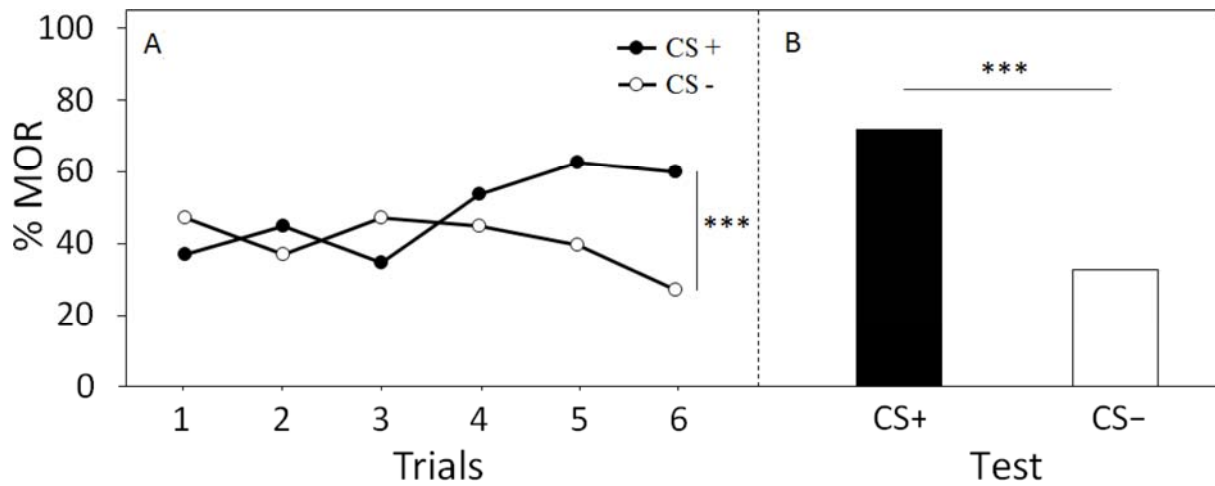


**Fig. 1. Experimental set-up.** **A)** Mandible opening response (MOR) of harnessed *Camponotus aethiops* ants. **B)** Lateral view showing the position of the ant, attached by two strings to the experimental holder made of a foam strip. The ant is fixed vertically, with its abdomen oriented forward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

