

## RESEARCH ARTICLE

# Formic acid modulates latency and accuracy of nestmate recognition in carpenter ants

David Baracchi<sup>1,2,\*</sup>, Martin Giurfa<sup>1,3</sup> and Patrizia d'Ettorre<sup>3,4</sup>

## ABSTRACT

Decision-making processes face the dilemma of being accurate or faster, a phenomenon that has been described as speed–accuracy trade-off in numerous studies on animal behaviour. In social insects, discriminating between colony members and aliens is subject to this trade-off as rapid and accurate rejection of enemies is of primary importance for the maintenance and ecological success of insect societies. Recognition cues distinguishing aliens from nestmates are embedded in the cuticular hydrocarbon (CHC) layer and vary among colonies. In walking carpenter ants, exposure to formic acid (FA), an alarm pheromone, improves the accuracy of nestmate recognition by decreasing both alien acceptance and nestmate rejection. Here, we studied the effect of FA exposure on the spontaneous aggressive mandible opening response (MOR) of harnessed *Camponotus aethiops* ants presented with either nestmate or alien CHCs. FA modulated both MOR accuracy and the latency to respond to odours of conspecifics. In particular, FA decreased the MOR towards nestmates but increased it towards aliens. Furthermore, FA decreased MOR latency towards aliens but not towards nestmates. As response latency can be used as a proxy of response speed, we conclude that contrary to the prediction of the speed–accuracy trade-off theory, ants did not trade off speed against accuracy in the process of nestmate recognition.

**KEY WORDS:** *Camponotus aethiops*, Cognition, Olfaction, Pheromones, Social insects, Speed–accuracy trade-off

## INTRODUCTION

The recognition of group members is important for the evolution of cooperation and the maintenance of social life (Hamilton, 1987). In social insects, discriminating colony members from aliens allows the regulation of appropriately altruistic behaviours without incurring the cost of cooperating with intruders. Moreover, recognizing and reacting promptly to social parasites, robbers or predators is vital for colony success. As a result, insects living in social groups typically excel in discriminating friends and foes (d'Ettorre and Lenoir, 2010). Social recognition systems are based on a multitude of cues from different sensory modalities, among which vision and olfaction play a significant role (Tibbetts, 2002; van Zweden and d'Ettorre, 2010; Baracchi et al., 2016). In ants, recognition systems are predominantly based on the layer of

hydrocarbons coating the cuticle of individuals, which defines the chemical signature of colonies (d'Ettorre and Lenoir, 2010; Bos and d'Ettorre, 2012; d'Ettorre and Lenoir, 2010). Cuticular hydrocarbons (CHCs) constitute a blend of many chemical compounds, mainly linear alkanes, alkenes and methyl-branched alkanes (van Zweden and d'Ettorre, 2010), which vary qualitatively among different species, and quantitatively among colonies of the same species, or even among individuals belonging to different morphological or physiological castes (Vander Meer and Morel, 1998; Monnin, 2006). The sophisticated olfactory system of ants detects CHCs at very short distance (Brandstaetter et al., 2008) and resolves the identity of opponent ants up to the individual level (d'Ettorre and Heinze, 2005), securing the nest from exploiters.

CHCs are not the only chemical cues that mediate social interactions in ants and other social insects. Volatile pheromones are also used to alert colony members to coordinate their defence against exploiters (Blum, 1969; Nouvian et al., 2016). Pheromones are intraspecific chemical messengers that trigger context and signal-specific, adaptive responses (Karlson and Lüscher, 1959; Wyatt, 2014). Their primary function is to convey a message to one or more receivers, thereby eliciting a fast, highly predictable and adaptive response. Yet, pheromones are not just chemical messengers. Recent work has uncovered a novel function for these substances; namely, the modulation of the subjective evaluation of reinforcing stimuli (e.g. reward or punishment). Pheromones can thus modify the responsiveness to aversive or appetitive stimuli (Urlacher et al., 2010; Baracchi et al., 2017, 2020; Rossi et al., 2018). Such a modulatory effect was also detected when *Camponotus* ants were pre-exposed to the alarm pheromone formic acid (FA) in the context of social interactions (Rossi et al., 2019). Despite the fact that FA could eventually be toxic in a defensive context, pre-exposure to FA improved the accuracy of nestmate discrimination by increasing aggressive behaviours towards aliens, while simultaneously decreasing aggression erroneously directed towards nestmates (Rossi et al., 2019). Although the exact neural mechanisms underlying this modulatory action of FA remain to be elucidated, it was suggested that this pheromone modulates attentional processes and thus the sensitivity to recognition cues (Rossi et al., 2019).

Although accuracy is certainly a crucial aspect of any recognition process, the speed of recognition is equally important (Heitz, 2014). Both variables are intimately connected in many decision-making processes (Wickelgren, 1977; Heitz, 2014) and may be traded off as decision accuracy depends on being well informed, which requires time. In contrast, being faster in a decision process could occur at the expense of being accurate. The relationship between speed and accuracy in decision making has been referred to as the speed–accuracy trade-off (Busmeyer and Townsend, 1993). Examples of these trade-offs have been described for several social insect species, in many different ecologically relevant tasks, including foraging, predator detection, prey choice and communication (Wickelgren, 1977; Franks et al., 2003; Ings and Chittka, 2008;

<sup>1</sup>Research Centre on Animal Cognition, Center for Integrative Biology, CNRS, University of Toulouse, F-31062 Toulouse, France. <sup>2</sup>Department of Biology, University of Florence, 50019 Sesto Fiorentino, Italy. <sup>3</sup>Institut Universitaire de France (IUF), 75231 Paris, France. <sup>4</sup>Laboratory of Experimental and Comparative Ethology, University Sorbonne Paris Nord, 93430 Villetaneuse, France.

\*Author for correspondence (david.baracchi@unifi.it)

© D.B., 0000-0003-1308-0612; M.G., 0000-0001-7173-769X

Trimmer et al., 2008; Chittka et al., 2009). For instance, when foraging bumblebees were tested in a colour discrimination task, some individuals made rapid choices but with low precision, while others were slower but highly accurate (Chittka et al., 2003).

Here, we focused on nestmate recognition in carpenter ants and studied whether the alarm pheromone FA affects not only the accuracy (Rossi et al., 2019) but also the speed of this process in an attempt to determine to what extent pheromones act on the trade-off between speed and accuracy. To this end, we pre-exposed individually harnessed ants to FA and quantified afterwards their mandible opening response (MOR) to a glass rod coated with alien (non-nestmate) or nestmate CHCs (Guerrieri and d'Ettorre, 2008). This stereotyped defensive response has already been used to study both within- and between-species aggression in various ant species and aversive associative learning of carpenter ants (Desmedt et al., 2017). We thus determined whether the speed and accuracy of the recognition process are traded off and affected by pheromone pre-exposure.

