Université Fédérale
Toulouse Midi-Pyrénées

THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)

Présentée et soutenue par :
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le jeudi 27 septembre 2018

Titre :

Pheromonal modulation as a drive for behavioral plasticity in two insects: honey bees and ants

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École doctorale et discipline ou spécialité :

ED BSB : Neurosciences

Unité de recherche :

Centre de Recherches sur la Cognition Animale (CRCA)

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Acknowledgments

I thank Martin Giurfa and Patrizia d'Ettorre for hiring me and for all the gates you opened to me during this thesis. Thank you both for provided me unrestrictedly finance support in terms of equipment and trips (to Paris, Buenos Aires, and congresses). Thank you Patrizia for continuously keeping in touch with me despite my reclusive personality and the distance between our labs. I didn't realize at that time but you are probably the one who saved me from my perfectionist obsession at the end of my first year. Thank you for the opportunity of collaboration with Roxana Josens you gave me and thank you for forcing me to take a step back from the PheroMod project, allowing me to be a little more creative, think aside of the project, and really appropriate my thesis as being mine and not just a project in which I was employed. Thank you for your humanity and for caring about your students, it really meant something to me and I now believe you when you said that you sometimes cry for your students as a supervisor because of your commitment to them, I really value that. Finally, thank you for always pushing me. Thank you Martin for the simple fact of integrating me in your lab, which allowed me to benefit from your aura as an internationally reputed researcher. Thank you for your investment in your corrections and scientific discussions. Thank you for the autonomy you gave me and the environment you indirectly provided me from which I learned a lot. Thank you for the valuable scientific and human spirit you injected in the lab and particularly in the Explain team, I really valued the quality of the invited speakers in the lab, as well as the different celebrations you instigated, which allowed to strengthen the links between colleagues in order to do good science (I hope) among friends.

I thank Roxana Josens from the University of Buenos Aires for allowing me to come in her lab to collaborate on a project on Argentine ants. Thank you for your kindness and warmth as well as for your sound scientific supervision. Thank you also for supporting my applications for post-doc grants, despite the complications…

I thank the examiners of this thesis, Sylvia Anton and Maria Cristina Lorenzi, for investing their time in reading and commenting my work, as well as for coming to Toulouse to discuss it during my PhD defense. I also thank Giovanni Galizia and Vincent Fourcassié for kindly accepted to be part of my PhD jury.

I thank the members of my PhD committee, Julie Batut and Claudio Lazarri, for their true commitment to me.

I thank David Baracchi, my post-doc collaborator in this project, for valuable feedbacks and discussions, as well as for all the collaborative work we did together in terms of developing set-ups, experiments, paper writing and so on.

I thank all the technicians, engineers, and administrative people of the CRCA and the LEEC. A special mention to Patrick Arrufat for his help and advices regarding the electrical devices I used along my PhD and for his constant good mood. I also thank Lucie Hotier for teaching me the bases of bee collection and discussions regarding beekeeping. Thank you for helping me collecting bees at the beginning of my PhD and for the barbeques in the apiary! I would also like to thank Gérard Latil for the passionate talks about ants and because he kindly agreed to share his equipment with me (besides feeding my ants every time I was away or, lest be honest, when I forgot). I thank Paul Devienne for his weirdness which brought a little bit of joy in the LEEC. Thank you as well for your assistance with the equipment and ants. I thank Chloé Leroy for her kind assistance with the GC-MS. I also thank Maud Comb and Alain Le Négrate for assistance with informatics. Finally, I thank Christine Renault for always standing for the students and dealing with all my missions.

I thank all the people of the LEEC for their kindness, and especially Heiko Rödel, Stéphane Chameron, Gilles Gheusi, Fabrice Savarit and Baptiste Piqueret for scientific inputs. I also thank all the people of the lab in Buenos Aires for their help and care: Walter, Diego, Cecilia, Flor, Sol, Ali, Maru, Andres, Emilia, y Alejo.

Thanks to the students who helped me with my experiments: Mélanie Picot, Mélanie Bazin, Alexia Bernadt, Elodie Navailles, and Jim Sigailov. I hope I wasn't too much of a mess as a supervisor.

I would like to emphasize the importance of communicating between teams and sharing points of view. I was a big fan of the doc/post-doc meetings we used to have every Tuesday evening and I think it's a shame that this great collective working spirit is not continuing. For that, I particularly thank Valentin Lecheval, David Vogel, Rémi Gouttefarde, Kévin Berthelot, Guillaume Fumery, Manon Azaïs and Patrick Schultheiss. Thank you for your inputs, especially Valentin, David and Manon for the discussions and help regarding statistics.

In the same spirit, I would like to thank the members of the Rainbio association we created in order to promote the students of the CRCA: Alexis Buatois, Vanessa Cattaud, Amélie Cabirol, Rémi Gouttefarde, Kévin Berthelot, David Vogel, Hélène Villain, and Petnoi

Petsophonsakul. I therefore thank the PhD students and post-docs who took over: Violette Chiara, Aurélie Muria, Maru Villar, Benjamin Portal, Christophe Rey, and Matthias Durrieu.

I obviously thank my current and former office colleagues for their great personalities and the fun we had: João Robazzi, Grégory Lafon, Beli, Morgane Nouvian and Paul Marchal.

A special thanks to Margot Perez, my "old sister" (same co-supervisors), for all the advices you provided me and all the great scientific discussions we had, you helped me a lot! All your suggestions always changed the game. In the same way, thanks to Lucie Desmedt who took the time to train me to the aversive conditioning in ants and always responded to my mails. Thanks to Rio Tedjakumala who kindly tried to teach us how to inject bees despite our insolence.

I thank my former partners in party/crime for the lot of fun we had: Pierre Lesne, Ugo Lopez, David Vogel, Kévin Berthelot, Rémi Gouttefarde, Margot Perez, Raphael, Valentin Lecheval, Bertrand Jayles, Morgane Nouvian, Simon Klein, Isabelle Massou and Amélie Cabirol. As well as my current ones: Antoine Wystrach, Cristian Pasquaretta, Mathieu Lihoreau, Tamara Gomez, Sabastian Schwarz, Aurélie Muria, Maru Villar, Hugo Merienne, Benjamin Portal, Iago Sanmartin Villar, and the two Manon.

A special thanks to Maru Villar for her genuine human qualities. Thank you for your interest and the feedbacks you gave me scientifically speaking but, most of all, thank you for being my friend.

Finally, a special mention for my PhD twin Alexis Buatois, without whom the writing of this thesis would have been much harder. Thank you for making me laugh every day, for all the discussions we had, for your creativity and for always having a 1000 projects in mind.

Thank you all for the great social environment you provide/provided each day, which always made me happy to get up and go working, which I think is a gift.

Contents

PREFACE

The chapters of this thesis constitute a set of papers ready for submission, submitted or published in peer-reviewed journals.

Publications included in this thesis

Rossi, N., d'Ettorre, P., & Giurfa, M. (2018). Pheromones modulate responsiveness to a noxious stimulus in honey bees. *Journal of Experimental Biology*, *221*(5), jeb172270.

- Included in Chapter 1. NR, PdE and MG designed the experiment – NR conducted the experiment – NR analyzed the data – NR, PdE and MG wrote the paper.

Rossi, N.*, Baracchi, D.*, Giurfa, M., & d'Ettorre, P. (2018). Pheromone-induced accuracy of nestmate recognition in carpenter ants: Simultaneous decrease of Type I and Type II errors. *The American Naturalist* (accepted). [*Co-first authors]

- Included as Chapter 2. NR, DB, MG and PdE designed the experiments – NR and DB conducted the experiments – NR and DB analyzed the data – NR, DB, MG and PdE wrote the paper.

Conference presentations during PhD

(Poster) Rossi, N., d'Ettorre, P., & Giurfa, M. (2017). An alarm pheromone increases responsiveness to aversive stimuli in the honey bee (*Apis mellifera*). 18ème rencontre du Club de Neurobiologie des Invertébrés, June 08-09, Dijon, France.

(Poster) Rossi, N., d'Ettorre, P., & Giurfa, M. (2018). Pheromones modulate responsiveness to a noxious stimulus in honey bees. The International Congress of Neuroethology (ICN2018), July 15-20, Brisbane, Australia.

(Poster) Josens, R., Rossi, N., Moauro, M.A., d'Ettorre, P. (2018). Sugar responsiveness varies with a synthetic trail pheromone pre-exposure in the ant *Linepithema humile*. X Congreso Argentino de Entomología, May 21-24, Mendoza, Argentina.

(Oral) Rossi, d'Ettorre, P., & Giurfa, M. (2016). Pheromones as general modulators of insect behavior. CBI symposium, January 15th, Toulouse, France.

(Oral) Rossi, N., Baracchi, D., Giurfa, M., & d'Ettorre, P. (2017). Formic acid makes ants better at discriminating. 2ème symposium des étudiants du CRCA, September 28th, Toulouse, France.

(Oral) Rossi, N., Baracchi, D., Giurfa, M., & d'Ettorre, P. (2018). Pheromone-induced accuracy of nestmate recognition in carpenter ants: Simultaneous decrease of Type I and Type II errors. International Union for the Study of Social Insects (IUSSI2018), August 5-10, Guarujá, Brazil.

ABBREVIATIONS

2H: 2-Heptanone

5-HT: Serotonin

AL: Antennal lobe

cAMP: cyclic adenosine monophosphate

CHC: Cuticular hydrocarbon

CN: Centrifugal neuron

CS: Conditioned stimulus

DA: Dopamine

EN: Extrinsec neuron

FA: Formic acid

HVA: Homovanillyl alcohol

IPA: Isopentyl acetate

LH: Lateral horn

MaLER: *Maxilla-labium* extension response

MB: Mushroom body

MGC: Macroglomerular complex

MOR: Mandible opening response

MP: Mammary pheromone

OA: Octopamine

OL: Optic lobe

OR: Olfactory receptor

ORN: Olfactory receptor neuron

PER: Proboscis extension response

PN: Projection neuron

QMP: Queen mandibular pheromone

SAP: sting alarm pheromone

SER: Sting extension response

US: Unconditioned stimuli

VUMmx1: ventral unpaired median neuron of the maxillary neuromere 1

INTRODUCTION

Behavioral plasticity

Nowadays, particular attention is paid to phenotypic plasticity because of human-related changes in the environment that lead living organisms to either adapt or die. Phenotypic plasticity is the property of a given genotype to produce different phenotypes in response to distinct environmental conditions (Pigliucci, 2001). This includes changes in all sets of observable traits of an organism, from morphology, to development, physiology, life history, and behavior. Behavioral plasticity differs from the other forms of plasticity in the sense that it only concerns animals. Human-induced environmental changes being rapid at the scale of evolution and placing organisms in novel and variable conditions, behavioral plasticity might be particularly observed given that it allows rapid adaptation contrary to developmental plasticity for instance that is quite slow because it requires the growth of some cells, tissues or organs. Included under the heading of behavioral plasticity are adjustment, learning, memory and changes in adult behavior as a result of experience during development (Binder et al., 2008).

Snell-Rood (2013) distinguished developmental from activational behavioral plasticity. Developmental behavioral plasticity is defined as a genotype expressing different developmental trajectories leading to behavioral phenotypes in different environments. It generally targets behaviors resulting from an underlying network having been the subject of costly changes at the level of the brain or muscles for instance. Activational behavioral plasticity on the opposite requires a pre-existing machinery/network. These processes differ in their costs and benefits and therefore in the types of environment they are more likely to evolve. Developmental behavioral plasticity, although being a slow process, has the benefit of often being related to changes in other traits of the phenotype for a more integrated plasticity in a given environment. This type of plasticity is more likely to evolve in environments changing across generations but not within a generation. Indeed, since this plasticity is hardly reversible, it would be too costly for an individual to grow and develop features in particular conditions, hence being particularly adapted to this specific environment, to then suffer unsuitability every time the conditions change. On the other hand, activational behavioral plasticity is a fast and reversible response particularly suited to changing environments during a lifespan (e.g; non-migratory, temperate species that are subjected to seasons).

Reflex behavioral responses and learning

We will concentrate in this thesis on two forms of behavioral plasticity concerning reflex behavioral responses and learning, the two being inter-correlated. Learning is a broad term designating associative and non-associative forms of learning, both consisting in acquiring new skills in order to be able to cope with the environment. Non-associative learning refers to habituation, the decremental response to a repeated stimulation, and it's exact opposite, sensitization, the incremental response to a repeated stimulation. Both cases have been studied in *Aplysia californica*: repeatedly touching the siphon of the individual led to the decrement of the gill-withdrawal reflex (Pinsker et al., 1970) but this defensive reflex could be sensitized if the animal was previously given noxious stimuli each day (Pinsker et al., 1973). These famous studies provided a system for analyzing the neural mechanism of behavioral modifications of intermediate complexity.

While habituation and sensitization involve acquiring and remembering information of a single stimulus, associative learning consists in establishing a relationship between two stimuli: a predictive one that can either be neutral (Pavlovian conditioning), or related to the animal's own behavior (operant conditioning), and another one eliciting reflex responses. In Pavlovian conditioning, the animal has to learn to associate a neutral stimulus yielding no specific response before learning (the conditioned stimulus, CS), and a biologically relevant one inducing characteristic reflex responses (the unconditioned stimulus, US). For this link between CS and US to be established, the two have to be presented with an overlap, and the CS should precede the US so as to predict the latter. If the animal has learned, the behavior usually observed during the US should be displayed to the CS alone (Fig. 1). This process reflects a change in the internal representation of the CS as it acquires a new significance for the animal. It should be emphasized that learning involves reflex behavioral responses that are associated with other stimuli, the association of the two leading to an acquired behavior.

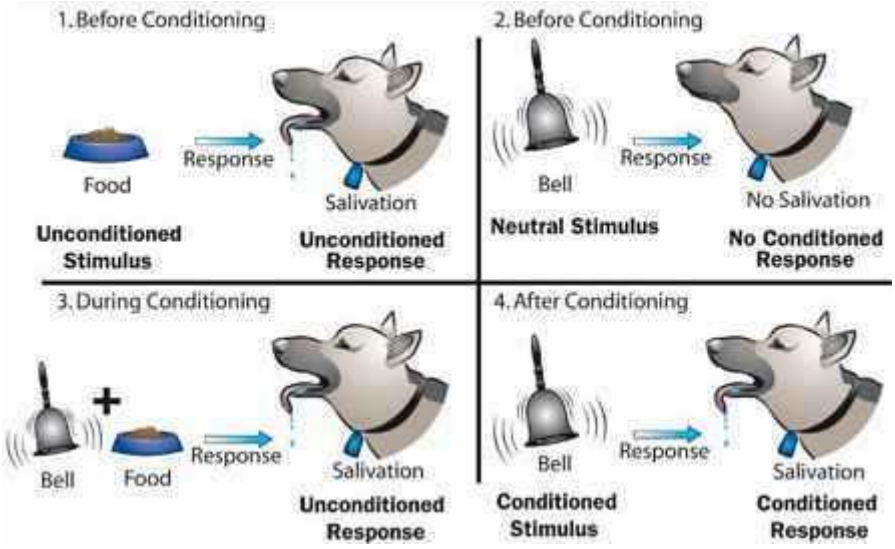

Figure 1: The famous example of dog's Pavlovian acoustic conditioning of the salivation response (Source: psychologywizard.net).