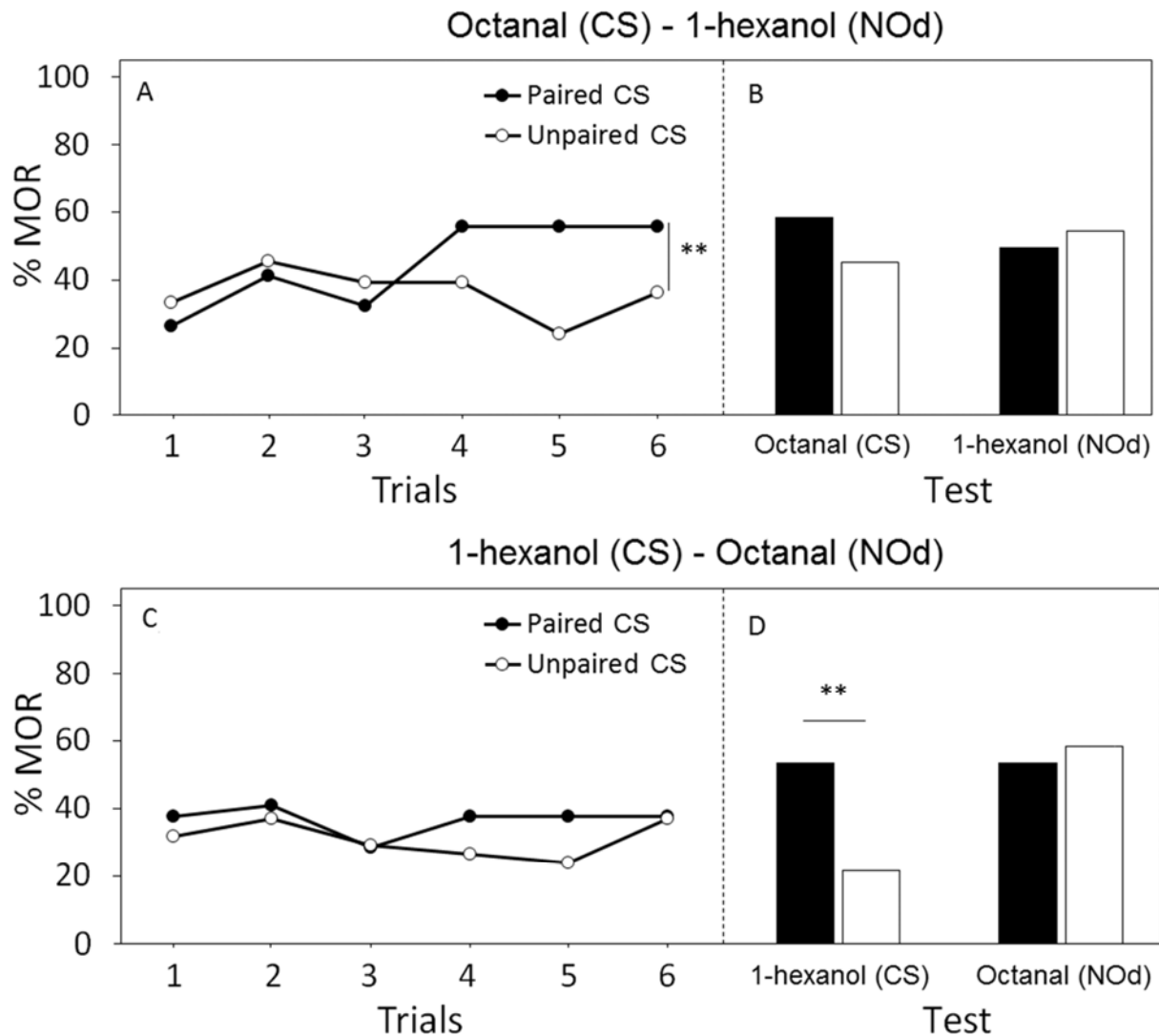




**Fig. 2: Unconditioned MOR: dependence on temperature intensity and localization of the thermal stimulation.** Percentage of ants displaying unconditioned MOR to successive thermal (*black circles*) and tactile (*white circles*) stimulations. Stimulations were applied to **A)** the dorsal abdomen (n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



**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  $75^{\circ}\text{C}$  (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, \*\*\*:  $p < 0.0001$ ).



**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*: \*\*:  $p < 0.0045$ ). (**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:  $n = 38$ .

## SUPPLEMENTARY MATERIAL

### Aversive learning of odor-heat associations in ants

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### Temperature effect on the latency to perform mandible opening response (MOR)

#### *Materials & methods*

The ants were prepared as described in the main text (Fig. 1) and placed individually under a stereoscopic microscope (Leica M80, ocular  $\times 10$ , zoom 8:1). An interval of 20 seconds was established before the presentation of the stimulus to avoid the contextual effects. Then, the ant 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, hind legs), the mandible opening response (MOR) was recorded over a period of 20 seconds in three different groups of ants (one per each body part). This time was considered sufficient to observe a response from all tested ants. The estimation of an average latency to perform MOR for the different temperatures tested and for each body zones allowed quantifying MOR as a binary response.

### *Statistical analysis*

Data were analyzed with R software version 3.3.0 (R Development Core Team, 2009). 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.