## MATERIALS AND METHODS

### Study species and housing

We used four queen-right colonies of *Camponotus aethiops* (Latreille 1798) collected in 2016 at Pompertuzat (Midi-Pyrénées, France). Colonies were kept under controlled laboratory conditions (25°C, 12 h:12 h light:dark, ~50% relative humidity). Each colony was housed in a plastic box (26×19×10 cm) with a plaster floor connected by a tube to another box conceived as a foraging arena (26×19×10 cm), containing sand on the floor. The nest box was covered with cardboard in order to make it dark, whereas the foraging arena was exposed to light. 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 twice a week with pieces of cricket and flour worms for proteins and honey/apple mix for carbohydrates and vitamins. Water was provided *ad libitum*.

### Nestmate recognition assay

We designed an experiment to determine whether the alarm pheromone FA (analytical grade, Sigma-Aldrich) modulates the speed and accuracy of the responsiveness to nestmate and non-nestmate odours. On each experimental day, medium size forager ants were gently collected with tiny forceps from the foraging arena of one of the four colonies.

Even if ants from the same colony are in principle not fully independent samples, each ant can be considered as an individual given the proved existence of different behavioural phenotypes (personalities), even within the same behavioural caste in ants (Chapman et al., 2011; d'Ettorre et al., 2017; Carere et al., 2018). Ants were immediately cold anesthetized on crushed ice for a few minutes and individually harnessed in small plastic holders. A small strip of adhesive tape between the head and the thorax was used to immobilize the ants, so that they could only freely move their antennae and mouthparts (Guerrieri and d'Ettorre, 2008). Once harnessed, ants were kept resting for 3 h in a dark and humid place at room temperature (about 60–70% relative humidity, 24±2°C) to let them acclimatize to the new restraining situation. After resting, ants were randomly allocated to either the control group and exposed to pure water (solvent) or the experimental group and exposed to FA.

Before exposure, a first assay (test 1) was performed to quantify basal responsiveness to nestmate and non-nestmate CHCs. Ant responsiveness to these chemicals was quantified using the MOR (Guerrieri and d'Ettorre, 2008). The test entailed eight presentation

trials for each ant: four nestmate trials (either A or B) and four non-nestmate trials (either B or A) in a pseudorandom sequence, such as ABABBABA, so that the same stimulus (A or B) was never presented more than twice consecutively. Stimuli (i.e. alien/nestmate odours) were presented blind to harnessed ants. A 12 min inter-trial interval was used. During each trial, one ant at a time was placed under a stereomicroscope (Leica S8 APO, magnification 10×) in order to better visualize its MOR. Each trial lasted 25 s and consisted of 10 s of familiarization with the experimental context, 10 s of stimulus (nestmate or non-nestmate CHCs) presentation and 5 s of post-stimulus resting in the setup. Each chemical stimulus was presented to the harnessed ant on a glass rod whose tip was previously coated with the CHC extract of either nestmate or non-nestmate ants (see below). The glass rod was carefully manoeuvred by means of a micromanipulator (WPI, M33) to avoid contamination. For stimulation, the rod was always placed at the same distance (2 mm from the head) of the antennae. Each stimulus was preceded by the presentation of a clean rod (presented by hand) in order to familiarize the ants with the visual component of this stimulus.

Then, 15 min after the end of this first assay (test 1), ants were exposed either to FA (experimental group) or to the solvent alone (pure water, control group) to determine whether pheromonal exposure modified their MOR. To this end, harnessed ants were individually confined for 15 min in a 50 ml plastic bottle containing a filter paper (1×5 cm) soaked in either the pheromone or pure water (Rossi et al., 2019). The entire procedure was performed under a hood. FA was diluted to 12% (3 µl pheromone+22 µl water, equivalent to one-third of the content of one poison gland; Stumper, 1952). Control ants were exposed to 25 µl of water. After exposure, ants were allowed to rest for an additional 30 min (Rossi et al., 2019) and then tested again (test 2) for responsiveness to nestmate and non-nestmate odours using the same procedure as in test 1.

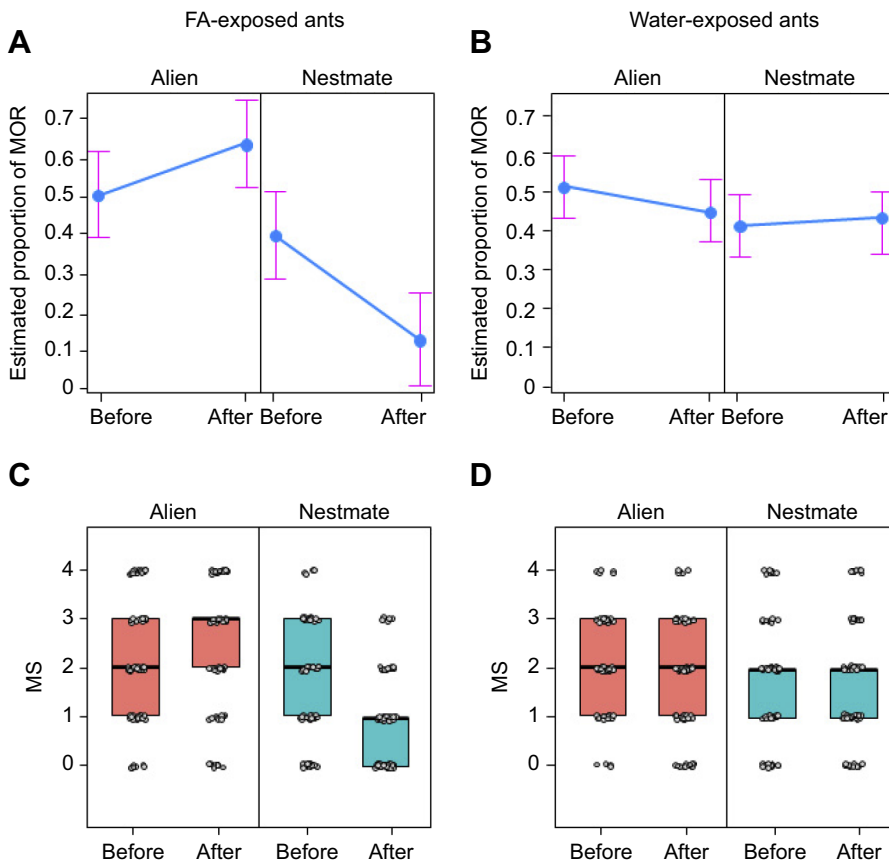
CHC extracts were obtained by washing pools of 5 nestmate or non-nestmate ants in 2.5 ml of solvent (pentane, HPLC grade, Sigma Aldrich) for 10 min (Rossi et al., 2019). The amount of nestmate and non-nestmate odour used in each presentation was equivalent to that of a single ant. The tips of the rods were coated by adding drops of the chemical extracts using a micropipette and the rods were allowed to dry for 1 h before starting the experiment to ensure that the solvent (pentane) had evaporated. To avoid real replicates during the eight presentation trials within each assay (test 1 and test 2), alien and nestmate extracts were obtained from 4 pools of alien and nestmate ants, respectively. In the case of non-nestmates, each pool belonged to a different colony. Each presentation was video recorded from above with an integrated microscope camera. The latency to display the MOR from the moment at which the rod was positioned 2 mm from the head, and the occurrence of the MOR (yes/no) to each stimulus presentation were quantified.