Modulation of behavioral plasticity

Learning can be modulated by varying the relationship between the CS and the US, through intensity, duration or overlap between the two stimuli. Besides affecting this relation, a plethora of factors can directly impact the reflex behavioral response (US) involved in learning by modulating the internal state of the animal. This involves the satiation state of the animal, its level of stress, immune system, physiological state, age, genotype, or balance of nutrients (non-exhaustive list). But external factors can also modulate reflex responses, as for instance stimuli providing a given context (appetitive, aversive, social…). A good example of modulation of innate behaviors by both internal and external factors has been studied in *Drosophila melanogaster*. Indeed, in this insect, external fruit odors can suppress the innate aversion for CO_2 which is usually released by agitated fruit flies but can also be released by ripe fruits which signal a potential mating site. In this case, the same component (CO_2) is evaluated differently by the fly according to the context in which it is perceived. In the case of internal factors, satiety regulates feeding behavior by enhancing olfactory and gustatory sensitivity and by regulating internal nutrient sensors (Su and Wang, 2014).

Recently, some pheromones have been shown to play a role as "modulators" of cognitive phenomena, such as associative learning (Coureaud et al. 2006; Bredy and Barad 2009). Pheromones are chemical substances released into the environment by an individual, which trigger stereotyped behaviors and/or physiological processes in individuals of the same species. This modulation corresponds to a previously unknown effect of pheromones as it targets behaviors that are different from the stereotyped responses typically controlled by a given

pheromone. In vertebrates, Bredy and Barad (2009) conditioned demonstrator mice to freeze when listening to a white-noise recording associated with electric shocks. They subsequently exposed congener observer mice to cotton balls impregnated with the urine of the demonstrator and showed that observer mice succeeded less in establishing the relationship between the white-noise and the electric shock. They attributed this effect on a putative stress-related anxiogenic pheromone released by the stressed familiar mouse which would have a decremental effect on the aversive conditioning of another mouse. Pheromone modulation of learning has also been reported in newborn rabbits in which exposure to a mixture of mammary pheromone (MP) and an initially neutral artificial odorant triggers a strong searching–grasping response when re-exposed to the odorant alone 24 h later (Coureaud et al., 2006). In other words, newborn rabbits then responded to the initially neutral odorant in the same way as to MP. In less controlled conditions, Denenberg and Landsberg (2008) showed that dog-appeasing pheromone increased learning performance in classes consisting of teaching puppies how to adjust to a new household in a manner that is acceptable to the family (training, socialization, and habituation to new stimuli).

Internal and external factors probably act on the attentional and/or motivational state of the animal, changing the subjective evaluation of what it perceives (e.g. the hedonic value it attributes to the US). Behavioral performance is determined to a large degree by an animal's attention and/or motivation. An optimal arousal level is required for proper cognitive and motor performance, and it is the result of an interaction between mechanisms controlling endogenous states and stimuli from the environment (Andretic et al., 2005).

Motivation and attention systems

There is little agreement among researchers to define motivation. For some, it is a generic term used for a variety of biological functions (hunger, thirst, sexual arousal…) which lead to the execution of a behavior toward specific goals (food, water, mates…). For others, it is the degree of activation or arousal in the nervous system (Grossman, 1979). I will not solve this problem of definition here and will refer to motivation as both as they seem to be complementary.

Internal drives of motivation often reflects part of a homeostatic system. For example, when an animal starts to be hungry there is a gradual increase in the motivation (or drive) to eat, with sensory inputs from the gut, changes in circulating hormones (e.g., insulin, leptin, ghrelin), changes in circulating nutrients, as well as intracellular metabolites, which signal "hunger" or "satiety," and thus modulate the motivation to eat. As internal signals of energy

deficiency increase, the animal's attention becomes directed toward the goal of obtaining food. After feeding to satiety, motivation abates and then gradually ascends again to the next meal (Palmiter, 2008).

Given the multiplicity of hats motivation wears, one can wonder if different behaviors are mediated by different forms of arousal or if there is one generalized arousal system that contributes to all behaviors (Van Swinderen and Andretic, 2011). It seems that common denominators to different behaviors such as sleep, locomotion, courtship and learning exist. Ungerstedt (1971) might have been the first to target dopamine (DA), a biogenic amine, as a central neural element to motivation. Indeed, after having completely denervated the nigro-striatal dopaminergic pathway in rats' brains, he observed "*long-lasting adipsia and aphagia, hypoactivity, difficulties to initiate activity and loss of exploratory behavior and curiosity*" in those rats. Other studies showed the same result: DA-depleted mice are not motivated to engage in goal-directed behaviors (Palmiter, 2008). Nevertheless, they still have learning capacities (such as learning the location of food) and a preference for sucrose, which shows that their lack of motivation does not come from a lack of detection or perception of stimuli. Interestingly, DA has also been targeted as a key element in setting arousal thresholds in invertebrates. Van Swinderen and Andretic (2011) defend that, in *Drosophila*, DA may involve perceptual suppression, a necessary component of both sleep and selective attention.

The role of biogenic amines

Neuromodulation provides one powerful mean to dramatically but reversibly reconfigure the function of a sensory circuit without changing the 'hard-wiring'. By definition, it should therefore be the major mechanism in action during activational behavioral plasticity. A neuromodulatory effect typically begins with the binding of a small molecule to a neurotransmitter receptor which triggers a cascade of biochemical reactions that ultimately changes the physiology of the cell and can elicit much more complex effects than the simple excitation or inhibition of classical neurotransmission. Biogenic amines are organic bases with low molecular weight that can act as neuromodulators and have a wide range of action, affecting many aspects of cell physiology and acting on distant targets. As such they can modulate every aspect of an animal's behavioral repertoire (in humans: Schildkraut and Kety 1967; rats: Matthews et al. 2001; worms: Chase 2007; lobsters: Livingstone et al. 1980; fruit flies: Yellman et al. 1997; honey bees: Scheiner et al. 2006; ants: Kamhi and Traniello 2013).

Neuromodulation is known to increase sensitivity of receptor neurons to their particular stimulus. A famous example of it has been shown in the male silkworm moth in which

octopamine (OA, a biogenic amine) enhances the sensitivity of neurons that detect pheromones by lowering their threshold for generating both action potentials and behavioral responses (Pophof, 2000). But the best-known example of invertebrate sensory modulation is the one mentioned earlier: the gill-withdrawal reflex of the sea slug *Aplysia californica*. This system has been extensively studied since the first discoveries and progress has been made in understanding all behavioral, network, cellular and biophysical levels (Kandel, 2001). Marinesco and Carew (2002) were thereby able to show that serotonin (5-HT) is the biogenic amine that mediates sensitization by modulating transmitter release at the sensory-motor synapse. Moreover, when series of noxious stimuli were applied, 5-HT promoted gene activation and the synthesis of new proteins induced the growth of new synapses leading to prolonged sensitization behavior (reviewed in Kandel 2001).

Two models: the honey bees and ants

Honeybees and ants contradict the notion that insect behavior tends to be relatively inflexible and stereotyped, as fine-grained variation models postulate (Snell-Rood, 2013). According to these models, reversible phenotypic plasticity exhibited in environments changing within the lifespan of an organism should occur in increased-brain-size, long-lived, non-migratory, temperate species that do not diapause nor hibernate. Obviously, this is not the case of these two eusocial insects which show small brains, short individual lives, and hibernation phases. However, although the life of an individual bee or ant worker is relatively short, that of the colony is potentially unlimited. This social lifestyle has led individual animals not to be innately programmed for a large part of their behaviors but on the contrary display extraordinary learning skills so as to be able to handle environmental variations occurring in a life time (e.g. characterize feeding places, the place of the colony and potential new nest sites). Moreover, because these relatively complex behaviors are controlled by a brain consisting of only 1 million or so neurons, insects offer an opportunity to study the relationship between behavior and cognition in neural networks that are limited in size and complexity (Menzel, 2012). Species as *Caenorhabditis elegans* and *Drosophila melanogaster* are privileged models in neurosciences because of the wide range of genetic tools available. However, little is known about their respective ecology. Honey bees and ants turn out to be excellent models to precisely make the links between the ecology of the animals and the neural mechanisms underlying their behaviors.

The ecology of honey bees and ants

Honey bees

The European honey bee, *Apis mellifera* L., is a hymenopteran (their hind wings are connected to their fore wings), which Latin name literally means "honey-bearing bee" and refers to the bees' habit of collecting nectar and producing stocks from it to allow colonies to survive dearth periods. There is still a debate on the origin of *A. mellifera* nowadays about whether it originated in Africa and expanded into Eurasia (Whitfield et al., 2006), or expanded out of Asia (Han et al., 2012). Peters et al. (2017) located the phylogenetic origin of bees (Anthophila) within the apoid wasp family ''Crabronidae'', which substantiates the idea that the switch from a predatory to an herbivorous lifestyle was a key to the tremendous diversification of bees (Peters et al., 2017). The authors estimate the origin of bees to have been in the Cretaceous, a result that is consistent with a close temporal link between the diversifications of bees and angiosperms (flowering plants, Peters et al., 2017). Honey bees are classified in the family Apidae which is characterized by the presence of a corbicula (pollen basket) on the outer surface of their hind tibia, mostly used to carry pollen. Within the *Apis* genus, four other species are counted: *A. dorsata*, *A. laboriosa*, *A. cerana*, and *A. florea*. These species are all found in India, which shows the greatest species diversity in the *Apis* genus, except for *A. mellifera*. This last one is now found in every part of the world because of beekeeping activities but is still called the European honey bee.

Honey bees must undergo a number of stages before finally emerging as adults, as most insects, but what is particular to social insects such as honey bees is the interactions that occur between brood and adults, which cooperatively care the brood. The life cycle of a honey bee begins with the queen laying eggs in cells she would have previously constructed. Fertilized eggs will develop in diploid females while unfertilized eggs will develop in haploid males (or drones) because of the haplodiploid sex-determination system of hymenopterans which is determined by the number of sets of chromosomes an individual receives. Before emerging as adults, eggs will develop into larvae, i.e. the feeding time, when bees gain in weight and grow in size. Once the queen and/or workers have capped the cells, larvae will develop into pupae, which will undergo developmental transformations to create future external and internal structures so as to produce adults (metamorphosis). When transformation is done, individuals will chew their way out of the cells. The whole process can take from two to three weeks depending on the caste and other factors (temperature, nutrition, genetics…) (Winston, 1987).

Fertilized eggs can develop into queens or workers depending on the type of cell they are laid in and some aspects of nutrition: workers are lightly fed, while queens are heavily fed with a particular food: royal jelly. Workers perform all the tasks in the nest except for laying eggs which is the role of the queen. Worker activities have a temporal basis: younger workers perform in-nest tasks, while older workers perform outside tasks. They tend to do groups of within-colony tasks in the order of cell cleaning, brood and queen tending, receiving nectar, packing pollen, building comb, cleaning debris, ventilating, guard duty, and foraging trips. Any of these jobs is highly specialized, yet a worker will perform many of these tasks in few hours, and nearly all of them during her lifetime. As such, worker bees are both generalists and specialists. They are also flexible in the ages at which they do these jobs and thus can adjust their work schedules to colony requirements (Winston, 1987).

It is likely that most outside tasks would require behavioral plasticity to deal with environmental variations. Foragers gain considerable information about the location and nature resource from following dances (e.g. round dance, waggle dance, Fig. 2). Besides having to adjust to external factors, honey bees also have to adjust to colony requirements and allocate foraging tasks to maximize gain, recruit resources, transfer information, and make decisions about when to switch resources (Winston, 1987).

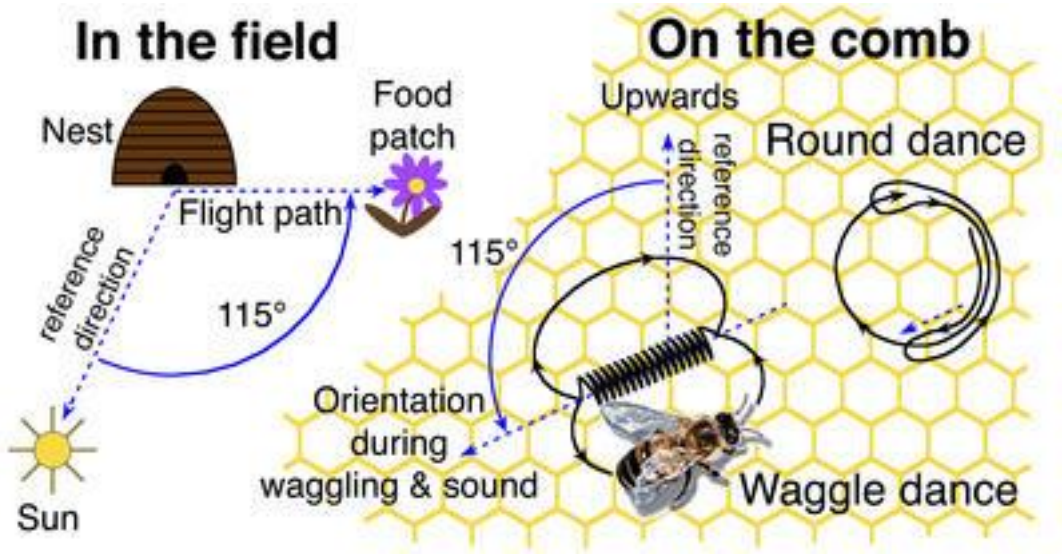

Figure 2: The round dance indicates a resource close to the nest. The waggle dance indicates the orientation of the resource. Here the food patch is 115° to the left of the sun, and the dance is 115° to the left of vertical (Credit: P. Kirk Visscher).

Decision making is also highly required during swarming, i.e. the division of a colony through the leave of the nest by an old or new queen with young workers to search for a new home. Swarming is elicited by the congestion of the colony due to worker population's rapid

growth upon leaving winter. Swarms are formed of several hundred bees working together to find a dozen or more candidate nesting cavities in trees and then selecting the best one of these options for their new home. This group intelligence is a product of a quorum (sufficient number of scout bees signaling for one site). By this quorum-sensing process, a scout bee "votes" for a site by spending time at it, somehow the scouts act and interact so that their numbers rise faster at superior sites, and somehow the bees at each site monitor their numbers there so that they know whether they've reached the threshold number (quorum) and can proceed to initiating the swarm's move to this site (Seeley et al., 2006).