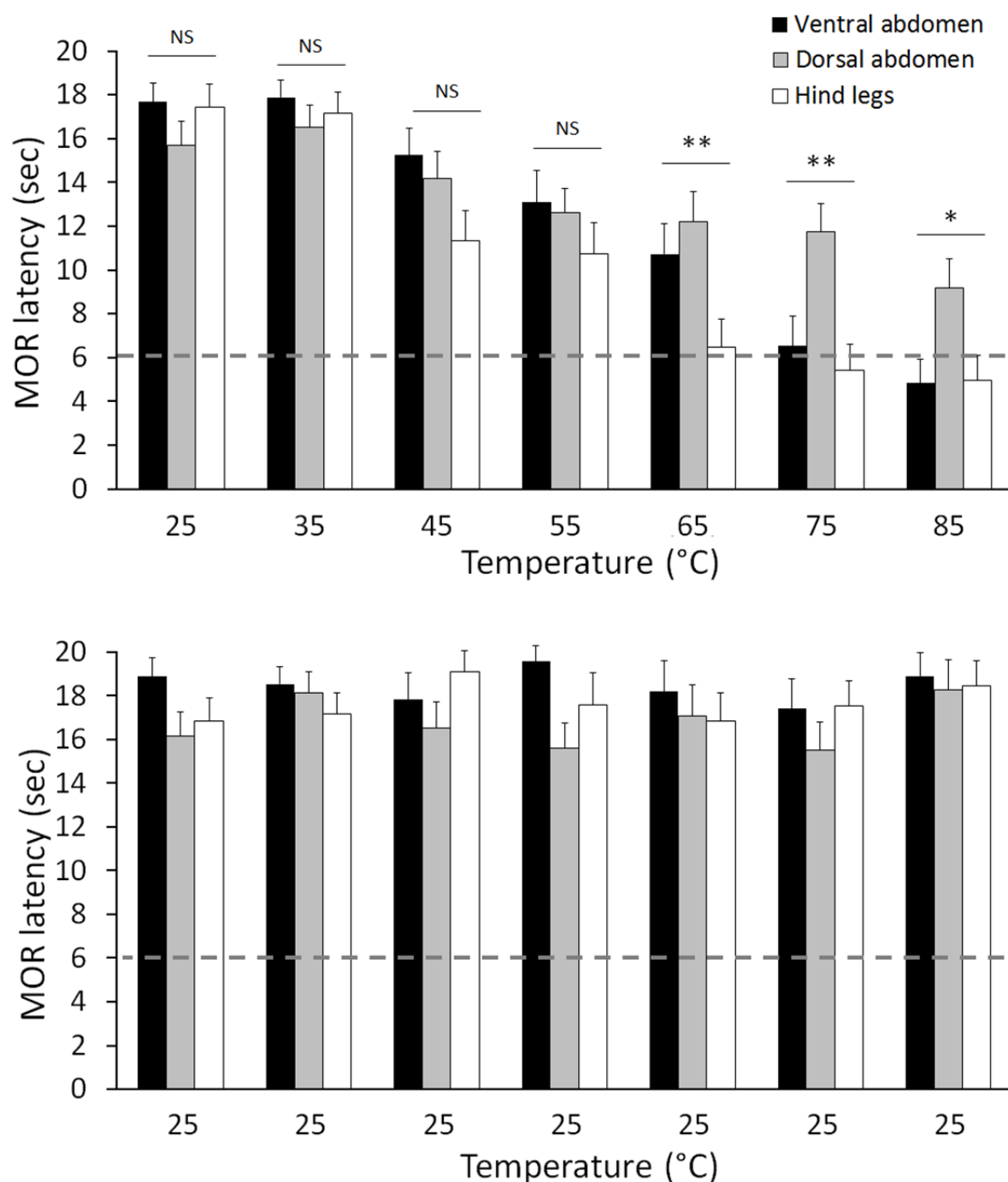
### *Results*

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:  $H = 10.6$ ,  $df = 2$ ,  $p = 0.005$ ), 75°C ( $H = 12.63$ ,  $df = 2$ ,  $p = 0.002$ ) and 85°C ( $H = 9.16$ ,  $df = 2$ ,  $p = 0.01$ ). For these three temperatures, the thermal stimulation of the hind legs triggered the MOR within six seconds (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 test:  $W = 702$ ,  $p = 0.002$ ;  $W = 637.5$ ,  $p = 0.026$ , 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 test:  $W = 719$ ,  $p = 0.001$ ;  $W = 670.5$ ,  $p = 0.01$ , respectively). No significant differences were observed when the ventral abdomen was stimulated (Mann-Whitney test:  $W = 524$ ,  $p = 0.54$ ;  $W = 529$ ,  $p = 0.49$ , respectively). Moreover, during a thermal stimulation at 75°C, the proportion of ants which responded with MOR in six seconds was 67.7% when stimulation was applied to the hind legs, compared with 64.5% and 32.3% when stimulation was applied on 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.



**Figure S1: Latency to perform MOR observed during 20 seconds after stimulation.** MOR latency was noted after **A)** successive thermal stimulations from 25°C to 85°C and **B)** successive tactile stimulations (25°C). The mandible opening response was observed after stimulation of three body parts: the ventral abdomen (n = 31), the dorsal abdomen (n = 31) and the hind legs (n = 31), (Kruskal-Wallis test, \*:  $p < 0.05$ ; \*\*:  $p < 0.005$ ).