### Locomotor activity assay

In order to determine whether FA merely affected motor responses, thus influencing the observed MOR results, we designed a simple assay to monitor the locomotor activity of free-walking ants, pre-exposed either to FA or to water, which is described in the Supplementary Materials and Methods. The results show that FA neither impaired nor modulated the locomotor activity of ants (Fig. S1).

### Data analysis and statistics

To study the effect of FA in terms of population response, the proportion of reacting ants and the speed of their response to



**Fig. 1. Effect of exposure to formic acid on the accuracy of the mandible opening response to nestmate/non-nestmate odour in carpenter ants.** (A,B) Interaction plots of fitted means for the factors 'Exposure' [before/after exposure to either formic acid (FA) or water] and 'Odour stimulus' (nestmate/non-nestmate odours). Error bars in panels show confidence intervals (95% CI). (A) Mandible opening response (MOR) was differently affected by FA ( $n=69$ ) exposure depending on the nature of the stimulus presented so that it did not increase towards non-nestmate (alien) odour (GLMM, Tukey *post hoc* test:  $P=0.09$ ) whereas it decreased towards nestmate odour (GLMM, Tukey *post hoc* test:  $P=0.0001$ ). (B) Ants exposed to water ( $n=73$ ) did not change their responsiveness either towards nestmates or to aliens (GLMM, Odour stimulus  $\times$  Exposure:  $P=0.27$ ). (C,D) Nestmate and non-nestmate MOR score (MS) of individual tethered ants exposed to either FA or water. Boxes represent median, quartiles and maximum and minimum (upper and lower whiskers) MS values. Grey dots represent individual ants. (C) FA exposure did not significantly increase the MS to non-nestmates ( $P=0.054$ ) whereas it decreased the MS to nestmates (Wilcoxon test,  $P<0.0001$ ). (D) Water exposure did not affect the ants' MS either towards nestmates ( $P=0.76$ ) or to aliens ( $P=0.13$ ).

nestmate and non-nestmate stimulations, after and before exposure, were analysed using ANOVA. For testing accuracy, individual ant responses (MOR: 1 or 0) were examined using generalized linear mixed models (GLMMs) with a binomial error structure – logit-link function – *glmer* function of R package *lme4* (Bates et al., 2015). The speed of the response was analysed using GLMMs fitted with Poisson family distribution and identity link function. Q-Q plots and scatterplots of the residuals of the model were checked visually for normal distribution and homoscedasticity. Water- and FA-treated ants were always analysed separately in two independent tests. In a first analysis, only pre-treatment observations were used (test 1), focusing on the predictor Odour stimulus (nestmates versus non-nestmates). In a second analysis, both pre- and post-treatment observations (test 1 and test 2) were used, focusing on the interaction between Odour stimulus and Exposure (before versus after exposure). Specifically, in the models run to test for nestmate discrimination before water or FA exposure, Ant response was entered as a dependent variable, Odour stimulus (nestmate/non-nestmate extract) as a fixed factor, and Trial as a covariate. In the subsequent models, aimed at testing the effect of water or FA exposure, Ant response was entered as a dependent variable, Exposure (before/after exposure to either water or FA) and Odour stimulus as fixed factors, and Trial as a covariate. In all models, Individual identity (ID) was considered as a random factor to allow for repeated-measurement analysis. Colony of origin was also entered as a random factor. When models failed to converge, they were optimized with the iterative algorithms BOBYQA or Nelder-Mead. In each analysis, several models were run in parallel and compared to identify significant interactions between fixed factors and/or covariates and the significant model with the highest explanatory power (i.e. the lowest Akaike information criterion,

AIC value) was retained. When AIC values were very similar, the most significant and informative model (i.e. the one containing the interaction) was selected. Significant interactions are reported in the text. Tukey's *post hoc* tests were used to detect differences between the different groups (*lsmeans* function from R package *lsmeans*; Lenth and Lenth, 2018).

To study the effect of FA at the individual level, for each tethered ant we calculated a nestmate and a non-nestmate MOR score (MS). The former was quantified as the sum of MORs to the four nestmate presentations while the latter was quantified as the sum of MORs to the four non-nestmate presentations. Thus, both MSs could range from 0 to 4. In the case of nestmates, higher MS values correspond to incorrect responses (i.e. aggressive display towards a nestmate). In contrast, in the case of non-nestmates, higher MS values correspond to correct responses (i.e. aggressive display towards an alien). MSs were calculated for test 1 (before exposure) and for test 2 (after exposure) and compared by means of Wilcoxon signed-rank tests. We also calculated a latency score (LS) for each individual ant presented with nestmate and with non-nestmate CHCs. In the case of nestmates, the LS corresponded to the mean latency of aggressive responses upon the four nestmate odour presentations while in the case of non-nestmates, the LS corresponded to the mean latency of aggressive responses upon the four non-nestmate odour presentations. Both LSs were calculated for test 1 (before exposure) and for test 2 (after exposure) and compared by means of a Wilcoxon signed-rank test. LS was not calculated for ants that did not display any MOR to the four nestmate odours or to the four non-nestmate odours.

Finally, to test for the existence of a latency versus accuracy trade-off in nestmate and non-nestmate recognition, Spearman rank tests were used to correlate MSs for nestmates and non-nestmates with



nestmate and non-nestmate LSs, respectively, after and before FA/water exposure. All statistical analyses were performed with R 4.0.3 (<http://www.R-project.org/>).