Drone bees are necessary to complete the reproductive cycle by mating. Although drones exist only to mate and perform no other useful functions in the nest, most die before mating, either because they get old or are thrown out of the nest by the workers. The few drones which succeed in mating queens can do so only once, since they die immediately after mating, when their abdomens and genital apparatus rupture. Nevertheless, drones spend most of their adult lives flying out to congregation areas with hundreds or thousands of other drones, competing for the favors of the few queens which make their way to the mating sites. Drones are produced and maintained only when colonies can support them and when queens are potentially available for mating. The mating system is designed so that queens can mate with many drones, most often with drones from other nests (Winston, 1987).

Ants

The ants are classified as a single family, the Formicidae, within the order Hymenoptera, which also includes the bees, wasps, sawflies, ichneumons, and similar forms. Peters et al. (2017) inferred ants (Formicoidea) as being the closest extant relatives of Apoidea and estimate their last common ancestor to have lived in the Jurassic or the Cretaceous (ca. 162 million years ago). All known living ants are eusocial, with strong physical differences separating the queen and worker castes. This remarkable diversification and unchallenged success of ants appears to be due to the fact that they were the first group of *predatory* eusocial insects that both lived and foraged primarily in the soil and in rotting vegetation on the ground. Termites live in the same places and also have wingless workers, but they feed almost exclusively on dead vegetation. Ants have a number of adaptations fitting them for their special way of life. One of the most striking is the elongation of the mandibles into working tools. A second important innovation is the metapleural gland, which produces phenylacetic acid, an anti-fungus and bacteria. Where bees and wasps protect their immature forms by constructing antibiotic-impregnated brood cells, ants appear to disseminate antibiotic secretions diffusely through the nest from the

metapleural gland. This innovation is likely to have played a role in the colonization of the moist, microorganism-ridden environment in which the great majority of ant species live (Hölldobler and Wilson, 1990).

As in honey bees, the ant colony is an almost exclusively female society with the males remaining in the nest only until the time of their invariably fatal nuptial flight. The colony life cycle can be analyzed as an orchestration of energy investments, in which workers are multiplied until it is profitable to convert part of the net yield into new queens and males. Like the life cycle of the individual ant, the life cycle of an ant colony can be conveniently divided into three parts. The founding stage begins with the nuptial flight. The virgin queen departs from the nest in which she was reared, leaving behind her mother, who is the queen of the colony, and her sisters, who are either sterile workers or virgin reproducers like herself. She meets one or more males and is inseminated. The males soon die without returning home, while the queen finds a suitable nest site in the soil or plant material and constructs a first nest cell. Here she rears the first brood of workers, drawing on her own tissue reserves to produce eggs and feed the growing larvae. Soon after reaching the adult stage, the workers take over the tasks of foraging, nest enlargement, and brood care, so that the queen may confine herself to egg laying. Over the coming weeks and months the population of workers grows, the average size of the workers increases, and new physical castes are sometimes added. The colony is now in the ergonomic stage: its activities are exclusively concerned with work devoted to colony growth, rather than with reproduction or dispersal. After a period that ranges according to species from a single warm season to five or more years, the colony begins to produce new queens and males (reproductive stage). The sexual forms go forth to start new colonies, and the new colony life cycle has begun. Colonies of all known ant species are perennial: they issue a crop of reproducers, then return to an interval of purely vegetative growth (workers) (Hölldobler and Wilson, 1990).

Substantial variation has been elaborated out of this cycle, especially regarding the mode of colony founding and the number of egg-laying queens that coexist during the several stages of the life cycle. Monogyny refers simply to the possession by a colony of a single queen, as opposed to polygyny, which is the possession of multiple queens. The founding of a colony by a single queen is referred to as haplometrosis; when multiple queens start a colony the condition is called pleometrosis. Monogyny can be primary, meaning that the single queen is also the foundress; or it can be secondary, meaning that multiple queens start a colony pleometrotically but only one survives. Polygyny can also be primary, in which multiple queens persist from a

pleometrotic association, or secondary, in which the colony is started by a single queen and supernumerary queens are added later by adoption or fusion with other colonies. Next, the mode of colony founding is subject to complicated variation among species. It can be accomplished by swarming, in which two or more forces of workers separate in the company of queens. Swarming can occur in two forms: budding, in which a group of workers departs from the main nest with one or more queens and start a new nesting unit; and fission, in which portions of the colony containing fertile queens separate from each other and go their own ways (Hölldobler and Wilson, 1990).

Two general patterns of division of labor are recognized in social insects: temporal polyethism, or age-correlated patterns of task performance as in honey bees, and morphological polyethism, in which a worker's size and/or shape is related to its performance of tasks. Temporal polyethism is widespread in social insects and invariably follows the pattern of young workers performing tasks within the nest and older workers performing outside tasks such as foraging and defense (reviewed in Robinson, 1992). Morphological polyethism is found in termites and in those ant species with distinguishable sub-castes within the worker caste (Oster and Wilson, 1978). Patterns of morphological polyethism are variable; one generalization that appears to hold is that the more extreme sub-castes, in either size or morphology, have more specialized behavior and narrow repertoires (Oster and Wilson, 1978). The most common specializations are for defense and foraging. Other roles of morphologically specialized workers include food processing and food storage (Hölldobler and Wilson, 1990).

Some ant species therefore display considerable variation in worker size leading some species to even have specialized soldier castes with particular features (e.g. massive mandibles) (Fig. 3). However, there are so many ant species (ca. 15000, Economo et al., 2018) and particularities that one cannot draw the list of this diversity. The important consideration becomes the best arrangement of castes and division of labor for the functioning and reproduction of the colony as a whole. The colony can be most effectively analyzed if it is treated as a factory within a fortress. Natural selection operates so as to favor colonies that contribute the largest number of mature colonies in the next generation. Hence the functioning of the workers in gathering energy and converting it into virgin queens and males is vital. This part of the colony's activities constitutes the factory. But the colony is simultaneously a tempting target for predators. The brood and food stores are veritable treasure houses of protein, fat, and carbohydrates. As a consequence colonies must have an adequate defense system, which often takes the form of stings, poisonous secretions, and soldiers. This set of adaptations

constitutes the fortress (Hölldobler and Wilson, 1990).

Figure 3: Major and minor workers of *Atta cephalotes* demonstrating the size extremes among worker ants in a single leafcutter ant colony.

Insect societies are characterized by colony closure which lies on the ability to discriminate between homocolonial individuals ("desirables") and heterocolonials ("undesirables"), and manifests itself by affiliative behaviors or agonistic ones, respectively. Social organization in ants is based, among other things, on colony closure that preserves its social integrity and prevents intrusion by alien individuals inside the society. The ability of discriminating between familiar and unfamiliar individuals implies the comparison of the perceived odor with an internal representation of the colony visa (Errard et al., 2006). According to the gestalt model (Crozier and Dix, 1979), the colony identity results from the homogenization of individual chemical cues, mainly cuticular hydrocarbons (Wagner et al., 2000). This homogenization is performed by trophallaxis, grooming, or physical contacts (Soroker et al., 2003). In this social system, the recognition of desirable cues plays a major role in the defense of colony resources. Nestmate recognition cues can be determined genetically (Provost, 1991; Ross et al., 1987; Vander Meer et al., 1985), but may also include environmental substances that blend into the colony odor (Jutsum et al., 1979; Obin, 1986; Obin and Vander Meer, 1988). Studies pointed out that the postpharyngeal gland is responsible for storing and mixing the recognition

chemicals and reapplying them on the cuticle, creating a unified colony odor (Soroker et al., 1994; Soroker et al., 1995). In addition, geographical distance between colonies has been shown to be correlated to intercolonial aggression (Nowbahari et al., 1990).

In order to provide the colony, ants developed many strategies, among which the adoption of aphids, mealybugs, and other hymenopterans as cattle to provide a steady source of honeydew. A few raid colonies of other species (or within a species) also acquire workers as domestic slaves (intraspecific dulosis, Le Moli et al., 1993), or utilize the odor trails of other species, or defend common nest sites. Just as frequently, alien species have been found to insinuate themselves into the colony as inconspicuous social parasites. Taken together, the hundreds of cases of interspecific symbioses among ant species encompass almost every mode of commensalism and parasitism. True cooperation between species, however, is rare or nonexistent (Hölldobler and Wilson, 1990). Another spectacular feature of ants, besides being livestock farmers, is their agriculture skills. Members of the myrmicine tribe Attini share with macrotermitine termites and certain wood-boring beetles the sophisticated habit of culturing and eating fungi. Indeed, Attini ants are known to be agricultural pests because they create holes in crops, cutting small pieces of vegetation and transporting them to garden chambers in the nest on which fungi can ramified and absorb organic matter; the fungus being a food source for the ants (Hölldobler and Wilson, 1990).

Between farming, agriculture, and the use of tools (Maák et al., 2017), ants are very likely to have evolved particular cognitive abilities, just like the honey bee.

The neurobiology of honey bees and ants

Sensory processing in an insect brain

The nervous system of insects is composed of the brain (also called supraesophageal ganglion, Fig. 4) and multiple segmental ganglia of the ventral chord in the thorax and abdomen. The brain processes second or higher order inputs from all sensory organs, and coordinates the behavioral output through descending premotor neurons or interneurons.

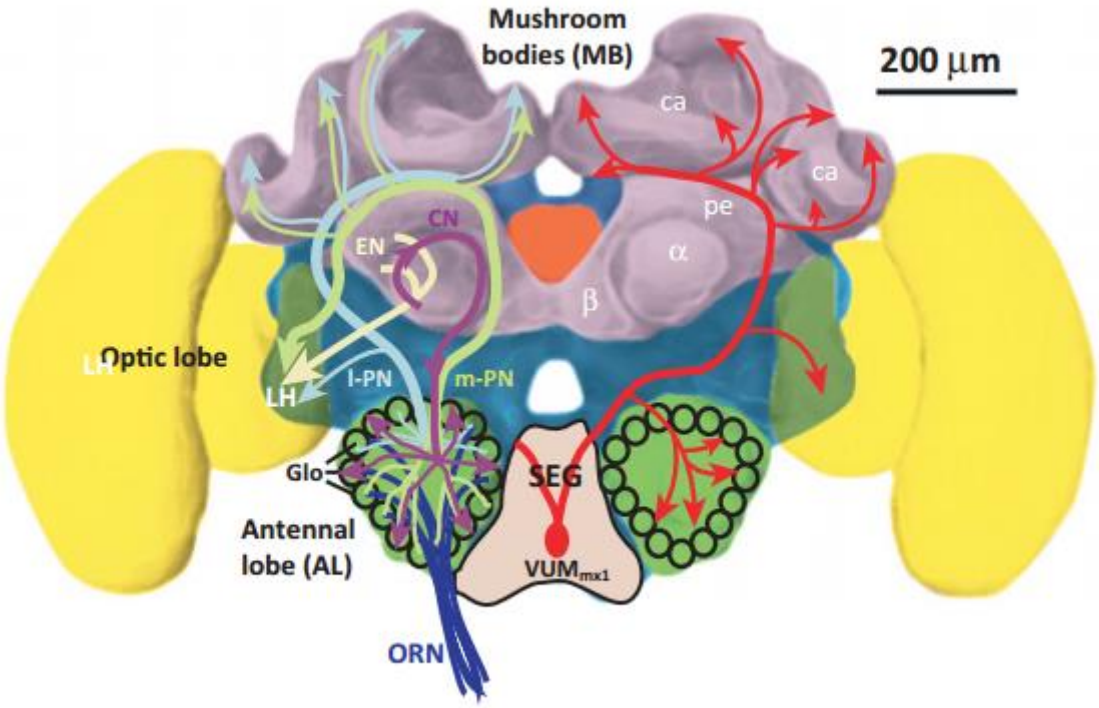

Figure 4: Neural pathways for conditioned stimulus (CS) and unconditioned stimulus (US) information in the honey bee brain. Left hemisphere – the olfactory (CS) circuit: ORN: olfactory receptor neurons; Glo: glomeruli; l-PN: lateral projection neuron; m-PN: median projection neuron; LH: lateral horn; CN: centrifugal neurons; EN: extrinsic neurons. Right hemisphere – the sucrose (US) circuit: VUMmx1: ventral unpaired median neuron of the maxillary neuromere 1; SEG: subesophageal ganglion; ca: calyces; pe: pedunculus; α: vertical lobe; β: horizontal lobe (Giurfa, 2013).

The first synaptically dense regions of the brain relaying information from sensory organs are called first-order neuropils and are associated with the different modalities sensory organs detect. The olfactory one in insects is called the antennal lobe (AL) and receives input from olfactory receptor neurons (ORNs) located within sensilla on the antenna. Odorant molecules bind to specific molecular olfactory receptors (ORs) in the ORN membrane. Through transduction, the chemical message is transformed into an electric message in the form of action potentials, which are transferred via the antennal nerve to the AL. The AL is composed of densely packed neuropils called glomeruli, which are sites of synaptic interaction between ORNs, inhibitory local neurons, and projection neurons (PNs). Odorants are encoded in the AL in terms of odor-specific spatio-temporal patterns of glomerular activation. Projection neurons constitutes the output of the AL and convey processed information via different tracts (l-PN and m-PN) to higher brains centers, the mushroom bodies (MBs) and the lateral horn (LH). A recent study in honey bees (Roussel et al., 2014) showed that coding at the input of the LH

follows a similar spatio-temporal logic as in the AL. The MBs are composed of Kenyon cells which have dendritic branches that arborize in the calyces (cup-shaped regions of the MBs). In the MBs, odors also evoke combinatorial activity patterns, but these patterns are sparser (Szyszka et al., 2005). Kenyon cells are highly odor specific and exhibit a temporal sharpening of their responses upon odor stimulation as a possible consequence of broader loops of neuronal inhibition (Szyszka et al., 2005).The calyx is segregated into modality-specific regions; the upper part is olfactory, the median part visual and the lower predominantly mechanosensory. At the base of the calyces, Kenyon cell axons come together and form a bundle known as the pedunculus. At the end of the pedunculus, Kenyon cell axons bifurcate and extend branches into the two output lobes of the MBs: the vertical lobe and the horizontal one. Within the MBs, feedback neurons project from the pedunculus and lobes back to the calyces, allowing inhibition of chosen inputs. Extrinsic neurons (ENs) take information from the pedunculus and the lobes and project to different parts of the protocerebrum (the part of the brain associated with eyes) and LH. Centrifugal neurons (CNs) provide feedback from the MBs to the ALs and are thought to be involved in retrograde modulation of AL circuits (Giurfa, 2013).