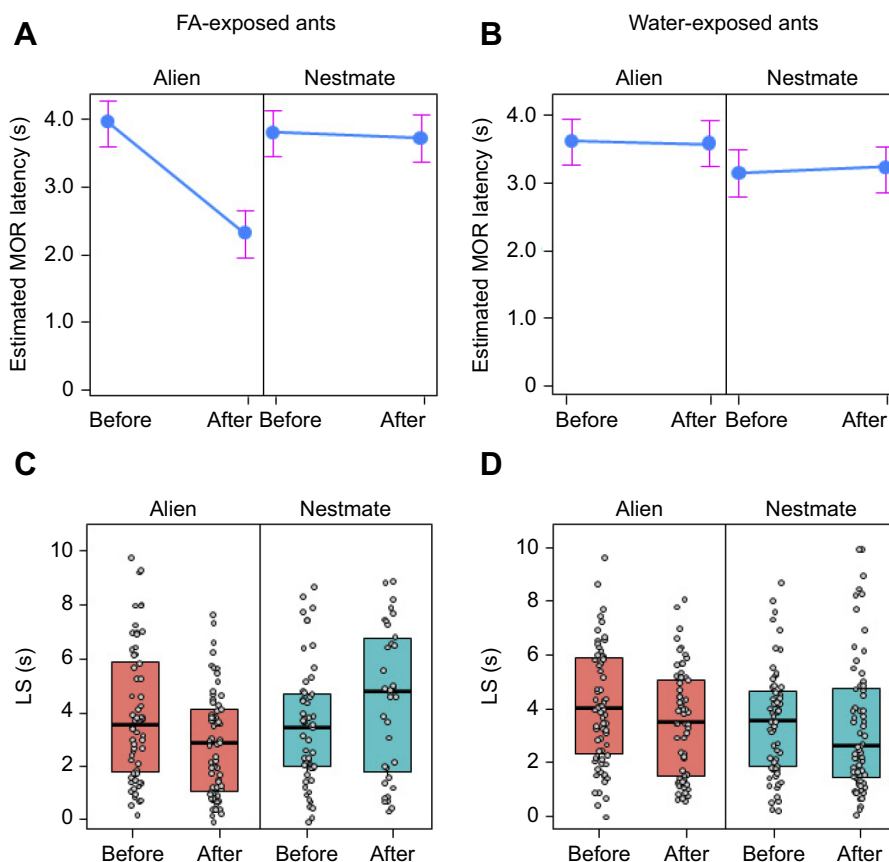
## RESULTS

In natural conditions, medium-size forager ants are typically aggressive towards alien ants and tolerant towards nestmates (Larsen et al., 2016). In the laboratory conditions in which the MOR bioassay was performed, harnessed ants reproduced this behaviour and displayed the MOR to alien CHCs. In test 1 (before pheromone exposure), both FA and water (control) groups, which were in principle identical at this point, reacted more aggressively towards the four non-nestmate presentations than to the four nestmate presentations (Fig. 1A,B: within each ant category, FA exposed and water exposed, compare the two proportions labelled as 'before'). This difference in the proportion of ants responding to either odour was significant (GLMM, water group: Odour stimulus:  $\chi^2=5.63$ , d.f.=1,  $P=0.018$ , Table S1A; FA group: Odour stimulus:  $\chi^2=5.93$ , d.f.=1,  $P=0.015$ , Table S1B; Fig. 1A,B; Fig. S2). In both groups, the proportion of ants showing MOR did not change over the four presentations (GLMM, water group: Trial:  $\chi^2=0.83$ , d.f.=1,  $P=0.36$ , Table S1A; FA group: Trial:  $\chi^2=0.03$ , d.f.=1,  $P=0.87$ , Table S2; Fig. 1A,B; Fig. S1B). Pheromone exposure induced a change in the proportion of ants responding with MOR to nestmate and non-nestmate odours (GLMM, Odour stimulus×Exposure:  $\chi^2=36.35$ , d.f.=1,  $P<0.0001$ , Fig. 1A; Fig. S2, Table S2A). In particular, FA exposure decreased erroneous MOR towards nestmates (GLMM, Tukey *post hoc* test:  $Z=-6.05$ ,  $P<0.0001$ , Fig. 1A) while it increased correct MOR towards aliens, albeit in a non-significant way (GLMM, Tukey *post hoc* test:  $Z=2.32$ ,  $P=0.09$ , Fig. 1A). In contrast, when ants were exposed to water, the

proportion of individuals responding to nestmates and to aliens did not vary significantly (GLMM, Odour stimulus×Exposure:  $\chi^2=1.21$ , d.f.=1,  $P=0.27$ , Fig. 1B; Fig. S2A, Table S2B).

In order to evaluate interindividual variability, we analysed responses in terms of individual MOR scores (MSs), which were computed both for responses to nestmate CHCs (i.e. the sum of responses to the four nestmate trials) and for those to non-nestmate CHCs (i.e. the sum of responses to the four non-nestmate trials). Fig. 1C shows that FA exposure caused a significant decrease of responses to nestmates (Wilcoxon test,  $n=69$ ,  $V=108$ ,  $P<0.0001$ , Fig. 1C) and an apparent increase in responses to non-nestmates, which was not significant (Wilcoxon test:  $n=69$ ,  $V=961$ ,  $P=0.054$ , Fig. 1C). In contrast, exposure to water did not affect the individual MS, either towards nestmates or to aliens (Wilcoxon test, nestmates:  $n=73$ ,  $V=805$ ,  $P=0.76$ ; alien:  $n=73$ ,  $V=572$ ,  $P=0.13$ , Fig. 1D).

At the population level, pheromone exposure induced a change in the mean latency of the MOR elicited by nestmate and non-nestmate odours (GLMM, Odour stimulus×Exposure:  $\chi^2=555.4$ , d.f.=1,  $P<0.0001$ , Table S3A; Fig. 2A,B; Fig. S3). In particular, ants exposed to FA had a shorter MOR latency towards alien CHCs (GLMM, Tukey *post hoc* test  $Z=-23.75$ ,  $P<0.0001$ , Fig. 2A) but did not change the MOR latency to nestmate CHCs (GLMM, Tukey *post hoc* test,  $Z=-0.98$ ,  $P=0.76$ , Fig. 2A). Overall, MOR latency towards alien CHCs decreased over the presentations and tests following FA exposure (GLMM, Trial:  $\chi^2=146.3$ , d.f.=1,  $P<0.0001$ ). In contrast, ants exposed to water did not change the latency of MOR towards nestmates or to alien CHCs (GLMM, Exposure:  $\chi^2=0.17$ , d.f.=1,  $P=0.68$ ; Odour stimulus×Exposure:  $\chi^2=1.01$ , d.f.=1,  $P=0.31$ , Table S3B; Fig. 2B; Fig. S3). Overall, MOR latency increased over the presentations and tests (GLMM, Trial:  $\chi^2=86.1$ , d.f.=1,  $P<0.0001$ ).