Different levels of specialization and segregation between pheromone and general odorants information have been found in the AL. In many insects, sex pheromones are processed within dedicated neural structures, while non-sexual pheromones are generally treated following neural strategies similar to those of non-pheromonal odors (Sandoz et al., 2007). In males of bees, and possibly ants, sexual female pheromones are processed in a macroglomerular complex (MGC), a sexually-dimorphic region of the AL constituted of hypertrophic glomeruli dedicated to respond to sex pheromone components (bees: e.g. Arnold et al., 1985; ants: e.g. Nishikawa et al., 2008 ; Stieb et al., 2011). Besides this specialization, typical of sex pheromones, there is usually no specific segregated processing in the AL for social (non-sexual) pheromones (Sandoz et al., 2007). In honey bees, for instance, several alarm pheromones as well as brood-related pheromones are processed following the same spatio-temporal glomerular activation logics of ordinary (non-pheromonal) odors (Galizia et al., 1999b; Sachse et al., 1999; Sandoz, 2006; Wang et al., 2008). In the ants *Atta sexdens* and *A. vollenweideri*, a macroglomerulus was found in large workers (Kleineidam et al., 2005), which does not seem to be associated with sex-pheromone processing but may be involved in the detection of a trail pheromone. Calcium-imaging studies in the ant *Camponotus rufipes* did not reveal a specific clustering of specialized glomeruli responsive to alarm pheromone, and the same glomeruli, or their direct neighbors, were shown to participate in responses to non-

pheromonal odors (Galizia et al., 1999a). A recent study showed that alarm-pheromone responsive PNs innervate a specific cluster of normally sized glomeruli within the AL, indicating some degree of anatomical segregation of pheromone processing in the AL of the ant *C. obscuripes* (Yamagata et al., 2007). However, calcium-imaging studies in *C. floridanus* (Zube et al., 2008) confirmed that non-sexual pheromones (e.g. alarm, brood pheromones) are encoded in the AL similarly to non-pheromonal odors. Beyond the AL, less is known about pheromone processing in the LH and MBs. In the fruit fly, the LH has been associated with a privileged and fast pheromone-processing role due to its premotor implication (Jefferis et al., 2007). Yet, calcium-imaging studies in the honey bee, in which pheromonal and non-pheromonal components were tested at the input of the LH, did not show such specialized segregation (Roussel et al., 2014). No information is available concerning specialized pheromone processing in MBs.

The first-order visual neuropil is the optic lobe (OL) and is constituted of three layers: the lamina, the medulla, and the lobula. Each of these layers receives input from photoreceptors located within the ommatidia (small lenses which focus light onto photoreceptors) of insect compound eyes. These photoreceptor cells can be grouped into classes according to the spectral sensitivity of the light-sensitive pigment (photopigment) they contain (e.g. UV, blue, green…) (Briscoe and Chittka, 2001). From the lamina, information is then conveyed to the next ganglion, the medulla, for further processing, and eventually to the lobula, where complex analysis of the image takes place, leading to the perception of color, shape, and motion (Srinivasan, 2010). Different tracts coming from each layer of the OL connect this first-order neuropil to the MBs for sensory integration and cognitive phenomena. Little is known about how visual information is then processed within the protocerebrum.

A least studied modality in "small insects" (as opposed to "big insects", e.g. locusts, cockroaches, crickets, and stick insects) is mechanosensory perception. Mechanosensation is allowed by the different types of insect mechanoreceptors which are traditionally divided into two functional groups: exteroceptors and proprioceptors. Exteroceptors directly detect mechanical forces generated in the external world and are generally enclosed in tactile hairs on the external body surface of an animal, while proprioceptors detect the position or movement of body parts and are generally located a bit more internally. Mechanoreceptors can be found all over the body, but within a given limb, different types of mechanoreceptors exist (Tuthill and Wilson, 2016). Since mechanosensation is mostly involved in motor reflexes, it is believed that the major processing of this modality is achieved in the thoracic ganglia instead of the brain

(Newland et al., 2000). However, Giurfa and Malun (2004) having introduced a form of associative mechanosensory conditioning of the proboscis extension reflex (PER) in honey bees, they showed that higher-order centers in the brain (e.g. MBs) could also be involved in the process. They argued that, since a class of interneurons has been shown to interconnect the dorsal lobe and the AL, and that these neurons target the same region of the AL, olfactory and mechanosensory information might be integrated into a common across-fiber activity pattern at the level of the AL output.

As highlighted in mechanosensation, stimulus transfer occurs between distinct sensory modalities (e.g. olfaction and mechanosensation). Therefore, a neural candidate should provide multimodal output consistent with response transfer to stimuli belonging to different domains. These requirements are met by the MBs. The MBs receive compartmentalized multisensory input (olfactory, visual, mechanosensory, gustatory) (Mobbs, 1982) and their extrinsic neurons respond to various stimuli including sucrose, odors, mechanosensory, and visual. Furthermore, the MBs are tightly related to reinforcement systems. In the bee, octopaminergic neurons such as the ventral unpaired median neuron of the maxillary neuromere 1 (VUMmx1), which serves as an appetitive reinforcement system, converge with the regions of olfactory input of the MBs. Activity in the VUMmx1 neuron substitutes for sucrose in the olfactory conditioning of the PER and thus may be the specialized reward system for olfactory cues. Also, dopaminergic neurons, which can act as a punishment system, converge with specific regions of the MBs, thereby mediating aversive memories of an odor-shock. Finally, MBs have been historically characterized as a substrate for associative memories, particularly long-term ones (Giurfa, 2013).

The cognitive architecture of insect mini-brains thus consists of a network of vertical modules (i.e. one input leads to one specialized output, e.g. sensory-motor routines, elementary processes in associative learning, or automatic processes of neuronal self-organization) that allows for stereotyped as well as flexible responses thanks to horizontal combinations between modules (i.e. central integration of diverse modules by the MBs) (Menzel and Giurfa, 2001).

Differences in neuroanatomy between honey bees and ants

Although the brain of the honey bee is small (about 0.4 to 0.6 mm3 with about 1 million neurons), it is large both in absolute and relative terms in comparison to other insect species. For example, the brain of desert ants (*Cataglyphis* spp.) is about 100-times smaller than the honey bee brain (Chittka and Niven, 2009) and indeed, Wehner et al. (2007) showed that ants have brains that are small not only in absolute but also in relative terms: the brain of an ant is

smaller than the brain of any ant-sized vertebrate would be. Based on comparisons of *Cataglyphis* species, Wehner et al. (2007) hypothesized that the significantly larger size of the brains in the large-colony species of *Cataglyphis* – as compared to their small-colony congenerics – is most likely correlated with the social interactions occurring inter-individually within the colony rather than with the outdoor activities of food retrieval and, in this context, navigation.

Since within-colony communication is mostly chemical in honey bees and ants, if we follow the above-mentioned hypothesis, honey bees should have bigger AL and therefore more glomeruli than ants. However, the opposite actually happens. In the carpenter ants *Camponotus japonicas* and *C. floridanus*, the worker and the queen each have ca. 430-434 glomeruli (Nishikawa et al., 2008; Zube and Rössler, 2008), whereas the honey bee worker only has ca. 166 glomeruli (Galizia and Rössler, 2010). In comparison, the fruit fly has 43 glomeruli (Vosshall and Stocker, 2007) and the moth *Agrotis ipsilon* 66 (Greiner et al., 2004). Recent studies suggest that the difference in number of glomeruli is, in large part, due to the presence or absence of glomeruli involved in social pheromone processing. The observation indicates that a larger number of olfactory receptor types have been evolved in ants than in other insects, including honey bees, which may be an adaptation to underground lifestyle relying heavily on olfaction rather than vision and also to allow for unusually sophisticated social communication (d'Ettorre, 2016; Mizunami et al., 2010).

Menzel (2012) argued that the possible explanation for the relatively large brain of honey bees compared to other insects would lie in the neural organization of the visual system and the MBs. Since honey bees and ants are both hymenopterans and that their MBs seem to be organized the same way (Strausfeld et al., 1998), it could be possible that vision plays a major role in honey bees compared to ants. However, it should be emphasized that there are 14,912 described ant species (Economo et al., 2018) and that exceptions must contradict the generalities drown here.

Because currently it is almost impossible to decide what would constitute a good measure of information-processing capacity (synaptic architecture, microcircuits, etc.), any comparison of "intelligence" or cognitive performances between species would be highly speculative.

Social organization and communication

Pheromones as regulators of the colony

Chemical communication is the oldest mode of communication (Wyatt, 2014) and, in insects,

although all other modes of communication occur, chemical cues and signals still dominate (Bradbury and Vehrencamp, 2011; Wyatt, 2014) (Fig. 5). In strictly solitary insects, communication is largely restricted to sexual context and involves signals that attract and inform mating partners. The most prominent and widespread examples for such signals are sex pheromones (single substances or blends of chemicals) (Wyatt, 2014), which are largely species and sex specific to ensure mating with an appropriate partner. As insects evolved higher levels of sociality, the information they needed to exchange between group members diversified to include division of labor, collaborative resource utilization, and collective defensive actions. This trend is reflected in the diversity of chemical signals required to maintain eusocial insect colonies (Leonhardt et al., 2016).

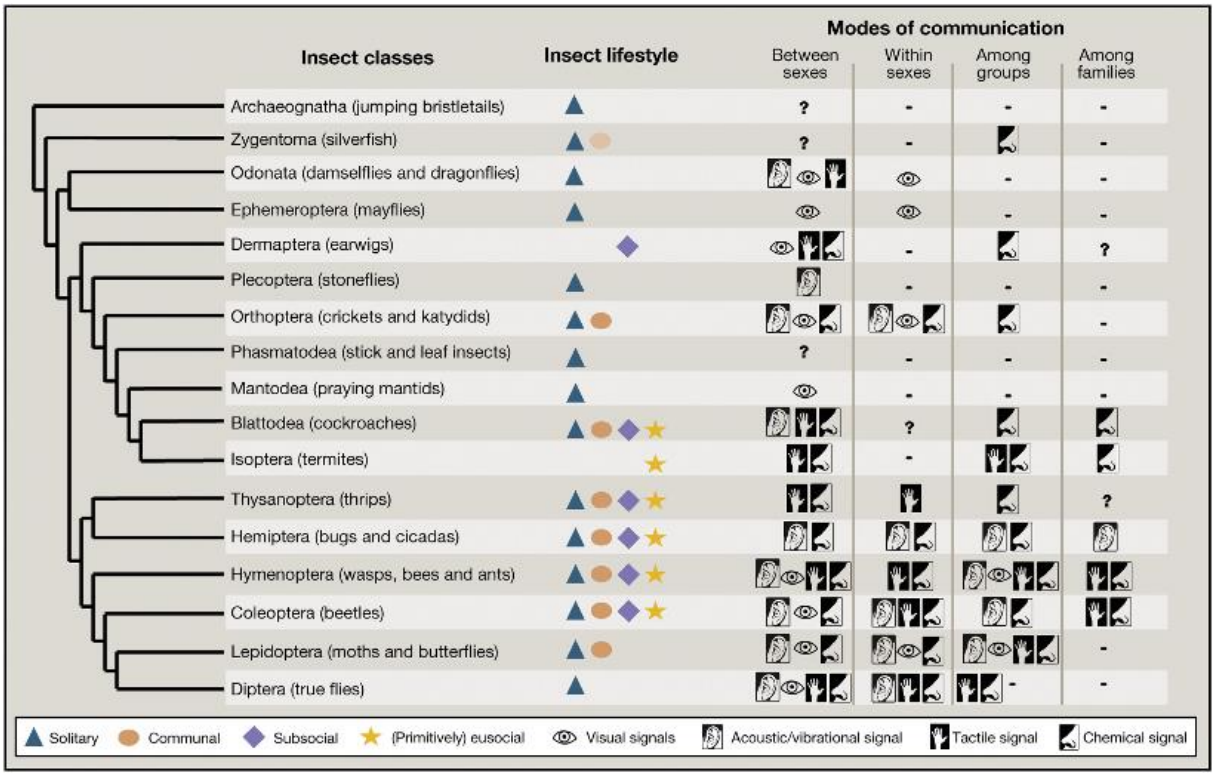

Figure 5: Types of social organization and communication modes employed by major clades in the Insecta (Leonhardt et al., 2016).

The word "pheromone" has been proposed by Peter Karlson and Martin Lüscher in 1959, following the discovery of the famous bombykol, the sex pheromone of silkworm moths (Butenandt et al., 1959). Since then, a plethora of studies have been published describing pheromones as eliciting innate stereotyped behaviors in same-species individuals (Wyatt, 2014). Pheromones are usually divided into two categories according to their effects: releaser pheromones induce an immediate behavioral response whereas primer pheromones alter behavioral repertoire as a result of altered physiology (Wilson and Bossert, 1963). However, it

should be kept in mind that such distinct separations do not exist in nature and that pheromones could exert both types of action (e.g. brood pheromone, Le Conte et al. 2001).

Honey bees

In honey bees, pheromones can be produced by workers, queens, and possibly drones, and are known to function for mating, alarm, defense, orientation, and integration of colony activities. Worker-produced pheromones are known to function for orientation as well as alarm and defense. The pheromones produced by the Nasonov gland are the most well-known orientation odors and are composed of several chemical compounds (Boch and Shearer, 1962; Butler and Calam, 1969; Pickett et al., 1980). The three major components of the Nasonov gland in a respective order are: geraniol, (E)-citral, and geranic acid. However, each component of the blend contributes to the attractiveness of the pheromone and the strongest responses are observed when using the full mixture. Workers expose the Nasonov gland by creating an opening between their 5^{th} and 6^{th} dorsal segment of the abdomen, and then disperse the pheromone by fanning their wings in a number of orientation situations, including nest entrance finding, forage marking, and swarming (Fig. 6). At the entrance, the Nasonov scent is important in guiding into the colony workers such as incoming foragers, workers on orientation flights, and disoriented workers following colony disturbance. Workers also release this pheromone when foraging for water, providing an odor to assist incoming water foragers in orientating to a relatively odorless resource, and at flowers. This orientation/aggregation pheromone is also particularly important during swarming, as well as after swarming to orient to the entrance of the new nest.

Figure 6: Examples of use of pheromones in honey bees and ants. Upper left: *Linepithema humile* ants following a trail connecting a food source to the nest. Lower left: a *Camponotus* ant in gaster flexing position, ready to spray formic acid (an alarm pheromone), and defending the entrance of her leaf nest. Center: a honey bee having stung a human being, thereby diffusing the alarm pheromone allowing congeners to orient towards the danger source (i.e. the human). Upper: homovanillyl alcohol (HVA), the major component of the queen mandibular pheromone which triggers retinue behavior; and isopentyl acetate (IPA), the major component of the alarm pheromone in honey bees, which signals danger. Right: honey bees exposing their Nasanov gland at the hive entrance and fanning their wings to diffuse the pheromone, which indicates the location of the nest to returning foragers.