**Fig. 2. Effect of exposure to FA on the latency of the MOR to nestmate/non-nestmate odour in carpenter ants.** (A,B) Interaction plots of fitted means for the factors 'Exposure' (before/after exposure to either FA or water) and 'Odour stimulus' (nestmate/non-nestmate odours). Error bars in panels show 95% CI. (A) MOR latency was differently affected by FA exposure ( $n=69$ ) in a stimulus-dependent manner so that it strongly decreased when ants were presented with non-nestmate odour (GLMM, Tukey *post hoc* test,  $P<0.0001$ ) but it did not vary when the same ants were presented with nestmate odour (GLMM, Tukey *post hoc* test:  $P=0.76$ ). (B) After water exposure ( $n=73$ ), ants did not change the latency of MOR towards either nestmates or aliens (GLMM, Odour stimulus × Exposure:  $P=0.31$ ). (C,D) Nestmate and non-nestmate latency score (LS) of individual tethered ants exposed to either FA or water. Boxes represent median, quartiles and maximum and minimum (upper and lower whiskers) LS values. Grey dots represent individual ants. (C) FA exposure decreased the LS to non-nestmates (Wilcoxon test,  $n=56$ ,  $P=0.002$ ) but not to nestmates ( $n=31$ ,  $P=0.12$ ). (D) After water exposure, ants decreased LS towards aliens ( $n=58$ ,  $P=0.043$ ) but not to nestmates ( $n=52$ ,  $P=0.37$ ).

At the individual level, FA exposure significantly decreased the latency score (LS) to non-nestmates (Wilcoxon test:  $n=56$ ,  $V=414$ ,  $P=0.002$ , Fig. 2C) but not to nestmates (Wilcoxon test:  $n=31$ ,  $V=328$ ,  $P=0.12$ , Fig. 2C). After water exposure, ants decreased their LS towards aliens (Wilcoxon test,  $n=58$ ,  $V=593$ ,  $P=0.043$ , but not to nestmates (Wilcoxon test,  $n=52$ ,  $V=566$ ,  $P=0.37$ , Fig. 2D).

We then combined our data on LS and MS to analyse the existence of a speed versus accuracy trade-off. While the quantification of MS provides a measurement of response accuracy when the ants are confronted with alien or nestmate odours, the latency of their response informs about the potential speed of their response; typically shorter latencies are associated with faster responses and higher speed while longer latencies are associated with slower responses and slower speed.

We found that the response latency was not affected by the accuracy of both nestmate and non-nestmate odour recognition in ants before exposure. In particular, individual nestmate MS did not correlate with nestmate LS (Spearman test,  $n=113$ ,  $\rho=0.09$ ,  $P=0.35$ ). A non-significant inverse correlation was found between individual non-nestmate MS and non-nestmate LS (Spearman test,  $n=130$ ,  $\rho=-0.17$ ,  $P=0.053$ ). After exposure to FA or water, no significant correlation was found when we analysed the LSs and MSs of exposed ants to nestmates (FA: Spearman test,  $n=35$ ,  $\rho=-0.19$ ,  $P=0.28$ ; water:  $n=63$ ,  $\rho=-0.05$ ,  $P=0.68$ ) and non-nestmates (FA:  $n=64$ ,  $\rho=-0.19$ ,  $P=0.14$ ; water:  $n=62$ ,  $\rho=0.04$ ,  $P=0.77$ ). Thus, ants did not trade-off these two aspects of the recognition process.

## DISCUSSION

The ability to discriminate between nestmates and intruders allows colony cohesion and nest defence in social insects (Hamilton, 1987). Accuracy is certainly a crucial aspect of the action component of the recognition process. Yet, the speed of the recognition is equally important (Heitz, 2014). Therefore, while the existence of a speed–accuracy trade-off necessarily imposes boundaries, the recognition process is expected to be as accurate and fast as possible (Wickelgren, 1977; Heitz, 2014). Over the course of evolution, the sensory systems of social insects have been strongly refined to achieve this goal (Stroeymeyt et al., 2010; van Zweden and d'Ettorre, 2010; Ozaki and Hefetz, 2014). Pheromones participate in this process as they have been naturally selected to facilitate communication and response coordination at the colony level (Blum, 1969). Alarm pheromones, in particular, coordinate defensive responses of social groups and allow individuals to react promptly with stereotyped responses towards imminent dangers, such as the presence of enemies (Blum, 1969; Nouvian et al., 2016).

In a previous study, we showed that FA, the alarm pheromone of several ant species, acts as a cognitive modulator by enhancing nestmate discrimination in *Camponotus aethiops* ants, even when it is no longer present in the surroundings of the targeted ant (Rossi et al., 2019). In this case, FA exposure also increased aggressive behaviours of ants walking in an arena and confronted with aliens, while it simultaneously decreased erroneous aggression towards nestmates. Here, we used a more controlled setup in which harnessed ants were exposed to CHCs of aliens or nestmates, and we confirmed our previous findings showing nestmate recognition was improved by FA exposure in carpenter ants. In addition, we evaluated the incidence of response latency in this process.

Our new results show that exposure to FA not only made ants more accurate in their aggressive responses but also modulated the latency of these responses. After FA exposure, those ants that still erroneously displayed MOR to nestmate odours did so with the same latency. In contrast, FA exposure reduced the latency of the

MOR towards non-nestmate odours. Thus, FA appears to act as a facilitator that speeds aggression towards the right targets. Most likely, these changes in response latency are relevant in natural scenarios, where faster attacks to non-nestmates would increase the probability of colony success.