Workers also produce numerous chemical compounds used for alarm and colony defense in the mandibular glands and in the Koschevnikov gland (sting). 2-heptanone is the only component of the alarm pheromone contained in the mandibular glands. The primary function of 2-heptanone may be to repel robbers and/or enemies at nest entrances since it is strongly repellent to foraging workers (Giurfa, 1993). On the opposite, the Koschevnikov gland contains a mixture of chemicals with isopentyl acetate (also called isoamyl acetate) as major compound (Fig. 6). These pheromones alert workers of a danger, lower their threshold of sensitivity for attacks, and assist workers in orienting to attackers which have already been stung. Alarm pheromones are however not sufficient to elicit full defensive behavior. Workers without moving visual stimulations become agitated, display characteristic aggressive postures, dash toward and crowd around the stimulation, but do not sting. The responses of workers depend on external factors (e.g. temperature, humidity) and their internal state (e.g. age) (Winston, 1987).

Contrary to workers, there are few chemicals involved in a multitude of functions in queens and it seems that all these functions are assumed by the pheromone produced in the queen mandibular glands. Indeed, the so-called queen mandibular pheromone controls the inhibition of queen raring and swarming, the prevention of worker ovary development, the attraction of drones for mating, the attraction of workers to swarms, the stabilization of swarm clusters, the stimulation of Nasonov pheromone release, the induction of worker foraging, and queen recognition (Winston, 1987).

There is some evidence that drones and brood may produce pheromones as well. Gerig (1971, 1972) showed that drone heads are attractive to flying drones. This is consistent with what has been observed in bumble bees (Kullenberg, 1956; Free, 1987). This behavior seems to be counter-adaptive from a male point of view as one would expect that males would do better by actively avoiding each other and establishing their own distinct routes in order to propagate their own genes. However, a system whereby many males are attracted to the same places may benefit females by providing an opportunity to choose high-quality males to reproduce with. Regarding the brood, Le Conte et al. (2001) showed that bees receiving brood pheromone initiated foraging at significantly older ages than did bees in control colonies in five out of five trials.

In summary, it is quite evident that pheromones exert considerable influence on honey bee behavior and that we are far from understanding all the effects and mechanisms involved.

Ants

Pheromones originate from exocrine glands. The number and diversity of these glands in social insects in general, and ants in particular, are impressive, with glands occurring in all parts of the body. Worker ants can easily contain 20 or more major glands in their small bodies, and hence can be considered as walking glandular batteries (Billen, 1991).

One of the more obvious and characteristic forms of behavior of some ant species is the sharing by many workers of a common path to and from a source of food (Morgan, 2009). Ants use trails to recruit workers of the same species to a food source, which use their antennae to follow the trails (Fig. 6). Particularly, recruits follow the trail made by a scout returning from a food source to the nest. Some species adopt a particular attitude when trail laying. Many lay a trail by dragging the tip of the abdomen along the ground or by touching the surface with the anal hairs or the tip of the lancet of the sting (Hölldobler and Wilson, 1990).

Ant alarm behavior is commonly released when ants detect a source of disturbance and potential danger. Alarm responses are released by alarm pheromones and generally involve an increase in movement coupled with an increase in aggressiveness. The exact nature of the overall alarm response tends to vary in both intensity and composition from species to species. The term 'alarm' is general and describes a mixture of diverse and complex responses. For example, alarm pheromones may release increased linear movement, increased sinuosity of movement, directional movement, widening of the mandibles, biting, extrusion of the sting, stinging, trail laying, recruitment, expulsion of exocrine products, attack, 'panic', etc. (e.g. Fig. 6). In some species of ant the overall alarm behavior has been shown to be a summation of a complex mixture of individual alarm responses to individual alarm pheromones. However, in most cases, authors merely report an overall alarm response to an exocrine gland secretion or specific components of the secretion (Parry and Morgan, 1979).

The fire ant (*Solenopsis invicta*) queen produces a series of pheromones that affect almost every aspect of colony life. These include primer pheromones that affect caste determination in female larvae, inhibit the production of sexuals (new queens and males, Vargo and Fletcher, 1986), suppress egg production in alates (winged females), and inhibit maturation and egg production in mated queens (Klobuchar and Deslippe, 2002). In polygyne colonies, the individual egglaying rate decreases as the number of queens increases owing to a primer pheromone produced by the various queens. A primer pheromone is also involved in the execution of extra queens by workers, but whether the same pheromone is responsible for all of these effects is unknown (Hölldobler and Wilson, 1990). Queen extract also possesses a primer pheromone activity that suppresses the production of juvenile hormone, which is sufficient to inhibit reproductive development (Brent and Vargo, 2003).

The success of social insect colonies lies in all members of the society acting in concert and in a well-organized manner. At the foundation of social insect self-organization are sophisticated means of communication, the chemical mode being at the center of it. The chemical language of social insects is complex. Many of the pheromones have multiple effects, acting as releaser and primer, and many other exocrine compounds that seem to not have a particular stand-alone function have a profound effect on the reaction of colony members to other pheromones. In summary, it is quite evident that pheromones play a crucial role in colony self-organization, affecting almost every aspect of social life, from the regulation of reproductive skew to division of labor and task allocation, and that we are far from understanding all the effects and mechanisms involved (Conte and Hefetz, 2008).

Pheromonal modulation of associative learning has been recently reported in the honey bee. The queen mandibular pheromone (QMP, mentioned in 3.1.), which is responsible for social cohesion around the queen, worker sterility and retinue behavior by young nurses, also suppresses aversive learning, as assessed by SER conditioning, in young but not in adult bees (Vergoz et al., 2007a). Young bees exposed to a queen, to extracts of QMP or to its main component homovanillyl alcohol (HVA) (Fig. 6), are incapable to learn the odor-shock association. However, they are not impaired in their capacity to learn appetitive associations such as odor-sucrose associations in harnessed conditions. This suggests that the QMP suppresses any aversive experience of young bees around the queen to strengthen the bonds between these bees, which care about the queen, and the queen itself as the target of attendance behavior. Beggs et al. (2007) highlighted the chemical structural similarity between HVA and DA and tested whether QMP-induced changes affect the responsiveness of brain tissues to DA. They showed that isolated MB calyces from QMP-exposed bees additionally exposed to either DA or HVA responded the same way, i.e. by a small reduction in levels of cyclic adenosine monophosphate (cAMP, second messenger used for intracellular signal transduction). Vergoz et al. (2007*a*) also showed that blocking of dopaminergic, but not octopaminergic, receptors suppresses aversive learning. It is therefore possible that HVA, the main component of QMP, would interact with dopamine receptors, possibly by down-regulating the expression of a particular type of dopamine receptor (Beggs et al., 2007). Yet the exact mechanisms of QMP modulation of aversive learning remain unknown.

Further research on honey bees has uncovered that appetitive olfactory learning is impaired by prior exposure to the sting alarm pheromone (SAP), which, when released by guards, recruits workers to defend the hive. This effect is mimicked by the SAP main component IPA, which is dose-dependent and lasts up to 24 h. Learning impairment is specific to alarm-signal exposure and is independent of the odor used for conditioning. These results suggest that learning impairment is a response to the biological significance of SAP as an alarm signal, which would distract bees from responding to any appetitive stimuli in a situation where such responses would be of secondary importance with respect to hive defense (Urlacher et al., 2010).

These examples show that, besides triggering stereotyped responses, pheromones can act on behaviors that can be modified through individual experience by affecting their intensity, success or probability of occurrence. As releaser pheromones are mostly used during outside

tasks, suited to adjust to changes in the environment, and require a pre-existing network which allows fast and reversible responses, they might have a role in the frame of activational behavioral plasticity.

Objectives

The general objective of this research project was to achieve an integrated understanding of how pheromones can act as modulators of reflex responses, learning, and decision making of insects, besides their specific known function of inducing stereotyped behaviors. We focused on three species that are models for fundamental and applied biological research with a capital importance in economic activities: the honey bee *Apis mellifera*, and the ants *Linepithema humile*, and *Camponotus aethiops*.

The first objective was to determine whether we could obtain modulations of appetitive or aversive responsiveness in *Apis mellifera* and *Linepithema humile*, using pheromones signaling very different contexts (**Chapter 1**).

In order to make the link between reflex responses and the natural conditions in which an organism can actually express these, we studied the modulation of decision making in the frame of nestmate discrimination by an alarm pheromone in ants (**Chapter 2**).

Given the inter-correlation between responsiveness and learning, we addressed the question of whether learning was modulated by pheromones and whether this modulation was consistent with the one of responsiveness (**Chapter 3**).

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CHAPTER 1: Pheromonal modulation of responsiveness

Pheromones modulate responsiveness to a noxious stimulus in honey bees

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Abstract

Pheromones are chemical substances released into the environment by an individual, which trigger stereotyped behaviors and/or physiological processes in individuals of the same species. Yet, a novel hypothesis has suggested that pheromones not only elicit innate responses but also contribute to behavioral plasticity by affecting the subjective evaluation of appetitive or aversive stimuli. To test this hypothesis, we exposed bees to three pheromonal components whose valence was either negative (i.e. associated with aversive events: isopentyl acetate and 2-heptanone) or positive (i.e. associated with appetitive events: geraniol). We then determined the effect of this exposure on the subjective evaluation of aversive stimuli by quantifying responsiveness to a series of increasing electric shock voltages before and after exposure. Two experiments were conducted varying the time lapse between shock series (15 min in experiment 1, and 24 h in experiment 2). In experiment 1, we observed a general decrease of shock responsiveness caused by fatigue, due to the short lapse of time between the two series of shocks. This decrease could only be counteracted by isopentyl acetate. The enhancing effect of isopentyl acetate on shock responsiveness was also found in experiment 2. Conversely, geraniol decreased aversive responsiveness in this experiment; 2-heptanone did not affect aversive responsiveness in any experiment. Overall, our results demonstrate that certain pheromones modulate the salience of aversive stimuli according to their valence. In this way, they would affect the motivation to engage in aversive responses, thus acting as modulators of behavioral plasticity.

KEY WORDS: Behavioral plasticity, Alarm pheromones, Aggregation pheromone, Aversive responsiveness, Sting extension response, Apis mellifera

Introduction

Pheromones are intraspecific chemical messengers playing a fundamental role in animal communication (Karlson and Lüscher, 1959; Wyatt, 2014). These signals are usually released into the environment, which trigger stereotyped behaviors and/or physiological processes in individuals of the same species that perceive them. Besides this well-documented pheromonal action, a novel hypothesis suggests that pheromones not only elicit innate responses but also contribute to behavioral plasticity by modulating innate responsiveness to reinforcement stimuli and thus the learning and memorization of cues predicting such reinforcements (Baracchi et al., 2017).

Honey bees are appropriate study organisms for testing this hypothesis. Their social lifestyle relies on a highly efficient division of labor among castes (Page et al., 2006; Wilson, 1971; Winston, 1987) and on sophisticated communication codes. The latter includes dances used to signal the presence of profitable food sources or nest sites (von Frisch, 1967), and a rich spectrum of pheromones, which regulate multiple social interactions and individual behaviors (Free, 1987). Several pheromones have been identified in *Apis mellifera*, and the neural circuits devoted to pheromone processing in the bee brain have also been studied (Carcaud et al., 2015; Roussel et al., 2014; Sandoz et al., 2007; Wang et al., 2008). Furthermore, innate responses to appetitive stimuli (Page and Erber, 2002; Scheiner et al., 2004) and aversive stimuli (Roussel et al., 2009; Tedjakumala and Giurfa, 2013) have been thoroughly characterized through standardized protocols in this insect, thus enabling the study of reinforcement responsiveness and the impact of pheromones on these responses.

Here, we focused on aversive responsiveness, which can be quantified through the sting extension response (SER) to electric shocks (Roussel et al., 2009; Tedjakumala et al., 2014; Tedjakumala and Giurfa, 2013), and on three pheromone components, which differ in valence and social context: geraniol, 2-heptanone (2H) and isopentyl acetate (IPA). Geraniol is the main component of the Nasanov gland, which elicits attraction and aggregation of receiver honey bee workers (Boch and Shearer, 1962; Butler and Calam, 1969). As this pheromone component signals valuable resources, triggers attraction and relates to an appetitive searching motivation, we characterize it as a 'positive-valence pheromone'. The alarm substance 2H is released by the mandibular glands of workers and exerts a repellent action on intruders and robbers from other hives (Shearer and Boch, 1965). Isopentyl acetate (also called isoamyl acetate) is the main component of the sting alarm pheromone released by the Koschevnikov gland of workers, which causes receiver bees to sting, attack (Boch et al., 1962) and stop foraging (Butler and

Free, 1952; Free et al., 1985). As 2H and IPA signal potential noxious or aversive situations/stimuli, and trigger attack or avoidance behaviors, we characterize them as 'negative-valence pheromones'.

Aversive responsiveness is quantified via the propensity to exhibit SER to a series of increasing voltages. SER can be systematically triggered in harnessed bees by the delivery of mild electric shocks (Lenoir et al., 2006; Núñez et al., 1997, 1983; Vergoz et al., 2007). Sting responsiveness to shocks varies among bees within a colony (Lenoir et al., 2006; Roussel et al., 2009). For instance, foragers exhibit higher sting extension responsiveness than guards when stimulated with a series of increasing voltages. Sensitivity to noxious stimulation determines behavioral specializations within the hive, thus contributing to the social organization of the colony (Roussel et al., 2009; Tedjakumala and Giurfa, 2013).

We studied the impact of geraniol, 2H and IPA on responsiveness to electric shocks in honey bee foragers. We measured shock responsiveness, exposed the same bees to pheromones and then re-measured their shock responsiveness. We hypothesized that negative- and positive-valence pheromones exert different modulatory effects on responsiveness assessed via SER: the former would increase SER by providing further aversive contextual cues while the latter would decrease it, as appetitive signals may detract the bees from aversive behaviors (Nouvian et al., 2015). According to this view, pheromones (and their main components) would modulate the bees' subjective evaluation of aversive stimuli, thus contributing to behavioral plasticity.

Materials and methods

Experiments were conducted at the Research Center on Animal Cognition, at the campus of the University Paul Sabatier (43°33′N, 1°28′E; 150 m above sea level). We used European honey bee female workers *Apis mellifera* L., typically 2–3 weeks old, collected at the apiary of our institute. Only nectar foragers caught at an artificial feeder containing 30% (w/w) sucrose solution were used as these bees are highly responsive to electric shocks (Roussel et al., 2009). Bees were captured in glass vials upon landing on the feeder and before they started feeding to control for the volume of liquid contained in their crop, which may influence electric conductivity and thus the subjective strength of electric shocks. They were then brought to the laboratory, which was maintained at a constant temperature of 25°C. Each bee was its own reference as aversive responsiveness was measured before and after pheromone exposure. Two experiments were performed in which the period of time between the two measurements of aversive responsiveness was varied: in experiment 1, it was 15 min, and in experiment 2, it was

24 h. Experiment 2 thus allowed for a recovery of aversive responsiveness between the two shock series and controlled for a possible effect of fatigue and/or sensory adaptation in the aversive responses measured after pheromone exposure. Fig. 1 summarizes the experimental procedure for the two experiments, which was the same except for the time elapsed between the two shock series.

Preparation of the bees

In the laboratory, bees were rapidly cooled on ice until they showed the first signs of immobility. Subsequently, they were harnessed with tape in holders consisting of two copper plates fixed to a plastic base, as previously described (Núñez et al., 1997; Vergoz et al., 2007). The bee's body thus made a bridge between the two plates, which facilitated the delivery of the electric shocks; 0.05 ml of EEG gel (Spectra 360 Electrode Gel, Parker Laboratories, Fairfield, NJ, USA) was placed between the copper plates to obtain a good contact between the plates and the thorax of the bee (neck and propodeum fitted into the notches of the plates). The bees were then fed with 5 µl of 50% (w/w) sucrose solution and placed in an incubator (at 28°C and 48% relative humidity) in the dark for 2 h. This resting time ensured that the bees adapted to the new harnessed situation. They were randomly assigned either to a control group that did not experience pheromone exposure or to an experimental group that was exposed to a given pheromone (one group per pheromone).

Measurement of shock responsiveness

Two identical set-ups were used in parallel, one for the control group and the other for the experimental group. Each set-up consisted of a Plexiglas box where a holder containing a bee could be connected to the output of an electric stimulator (50 Hz AC current). An air extractor was placed behind each holder to avoid the potential accumulation of alarm pheromone released by the bee upon shock delivery. When the holders were plugged into the setups, a timer was triggered and a series of 2 s electric shocks of increasing voltage was delivered to the bee, with a 2 min inter-shock interval to avoid sensitization. Voltages followed an ascending log series of 0.25, 0.5, 1, 2, 4 and 8 V (Roussel et al., 2009). Between and during shocks, the occurrence of SER was recorded as a binary variable (1 when the sting length exceeded that of the last two segments of the abdomen and 0 when this was not the case). If the bee responded several times during a single shock, only one response was noted. Bees that did not respond to any of the six voltages (7 out of 472 bees, i.e. 1.48%) were excluded from the analyses (pre-established, standard criterion). From these bees, only 4 did not respond after a specific treatment: 2 after 2H exposure, 1 after IPA exposure and 1 after mineral oil exposure. In experiment 1, where the

lapse of time between the two shock series was 15 min, bees were exposed to the pheromone immediately at the end of the first shock series (Fig. 1).

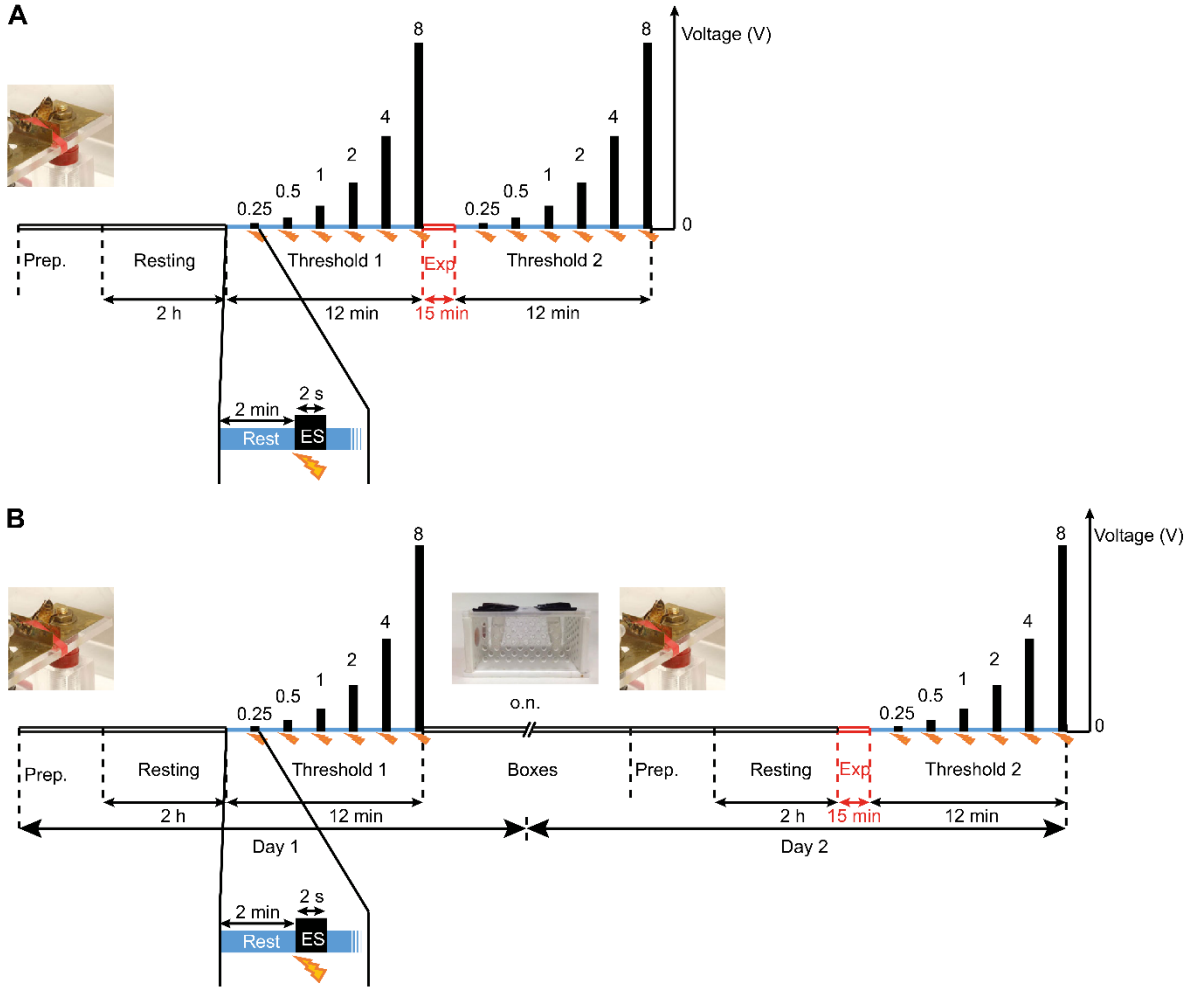

Figure 1. Experimental schedule of experiments 1 and 2. (A) Experiment 1; (B) experiment 2. In both cases, bees were captured at the beginning of the experiment and randomly assigned either to a control or to an experimental group run in parallel (see Materials and methods for more details). Prep.: preparation of the bees; ES: electric shock; o.n.: overnight; Exp.: exposure (to pheromone in the experimental group and to mineral oil in the control group). The pictures show a harnessed bee in the shock delivery setup (Vergoz et al., 2007) and a beekeeping box in which bees stayed overnight in experiment 2.

Thus, pheromone exposure occupied the 15 min lapse of time between shock series. In experiment 2, where the lapse of time was 24 h, bees were placed back in the incubator after the end of the first shock series. At the end of the day, they were released and individually placed in boxes with water and 50% (w/w) sugar solution *ad libitum*; boxes were then placed in the incubator. The following day, bees were cooled on ice and harnessed again. Harnessing was followed by a subsequent resting period in the incubator, which lasted 2 h. Bees were exposed to pheromone or mineral oil after this rest period. Then, the second series of shocks took place. Care was taken to ensure that shocks were delivered during the same hours as the

previous day to avoid any circadian effect on responsiveness. In all cases, we kept track of the identity of each bee. In both experiments, once the second series of electric shocks was finished, bees were killed by placing them in the freezer (−22°C). At the end of the day, glass vials were cleaned with detergent and water, and holders and set-ups were cleaned with ethanol to avoid odor marks.

Pheromone exposure

Bees belonging to the control group were exposed to 25 µl of mineral oil while experimental groups were exposed to one of the three pheromone components: geraniol, IPA or 2H. All chemicals were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Bees were individually confined for 15 min in a 137 ml glass vial containing a filter paper (1×5 cm) soaked with the pheromone component placed under a hood (Baracchi et al., 2017). The entire exposure process took place under a hood to avoid contamination between controls and pheromone-exposed bees. All pheromone substances were diluted to 24% (6µl pheromone+19µl mineral oil) (Baracchi et al., 2017; Urlacher et al., 2010). For IPA, this volume corresponded to the amount of IPA contained in 3–10 sting glands (Hunt et al., 2003). For 2H, we used the amount corresponding to that found in 1–3 mandibular glands of foragers (Vallet et al., 1991). In the case of geraniol, which is produced by the Nasanov gland, we used the same amount as for the other two pheromones as this gland has no reservoir (Snodgrass, 1956). In all cases, the amount of pheromone chosen corresponds to natural aversive or appetitive situations recruiting several bees at the same time.

In both experiments, a treatment consisted of a pheromone-exposed group and of its control run in parallel. In experiment 1, six replicates were performed for the geraniol treatment (n=86 bees; 43 for geraniol-exposed and 43 for mineral oil-exposed), 2H treatment (n=96 bees; 48 for 2H-exposed and 48 for mineral oil-exposed) and IPA treatment (n=96 bees; 49 for IPA-exposed and 47 for mineral oil-exposed). In experiment 2, we performed four replicates for geraniol treatment (n=48 bees; 25 for geraniol-exposed and 23 for mineral oil-exposed) and 2H treatment (n=56 bees; 28 for 2H-exposed and 28 for mineral oil-exposed) and six replicates for IPA treatment (n=83 bees; 42 for IPA-exposed and 41 for mineral oil-exposed). After the 15 min of pheromone/mineral oil exposure, bees were directly placed in their respective set-ups for assessment of aversive responsiveness.

Statistical analysis

We performed between-group comparisons to determine whether differences existed between bees exposed to mineral oil (control group) and bees exposed to one of the three pheromone components (experimental group). Furthermore, we performed within-group comparisons to determine whether differences could be detected before and after exposure in the same group of bees. We conducted three distinct analyses for each treatment (geraniol, 2H, IPA). The response data acquired from SER during both shocks and inter-shock intervals were fitted to general linear mixed models (GLMMs) using the *glmer* function of the *lme4* package (Bates et al., 2015). SER served as a binary-response variable (binomial family, 'logit' link), while group (control/experimental) and exposure (before/after) were entered as fixed effects and voltage as covariate. We included the bees' identity as a random effect, to account for the repeated measurements performed, and nested it into the replicates to account for the fact that bees tested within a given replicate were probably more affected by similar conditions (weather, pressure, etc.) than those tested in different replicates.

Previous papers have shown that SER increases with voltage (Balderrama et al., 2002; Núñez et al., 1997; Roussel et al., 2009; Tedjakumala et al., 2014), an effect that was found in all groups of our experiments (Figs 2 and 3). Therefore, we did not focus on the interaction of voltage with other factors but instead focused on the interaction of group with exposure in order to achieve between group and within-group comparisons (see above). To this end, we used a least-squares means (LSM) post hoc procedure with Bonferroni correction for multiple comparisons (*lsmeans* function from R package *lsmeans*; Lenth, 2016). In all cases, data met the assumptions of the tests used. All statistical analyses were performed with the open software R-3.3.1 (http://www.R-project.org/). The entire datasets are available upon request from the corresponding author (M.G.).

Results

Experiment 1

We evaluated SER responsiveness to a series of increasing voltages before and after pheromone or mineral oil exposure. Bees were exposed to their respective substance immediately after the end of the first shock series and the lapse of time between the two shock series was 15 min. Fig. 2 shows the responses of bees exposed to geraniol, 2H and IPA, and of their respective controls.

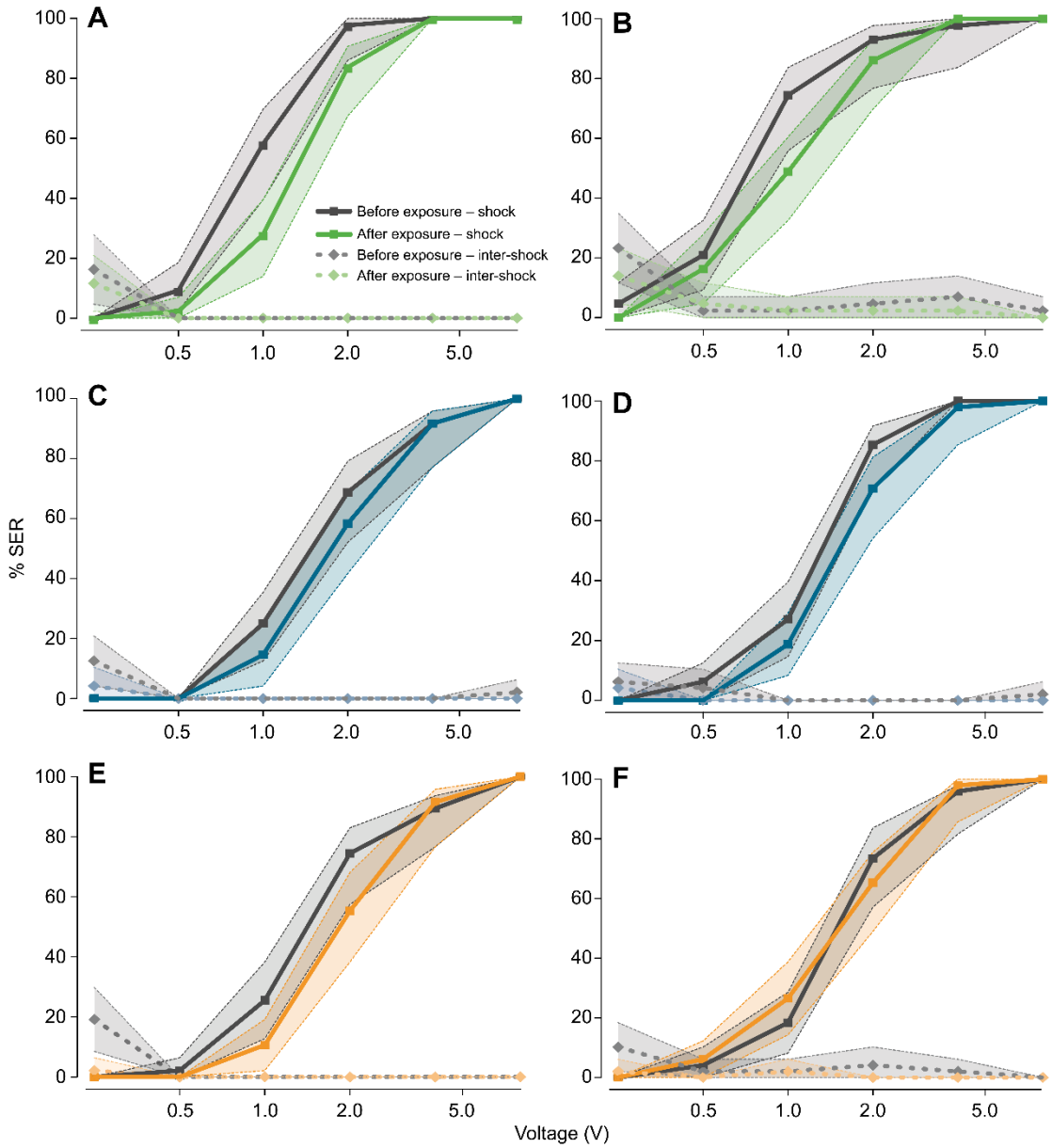

Figure 2. Experiment 1: effect of pheromone exposure on population shock responsiveness with 15 min between shock series. Shock responsiveness was recorded as the number of bees responding with SER to a given voltage. The abscissa is represented on a logarithmic scale. Solid lines represent responses to electric shocks (dark gray: before exposure; colored: after exposure). Dashed lines represent responses during inter-shock intervals (i.e. in the absence of shock; gray: before exposure; colored: after exposure). Curves are shown with their bootstrapped 95% confidence intervals (*BCa* function of the *bootBCa* package). (A,C,E) Control groups exposed to mineral oil between the two shock series; (B,D,F) groups exposed to pheromone between the two shock series. (A,B) Control (n=43, A) and geraniol (n=43, B). (C,D) Control (n=48, C) and 2H (n=48, D). (E,F) Control (n=47, E) and isopentyl acetate (IPA, n=49, F). While inter-shock responsiveness remained low and was not affected by pheromone/mineral oil exposure, shock responsiveness varied between the two series of shocks. The short lapse of time between these two series induced a general decrease of responsiveness in both control (A,C,E) and pheromone-exposed groups (B,D), which was due to fatigue. Only in the case of IPA (F) did shock responsiveness remain unchanged, thus showing that this pheromone component was able to counteract the fatigue effect, restoring responsiveness to original levels.