The theory of speed–accuracy trade-off (Wickelgren, 1977; Heitz, 2014) predicts that correct decisions take longer while fast decisions are more error prone. Although we did not quantify response speed (i.e. the speed of a triggered MOR), we measured the latency of MOR, which can be used as a proxy of MOR speed (i.e. a shorter latency corresponds to a faster response completion, while a longer latency corresponds to a slower response completion). Our results show that carpenter ants did not trade off speed (latency) against accuracy, either before or after exposure to the alarm pheromone FA. The observed increased accuracy was not affected by the speed of the responses, as FA exposure enhanced both the accuracy and the latency of the responses. Although a trade-off between speed and accuracy has been described in various contexts involving decision making, and in different modalities (Chittka et al., 2009), there are cases in which a correlation between accuracy and sampling time has not been found both in insects (Ditzen et al., 2003) and in mammals facing olfactory discrimination problems (Uchida and Mainen, 2003). Notably, in a study on nestmate recognition by hover wasps, no trade-off between speed and accuracy was found (Baracchi et al., 2015), suggesting that in this particular context a speed–accuracy trade-off may be uncommon.

The increased accuracy in nestmate recognition induced by FA exposure may be explained by an enhanced sensitivity to CHCs (Rossi et al., 2019). It has been proposed that FA increases the amount of information (e.g. the number of detected CHCs) available to the ants, thus decreasing the perceived phenotypic overlap between nestmate and non-nestmate recognition cues (Rossi et al., 2019). Changes in recognition speed, which determined changes in response latency, cannot be explained by changes in motor ability as the general locomotor activity of ants was unaffected by exposure to the pheromone (see Supplementary Materials and Methods). A possibility would be that FA affected attentional processes and enhanced motivation for the defensive task by acting on brain levels of neurotransmitters that have been associated with enhanced attention and aggressive responses. Attentional processes, similar to those described in vertebrates, have been characterized in insects both at the behavioural and neurobiological levels (Dyer and Chittka, 2004; Giurfa, 2004; Miller et al., 2011; van Swinderen, 2011; Van Swinderen and Andretic, 2011). In the fruit fly *Drosophila melanogaster*, visual attention for moving bars is mediated by a transient increase in a 20–30 Hz local field potential recorded in a region of the brain called the medial protocerebrum (van Swinderen and Greenspan, 2003). Current views relate dopamine levels in the insect brain with arousal levels (Van Swinderen and Andretic, 2011). In consequence, attenuation of dopamine release in fly mutants attenuates the 20–30 Hz responsiveness to the visual object. In contrast, a pharmacological increase of dopamine rescues this responsiveness (Andretic et al., 2005). Thus, FA may upregulate dopamine levels in the brain, thereby enhancing attention in the context of nestmate discrimination. This hypothesis is sustained by findings on defensive responses in honey bees, which are triggered by the sting alarm pheromone component isoamyl acetate (IAA) (Tedjakumala et al., 2014; Nouvian et al., 2018). Exposure to IAA increases defensive responses and upregulates dopamine and serotonin levels in the bee brain. While serotonin has been directly related to aggressive responses in invertebrates (Kravitz, 2000; Dierick and Greenspan, 2007; Alekseyenko et al., 2010, 2019;

Alekseyenko and Kravitz, 2014; Tedjakumala et al., 2014), the dopamine component of the response may reflect the enhanced attention required to appropriately direct an attack that may have lethal consequences for the defender bee (Tedjakumala et al., 2014).

In conclusion, we found that FA improved nestmate recognition in *Camponotus aethiops* by acting both on the accuracy (reducing erroneous responses) and on the latency (reducing the latency of appropriate attacks) of aggressive responses. Our behavioural experiments do not allow identification of the mechanism of action of FA, and neural analyses are necessary to determine whether and how exposure to FA upregulates levels of biogenic amines that have been associated with aggressive responses and with attentional processes. Future research aimed at quantifying biogenic amine levels upon FA exposure and at specifically blocking/activating biogenic amine receptors might help to shed light on the underlying mechanisms of FA action. Our findings add to new perspectives developed recently positing that pheromone functions exceed the traditional framework of intraspecific communication for which they have been selected. Pheromones do more than conveying specific messages to members of the same species. In insects, for instance, they can modulate in the long-term responsiveness to relevant stimuli (appetitive, aversive) in contexts that differ from those for which the pheromone is used as a messenger (Baracchi et al., 2017, 2020; Rossi et al., 2018, 2019, 2020; Hostachy et al., 2019; Murmu et al., 2020; Oberhauser et al., 2020). Further studies are needed to clarify these novel functions of pheromones as neuromodulators and to understand their implications for the functioning of recognition systems in general.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: D.B., P.d.; Formal analysis: D.B.; Investigation: D.B.; Data curation: D.B.; Writing - original draft: D.B., M.G., P.d.; Writing - review & editing: M.G., P.d.; Supervision: P.d.; Project administration: P.d.; Funding acquisition: P.d.

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#### Data availability

The datasets generated during this study are available from the figshare repository: <https://doi.org/10.6084/m9.figshare.15035130.v1>