As expected, SER to electric shocks (Fig. 2, solid lines) increased significantly in all

bees as voltage increased (Fig. 2A,B: geraniol treatment including experimental and control groups; χ^2=60.63, d.f.=1, P<0.001; Fig. 2C,D: 2H treatment including experimental and control groups; χ^2=90.72, d.f.=1, P<0.001; Fig. 2E,F: IPA treatment including experimental and control groups; χ^2=138.99, d.f.=1, P<0.001). A comparison of responses between the first and second series of shocks (dark-gray versus colored solid lines) revealed a decrease of responsiveness during the second series in both the geraniol treatment (Fig. 2A,B: χ^2=16.48, d.f.=1, P<0.001) and the 2H treatment (Fig. 2C,D: χ^2=4.13, d.f.=1, P=0.04). For these bees, the interaction between group and exposure was not significant (geraniol treatment: χ^2=1.15, d.f.=1, P=0.28; 2H treatment: χ^2=0.13, d.f.=1, P=0.72), thus showing that control and experimental bees exhibited the same decrease of responsiveness between the two shock series. In the case of IPA treatment (including experimental and control groups), the interaction between group and exposure was significant (Fig. 2E,F: χ^2=6.25, d.f.=1, P=0.01). Significance was due to the fact that control bees decreased their responsiveness during the second series of shocks (LSM post hoc with Bonferroni correction, before versus after: P=0.01), while IPA-exposed bees maintained the same responsiveness (LSM post hoc with Bonferroni correction, before versus after: P=1).

During inter-shock intervals (i.e. in the absence of shock), bees exhibited a low responsiveness (Fig. 2, dashed lines). However, this responsiveness was not the same at each inter-shock interval (GLMM, χ^2=93.33, d.f.=5, P<0.001). A high percentage (up to 20%) of bees responded during the 2 min before the first shock, which corresponded to the stressful period following placement in the set-up. Thereafter, SERs decreased significantly during the other inter-shock intervals (1st versus 2nd: P<0.001, 1st versus 3rd: P<0.001, 1st versus 4th: P<0.001, 1st versus 5th: P<0.001, 1st versus 6th: P<0.001). None of the treatment groups exhibited a significant interaction between group (control/experimental) and exposure (before/after) (Fig. 2A,B: χ^2=0.14, d.f.=1, P=0.71; Fig. 2C,D: χ^2=0, d.f.=1, P=0.97; Fig. 2E,F: χ^2=0.08, d.f.=1, P=0.78), thus showing that pheromones did not affect inter-shock responsiveness.

Taken together, the results of experiment 1 show that the short lapse of time between the two shock series induced a general decrease in shock responsiveness, which may have been due to fatigue. Only IPA was able to counteract this effect by keeping general responsiveness at the same level as that observed prior to pheromone exposure.

Experiment 2

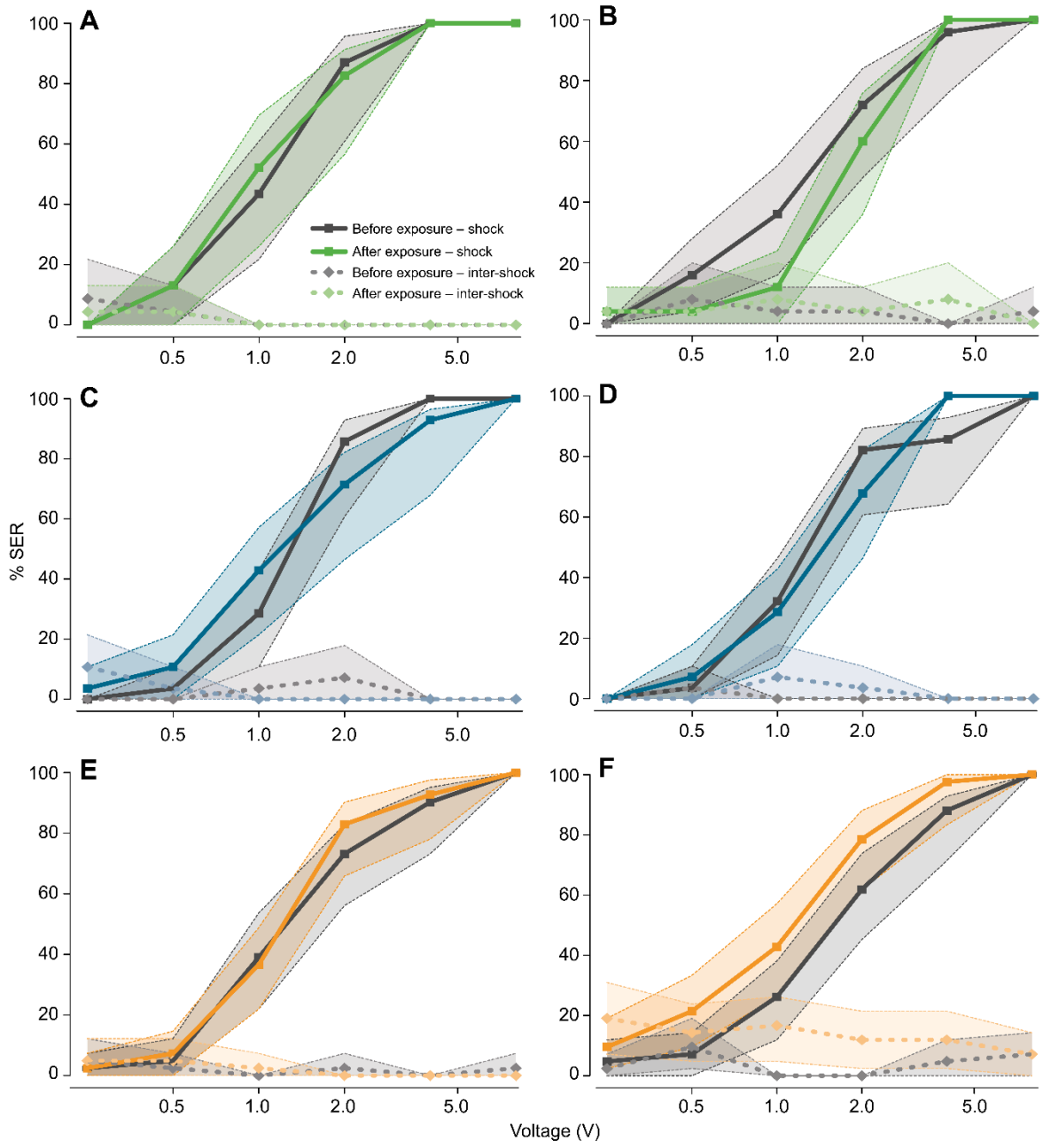

Figure 3. Experiment 2: effect of pheromone exposure on population shock responsiveness with 24 h between shock series. Shock responsiveness was recorded as the number of bees responding with SER to a given voltage. The abscissa is represented on a logarithmic scale. Solid lines represent responses to electric shocks (dark gray: before exposure; colored: after exposure). Dashed lines represent responses during inter-shock intervals (i.e. in the absence of shock; gray: before exposure; colored: after exposure). Curves are shown with their bootstrapped 95% confidence intervals (*BCa* function of the *bootBCa* package). (A,C,E) Control groups exposed to mineral oil between the two shock series; (B,D,F) groups exposed to pheromone between the two shock series. (A,B) Control (n=23, A) and geraniol (n=25, B). (C,D) Control (n=28, C) and 2H (n=28, D). (E,F) Control (n=41, E) and IPA (n=42, F). Inter-shock responsiveness remained low and was not affected by mineral oil, geraniol or 2H exposure (A–E). However, IPA significantly enhanced responsiveness in the absence of shock (F). Responsiveness to electric shocks varied depending on the pheromone to which the bees were exposed. After geraniol exposure (B), bees responded less to electric shocks, while they increased their responses after IPA exposure (F).

In this experiment, bees were exposed to their respective treatment after a lapse of time of 24 h. Fig. 3 shows the responses of bees exposed to geraniol, 2H and IPA, and of their respective controls exposed to mineral oil. As in the previous experiment, all bees exhibited a significant increase of SER with voltage (Fig. 3A,B; geraniol treatment including experimental and control groups; χ^2=109.47, d.f.=1, P<0.001; Fig. 3C,D; 2H treatment including experimental and control groups; χ^2=96.63, d.f.=1, P<0.001; Fig. 3E,F: IPA treatment including experimental and control groups; χ^2=97.27, d.f.=1, P<0.001). A comparison of responses between the first and second series of shocks (Fig. 3; dark-gray versus colored solid lines) revealed that exposure to mineral oil did not affect responsiveness (LSM post hoc with Bonferroni correction, before versus after, P=1 for geraniol, 2H and IPA controls). Thus, in the control groups, the 24 h lapse of time allowed recovery from the first series of electric shocks. In the experimental groups exposed to pheromones, different patterns of responses were observed. Bees exposed to 2H did not change their shock responsiveness, as shown by a non-significant interaction between group and exposure (Fig. 3C,D; solid lines; χ^2=0.02, d.f.=1, P=0.89). In contrast, bees exposed to geraniol and IPA varied their shock responsiveness and in consequence the interaction between group and exposure was significant (Fig. 3A,B; geraniol: χ^2=4.26, d.f.=1, P=0.04; Fig. 3E,F; IPA: χ^2=5.20, d.f.=1, P=0.02). Specifically, in the geraniol treatment, control and experimental groups behaved differently after exposure (LSM post hoc with Bonferroni correction, P<0.05) as experimental bees tended to respond less after geraniol exposure than before (LSM post hoc with Bonferroni correction, P=0.07). In the case of IPA, experimental bees responded more after exposure than before (LSM post hoc with Bonferroni correction, P<0.001). However, control and experimental bees reached similar levels of response after exposure (LSM post hoc with Bonferroni correction, P=1).

The analysis of responsiveness during the inter-shock intervals (Fig. 3, dashed lines) revealed again that responsiveness was low and decreased in the absence of shock (χ^2=11.85, d.f.=5, P=0.04). In control bees (Fig. 3A,C,E), inter-shock responsiveness was not affected by mineral oil exposure (gray versus colored dashed lines; LSM post hoc with Bonferroni correction, before versus after, P=1 for geraniol, 2H and IPA controls). Similarly, exposure to geraniol and 2H (Fig. 3B,D) did not change inter-shock responsiveness as shown by the non-significant interaction between group and exposure (geraniol: χ^2=0.51, d.f.=1, P=0.48; 2H: χ^2=0.37, d.f.=1, P=0.54). This interaction was only significant for IPA (Fig. 3F; χ^2=4.32, d.f.=1, P=0.04). Bees exposed to IPA behaved differently after exposure when compared with their control group (LSM post hoc with Bonferroni correction, P<0.01). Moreover, experimental

bees increased their responsiveness after exposure to IPA (LSM post hoc with Bonferroni correction, $P<0.001$).

Taken together, the results of experiment 2 show that the long lapse of time (24 h) between the two shock series restored shock responsiveness and that IPA and geraniol exerted opposite effects on aversive responsiveness; IPA enhanced it and geraniol decreased it.

Discussion

Our study aimed at investigating the role of pheromones as modulators of bees' subjective evaluation of aversive stimuli and thus at uncovering a non-canonical function of pheromones as key components of behavioral plasticity. To this end, we exposed bees to three pheromonal components of different valence (two negative, i.e. associated with aversive events, and one positive, i.e. associated with appetitive events) and determined the effect of this exposure on shock responsiveness using a within-group approach (comparison of SER responsiveness before and after exposure to two electric shock series of increasing voltage). As SER responsiveness to electric shocks provides a reliable readout of the bees' subjective evaluation of punishment (Roussel et al., 2009; Tedjakumala et al., 2014; Tedjakumala and Giurfa, 2013), changes in responsiveness following pheromone exposure show that pheromones are capable of behavioral modulation beyond the specific context in which they are released.

Two experiments were conducted to assess this effect with time lapses of either 15 min (experiment 1) or 24 h (experiment 2) between the two shock series. In both experiments we found a consistent enhancing effect of IPA on shock responsiveness. This enhancement even affected inter-shock responsiveness in experiment 2. Conversely, geraniol decreased aversive responsiveness in experiment 2 but not in experiment 1, although this may have been hidden by a fatigue effect due to the short lapse of time (15 min) between the two series of shocks. In this experiment, the only group not showing a decrease of responsiveness between shock series was the one exposed to IPA, thus indicating that this pheromone was able to counteract the fatigue-based decrease through its enhancement of aversive responsiveness. In both experiments, no effect of 2H on aversive responsiveness was found.

The effect of a positive-valence pheromone on the SER

Our results reveal the novel finding that geraniol, an appetitive pheromone component, has the capacity to modulate the subjective evaluation of aversive stimuli. Exposure to this substance decreased aversive responsiveness to electric shock, thus showing that it diminished the perceptual impact of shock in bees.