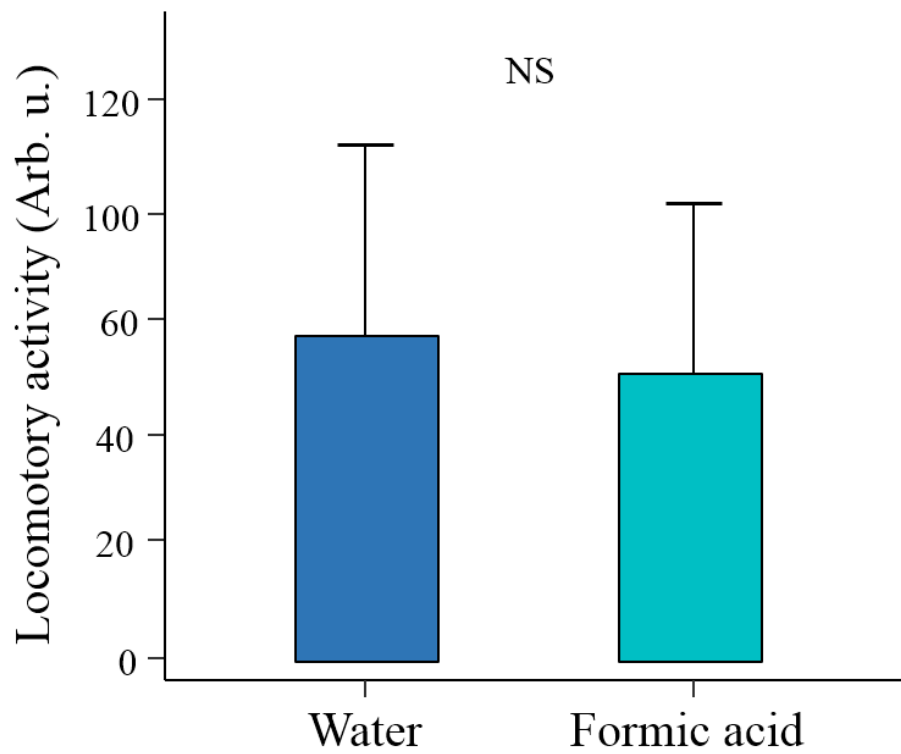
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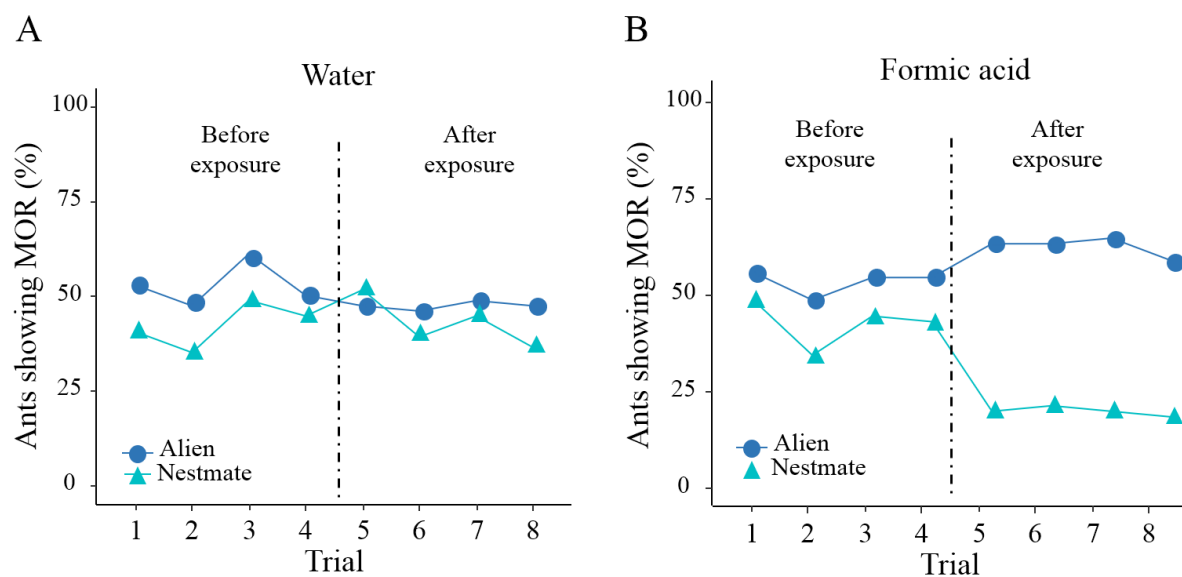


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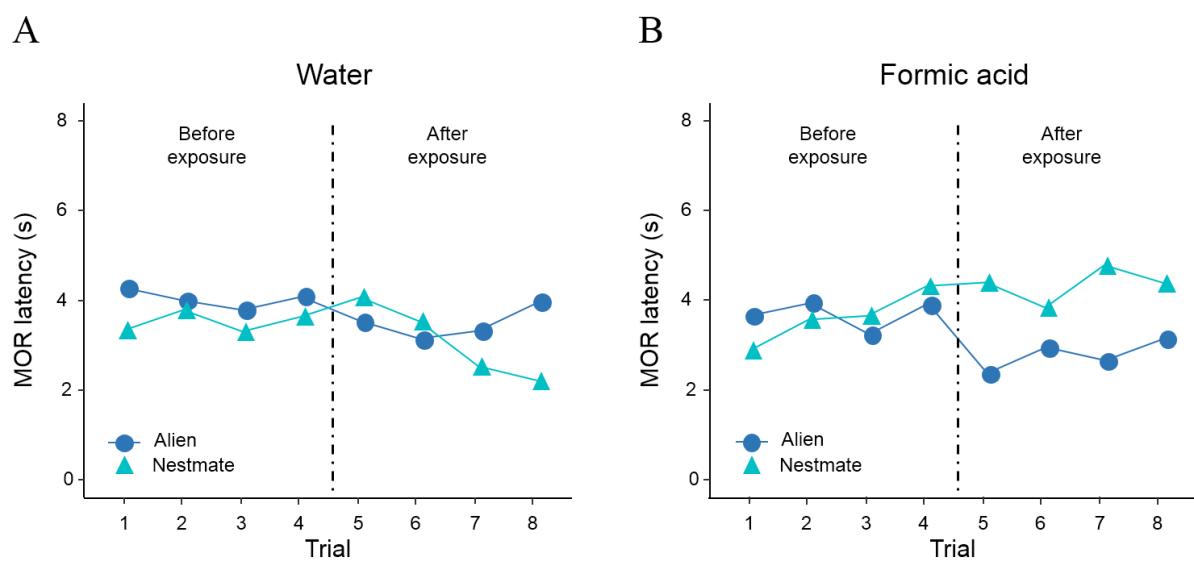


**Fig. S1.** Mean locomotor activity of ants previously exposed to either FA (pale blue) or water (dark blue). Error bars show standard deviation.





**Fig. S2.** Effect of FA and water exposure on MOR accuracy. Percentage of ants showing mandible opening response (MOR) when stimulated with eight presentation trials (four with nestmate odours and four with non-nestmate odours) before and after FA ( $n = 69$ ) or water ( $n = 73$ ) exposure. **(A)** Water neither affected MOR accuracy to nestmate odours nor to non-nestmate odours (GLMM, *Odour stimulus* \* *Exposure*:  $p = 0.27$ ). **(B)** FA made ants slightly more responsive to non-nestmate odours (GLMM, *Tukey post-hoc test*:  $p = 0.09$ ) and significantly less responsive to nestmate odours (GLMM, *Tukey post-hoc test*:  $p < 0.0001$ ).



**Fig. S3.** Effect of FA and water on MOR latency. Mandible opening response (MOR) latency showed by ants when stimulated with eight presentation trials (four with nestmate odours and four with non-nestmate odours) before and after FA ( $n = 69$ ) or water ( $n = 73$ ) exposure. **(A)** Water affected neither the latency of the aggressive response (MOR) to nestmate odours nor to non-nestmate odours (GLMM, *Odour stimulus* \* *Exposure*:  $p = 0.31$ ). **(B)** Ants exposed to FA had a shorter MOR latencies towards alien CHCs (LMM, *Tukey post-hoc test*:  $p < 0.0001$ ) but did not change the MOR latency to nestmate odours (GLMM, *Tukey post-hoc test*:  $p = 0.76$ ).

**Table S1.** (A) Anova output of the GLMM analysis of the Water group' accuracy performance in the test 1 (before water exposure). (B) Anova output of the GLMM analysis of the FA group' accuracy performance in the test 1 (before pheromone exposure).

<b>(A)</b> glmer (resp ~ stimulus + trial + (1   ID) + (1   Col), family = binomial (link = "logit"), data = ASSAY1W, na.action = na.omit, control = glmerControl (optimizer = 'bobyqa'))			
Dep var: <i>resp</i>	$\chi^2$	Df	p-values
<i>Intercept</i>	0.1169	1	0.68286
<i>Stimulus</i>	5.6301	1	0.01765*
<i>Trial</i>	0.8292	1	0.36251
<b>(B)</b> glmer (resp ~ stimulus + trial + (1   ID) + (1   Col), family = binomial (link = "logit"), data = ASSAY1FA, na.action = na.omit, control = glmerControl (optimizer = 'bobyqa'))			
Dep var: <i>resp</i>	$\chi^2$	Df	p-values
<i>Intercept</i>	0.3393	1	0.56022
<i>Stimulus</i>	5.9364	1	0.01483*
<i>Trial</i>	0.0287	1	0.86556

**Table S2.** (A) Anova output of the GLMM analysis of the FA group' accuracy performance before and after pheromone exposure. (B) Anova output of the GLMM analysis of the Water group' accuracy performance before and after water exposure.

<b>(A)</b> glmer (resp ~ Exposure + Stimulus + Exposure:Stimulus + (1   ID) + (1   Col), family = binomial (link = "logit"), data = LongdataFA, na.action = na.omit)			
Dep var: <i>resp</i>	$\chi^2$	Df	p-values
<i>Intercept</i>	6.0444	1	0.01395
<i>Exposure</i>	5.3857	1	0.02030
<i>Stimulus</i>	99.6940	1	< 0.0001
<i>Exposure*Stimulus</i>	36.3515	1	< 0.0001
contrast	estimates	z-ratio	p-values
<i>after A - before A</i>	0.433	2.321	0.0933
<i>after A - after N</i>	2.138	9.985	< 0.0001
<i>after A - before N</i>	0.877	4.658	< 0.0001
<i>before A - after N</i>	1.705	8.126	< 0.0001
<i>before A - before N</i>	0.444	2.399	0.0772
<i>after N - before N</i>	-1.260	-6.048	< 0.0001
<b>(B)</b> glmer (resp ~ Exposure + Stimulus + Exposure:Stimulus + (1   ID) + (1   Col), family = binomial (link = "logit"), data = LongdataW, na.action = na.omit)			
Dep var: <i>resp</i>	$\chi^2$	Df	p-values
<i>Intercept</i>	1.1472	1	0.2841
<i>Exposure</i>	1.9195	1	0.1659
<i>Stimulus</i>	0.6138	1	0.4334
<i>Exposure *Stimulus</i>	1.2108	1	0.2712



**Table S3. (A)** Anova output of the GLMM analysis of the FA group' latency of the MOR before and after pheromone exposure. **(B)** Anova output of the GLMM analysis of the Water group' latency of the MOR before and after water exposure.

<b>(A)</b> glmer (resp ~ Exposure + Stimulus + trial + Exposure:Stimulus + (1 ID), family = poisson, data = Longdata1FA, na.action = na.omit)			
Dep var: <i>resp</i>	$\chi^2$	Df	p-values
<i>Intercept</i>	3610.95	1	< 0.0001
<i>Exposure</i>	563.99	1	< 0.0001
<i>Stimulus</i>	749.95	1	< 0.0001
<i>Trial</i>	146.34	1	< 0.0001
<i>Exposure *Stimulus</i>	555.39	1	< 0.0001
contrast	estimates	z-ratio	p-values
<i>after A - before A</i>	-0.5348	-23.749	< 0.0001
<i>after A - after N</i>	-0.4612	-27.385	< 0.0001
<i>after A - before N</i>	-0.4854	-21.204	< 0.0001
<i>before A - after N</i>	0.0737	3.000	0.0144
<i>before A - before N</i>	0.0494	3.599	0.0018
<i>after N - before N</i>	-0.0242	-0.975	0.7637
<b>(B)</b> glmer (resp ~ Exposure + Stimulus + trial + Exposure:Stimulus + (1 ID), family = poisson, data = Longdata1W, na.action = na.omit)			
Dep var: <i>resp</i>	$\chi^2$	Df	p-values
<i>Intercept</i>	6874.0159	1	<0.0001
<i>Exposure</i>	0.1724	1	0.6780
<i>Stimulus</i>	63.7745	1	<0.0001
<i>Trial</i>	86.0800	1	<0.0001
<i>Exposure *Stimulus</i>	1.0147	1	0.3138

## Supplementary Materials and Methods

### *Locomotor activity assay*

Medium size forager ants were gently collected from the foraging arena of their colony. Ants were immediately exposed either to 25  $\mu$ L of formic acid (FA) (12 % in water) or 25  $\mu$ L of the solvent alone (pure water). To this end each ant was individually confined in a small glass vial (3 ml) glued in the middle of a 50 ml plastic flasks containing a filter paper soaked either with FA or water. The outer part of the glass vial was coated with Fluon® so that the ant was free to move inside the vial but without any possibility to escape and enter in direct contact with the pheromone/water. Immediately after the insertion of the ant and the pheromone/water into the device, the 50 ml flask was closed and placed under a hood. The exposure lasted 15 mins. After exposure, each ant was moved into a Fluon-coated ring (h = 3 cm,  $\varnothing$  = 2.2 cm) positioned in the middle of a testing arena (h = 5.3 cm,  $\varnothing$  = 8 cm) with Fluon-coated walls. After a 30-minute resting time (the same delay used in the MOR experiments), the small cylinder was removed, and the ant was free to explore the arena for additional 30 minutes. The floor of the arena was covered by a clean filter paper with 2 orthogonal drawn lines passing for the centre and delimiting four quadrants. All trials were videotaped. A total of 24 ants from two different colonies were tested (12 ants exposed to FA and 12 ants exposed to water). The number of times each ant crossed the lines was recorded and used as a proxy of locomotor activity. The results show that FA did not impair nor modulate the locomotor activity rate of ants (Two-Sample t test,  $p = 0.64$ ).