A recent study also found that innate appetitive floral odors (linalool and 2-phenylethanol), but not citral (another component of the Nasanov gland), diminish defensive responses (attack of a moving dummy) of honey bees (Nouvian et al., 2015). This could have been due to the lower concentration of citral used or to the caste employed (guards in their case, foragers in ours) as the function of the Nasanov gland changes with age (Boch and Shearer, 1963). Yet, the coincident fact is that an innate appetitive signal, geraniol in our case or two floral odors in Nouvian et al. (2015), down-regulated aversive responsiveness.

At first sight, this detraction of aversive behaviors by appetitive signals may appear counter-adaptive. Indeed, even though a food shortage might affect colony fitness on the long term, an alarm pheromone indicates an immediate danger, which might affect colony survival. It was thus suggested that appetitive floral odors, which are usually encountered away from the colony during foraging, could act as markers of distant foraging locations, thus detracting bees from their aggressiveness (Nouvian et al., 2015). This hypothesis provides a partial account of the geraniol effect, as this pheromone component is indeed released at attractive food sources (Free, 1987) but also at the nest entrance to orient returning foragers (Ribbands and Speirs, 1953) and swarms (Schmidt, 1994). Thus, rather than a location effect, the conflict between an appetitive signal (attractive floral odors, geraniol) and an aversive signal or context (enemy, electric shock) seems to be responsible for downregulating aversive responsiveness.

The effect of negative-valence pheromones on the SER

IPA and 2H are released in response to potential aversive situations (Boch et al., 1962; Shearer and Boch, 1965), although alternative functions have been reported for 2H (see below). It could be expected, therefore, that unlike geraniol, both pheromones provide a relevant alarm context enhancing aversive responsiveness. This hypothesis was only confirmed for IPA but not for 2H: the former increased shock responsiveness (or restored it to basal levels against fatigue) while the latter did not influence shock responsiveness.

The enhancement of shock responsiveness induced by IPA is similar to the one observed in Africanized honey bees (*Apis mellifera scutellata*) exposed to small amounts of this substance (0.3 µl versus 6 µl in our experiments; Balderrama et al., 2002). However, Africanized bees also decreased their shock responsiveness after being exposed to larger amounts of IPA (2.5, 5, 10 and 12.5 µl; Balderrama et al., 2002; Núñez et al., 1997). These values underline the known differences in aversive sensitivity between Africanized and European bees (Collins et al., 1982): the former are more defensive and react faster to smaller amounts of IPA while the latter are slower and require higher amounts to respond defensively.

The fact that we observed an enhancement of aversive responsiveness with 6 µl of IPA in our European bees, while only 0.3 µl was required in Africanized bees to induce a similar effect, is consistent with the reported variation in defensive behavior between these two races. As amounts above 2.5 µl induced an opposite effect (i.e. decreased shock responsiveness) in Africanized bees, amounts above a threshold value higher than 6 µl could produce a similar effect in European bees. Such a decrease has been explained by the activation of an opioid-like system by IPA, which would induce an analgesia-like state, depressing responsiveness to a noxious stimulus (Núñez et al., 1997). According to Núñez et al. (1997), 'the resulting stress-induced analgesia in the defender bee would reduce its probability of withdrawal thus increasing its efficiency against enemies'. This would be of particular importance in the context of a massive attack where all forces should be mobilized.

Unlike IPA, 2H did not affect shock responsiveness in our experiments. In the case of Africanized bees, Balderrama et al. (2002) found that large amounts (12.5 µl) of 2H increased shock responsiveness while small amounts (0.3 µl) did not affect it. Given the different sensitivity of Africanized and European bees to alarm signals, the intermediate amount of 2H we used (6 µl) could correspond to the small amounts assayed in Africanized bees. Furthermore, these values suggest that 2H is not directly associated with stinging responsiveness except if provided in massive doses. This is consistent with the results of Boch et al. (1970), who found that IPA is 20–70 times more efficient than 2H in eliciting alarm behavior at the hive entrance. Our results thus confirm the conclusion that IPA and 2H have different functions (Balderrama et al., 2002). IPA would act as a 'true' alarm pheromone, triggering SER, while 2H could act as an alarm signal, which would be insufficient to trigger SER. Interestingly, alternative functions have been suggested for 2H; it has been identified as an eventual paralyzing agent of enemies bitten by the bees (Papachristoforou et al., 2012) and as a potential negative scent mark to label recently visited and depleted food sources (Giurfa, 1993; Vallet et al., 1991). This multiple functionality could attenuate the impact of 2H on shock responsiveness.

Pheromone modulation contributes to behavioral plasticity

Our findings underline the role of pheromones as potential modulators of different behaviors, depending on their valence and dose. Such modulation could take place at two basic levels: the perceptual one, thus affecting the evaluation of the shock, and/or the motor-output one, thus affecting the production of SER. Distinguishing between these alternatives is difficult based on behavioral evidence; neural analyses would be necessary to determine whether and how their corresponding neural circuits are affected by pheromone exposure. In a recent study, Nouvian

et al. (2018) analyzed the stinging attacks of bees towards a rotating dummy, which could be in part assimilated to the stinging response measured here. This response is triggered by IPA, which is consistent with the enhancement of SER found in our work. Nouvian et al. (2018) quantified the levels of biogenic amines in the brain of stinging bees exposed to IPA and found that serotonin (5-HT) and dopamine (DA), but not octopamine (OA), were increased upon IPA exposure. As these two biogenic amines have been related to aggression and attentional processes (Tedjakumala et al., 2014; Tedjakumala and Giurfa, 2013), this finding can be linked to a modulatory effect of IPA on noxious-stimulus perception. At the motor-output level, analyses performed on isolated terminal abdominal ganglia of bees have shown that OA is a crucial modulator of SER (Burrell and Smith, 1995). This ganglion receives innervation from dorsal and ventral unpaired neurons, which are major releasers of OA (Stevenson and Sporhase-Eichmann, 1995). Not surprisingly, therefore, OA modulates several motor components of SER (Burrell and Smith, 1995). The fact that IPA exposure does not affect brain levels of OA (Nouvian et al., 2018) seems to favor the hypothesis that the modulatory effect of pheromones found in our work occurs at the perceptual rather than the motor level. Alternatively, the two levels could be affected sequentially with extremely short delays. Whether and how the increase in 5-HT and DA found upon IPA exposure translates into a major release of OA for motor control of SER remains to be determined.

The pheromonal modulation of noxious-stimulus perception is consistent with a new model describing the decision-making process underlying the defensive response of bees (Nouvian et al., 2015). In this model, an individual defensive threshold resulting from the integration of intrinsic (e.g. genetic traits, caste, age, etc.) and extrinsic (e.g. weather, season, available resources, etc.) factors would be weighed against an internal score to determine whether the bee engages in colony defense (Fig. 4A). We suggest that pheromones change this threshold, and that this change depends on pheromone valence. Negative pheromones, associated with aversive, dangerous events, would move the threshold closer to the score that needs to be overcome to elicit defensive responses, while positive pheromones would move the threshold away from the score, thus detracting bees from defensive behaviors (shaded arrow and red bar in Fig. 4A). A similar scheme can be proposed for appetitive behaviors such as foraging (Fig. 4B). In this case, an appetitive threshold determined by intrinsic and extrinsic factors would be weighed against an internal score to decide whether a bee engages in appetitive search behavior. In this case, positive pheromones would move the threshold closer to the score value, thus facilitating foraging, while negative pheromones would move it away from the

score, thus inhibiting foraging (shaded arrow and green bar in Fig. 4B).

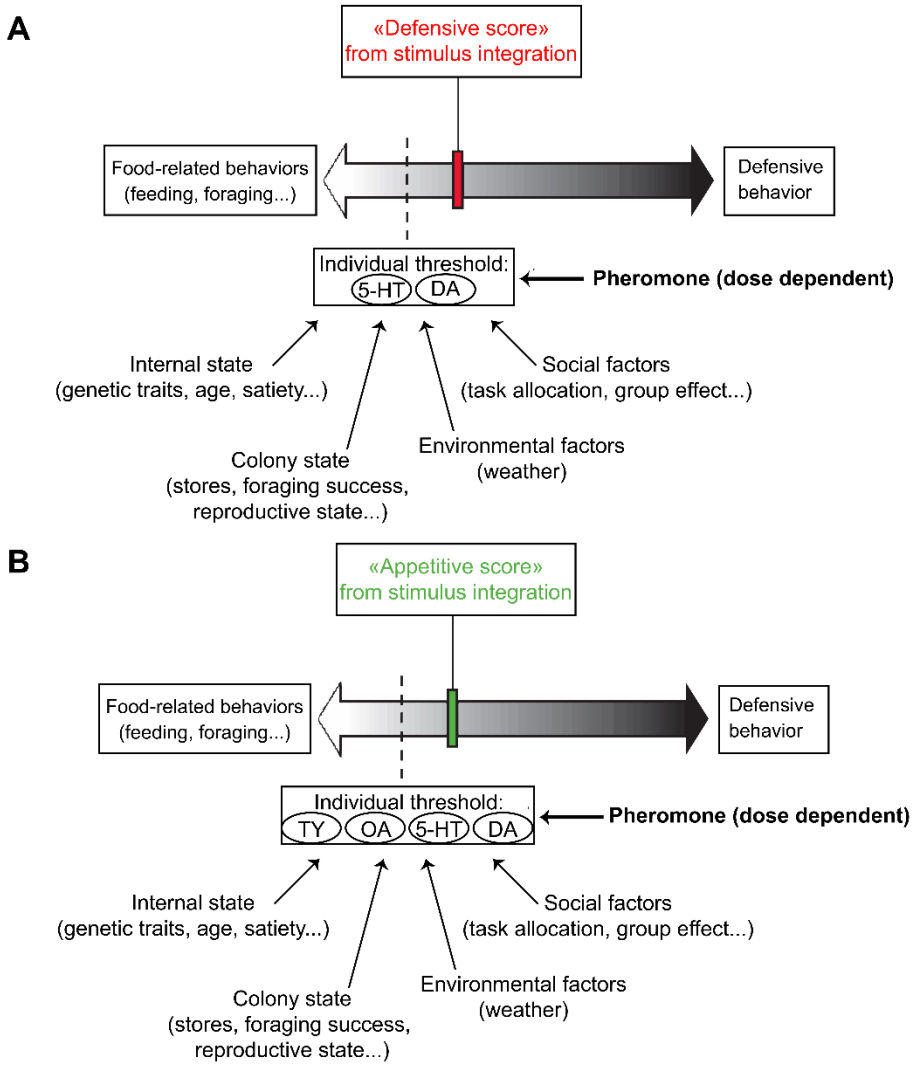

Figure 4. A model accounting for the modulatory effect of pheromones on decision making in honey bees. The model (adapted from Nouvian et al., 2015) postulates that each individual is characterized by a defensive and an appetitive threshold, which are determined by extrinsic and intrinsic factors. Pheromones may act on this threshold, moving it away from or towards a score that needs to be overcome to elicit a specific behavior. (A) Defensive score and its relationship with a defensive-response threshold. Pheromones modify the threshold depending on their valence (shaded arrow and red bar). Positive, appetitive pheromones move it away from the score, thus decreasing the probability of a defensive response. Negative, aversive pheromones have the opposite effect, moving the threshold towards the score and thus increasing the probability of a defensive response. (B) Appetitive score and its relationship with an appetitive-response threshold. Pheromones modify the threshold depending on their valence (shaded arrow and green bar). Positive, appetitive pheromones move the threshold towards the score, thus increasing the probability of an appetitive response. Negative, aversive pheromones have the opposite effect, moving the threshold away from the score, thus decreasing the probability of an appetitive response. 5-HT, serotonin; DA, dopamine; TY, tyramine; OA, octopamine.

The appetitive scenario proposed (Fig. 4B) is consistent with the findings of a recent paper, which reported the effect of the same pheromones used in our work (at the same concentration) on an appetitive innate response, the proboscis extension response (PER), which is triggered by

the contact of sucrose receptors on the antennae with sucrose solution (Baracchi et al., 2017). The authors investigated whether geraniol, 2H and IPA modulate appetitive responsiveness to sucrose and habituation to sucrose stimulation. Pheromones associated with an aversive context induced a significant decrease of sucrose responsiveness as 40% and 60% of bees exposed to IPA and 2H, respectively, did not respond to any sucrose concentration. In bees that responded to sucrose, geraniol enhanced sucrose responsiveness while 2H, but not IPA, had the opposite effect. Taken together, our results and those of Baracchi et al. (2017) show that IPA increases shock responsiveness and suppresses sucrose responsiveness. In contrast, geraniol enhances sucrose responsiveness and decreases aversive responsiveness. These results demonstrate that the same pheromone, at the same concentration, can have different effects according to the context (i.e. appetitive or aversive) in which it is released. The case of 2H seems more complex because of the possible multiple roles of this pheromone (see above): Baracchi et al. (2017) found that 2H suppressed sucrose responsiveness in 60% of the bees and down-regulated this responsiveness in the remaining 40%; in our case, no effect on aversive responsiveness was detected.

The modulatory effect of pheromones might be based on the action of these chemicals on different aminergic circuits modulating behavior. In the honey bee, several studies have shown that OA acts as a crucial neuromodulator of appetitive responses (Hammer, 1993; Scheiner et al., 2002) while DA and 5-HT are involved in aversive responses (Tedjakumala et al., 2014; Vergoz et al., 2007). Recent studies in the bee have cast doubt about the validity of such a clear separation between OA and DA in appetitive and aversive reinforcement signaling, respectively (Klappenbach et al., 2013). Irrespective of this, pheromones could regulate the balance of the biogenic amines contained in the bee brain, enhancing or depressing responsiveness to different kinds of stimuli according to their valence and context of release.

Through this non-canonical action (in the sense of not being associated directly with the response modulated, like the effect of geraniol on SER or of 2H on PER), pheromones would act on an animal's motivation to engage in a given behavior. Moreover, as pheromones change the subjective perception of stimuli, being attractive (sucrose) or aversive (electric shock), they may also have an impact on the capacity to learn about these stimuli. Bees that exhibit high responsiveness to sucrose solutions of variable concentration are better learners in olfactory and tactile conditioning protocols that use sucrose solution as a reward (Scheiner et al., 2001a,b). Similarly, the more sensitive bees are to an electric shock, the better they learn about that shock (Roussel et al., 2009). Therefore, the effect of pheromones might not only be restricted to

responsiveness and motivation but also could affect learning and memory via the modulation of the salience of an unconditioned stimulus. Thus, besides conveying stereotyped messages, pheromones have an important role as modulators of behavioral plasticity.

Acknowledgements

We thank Jean-Marc Devaud, David Baracchi, Margot Perez and Maria Eugenia Villar for valuable feedback on the experimental protocol and on a preliminary version of the manuscript. We also thank Lucie Hotier for beekeeping activities, Patrick Arrufat for his guidance and maintenance of the electric setups, Valentin Lecheval for advice on bootstrapping and Cristian Pasquaretta for advice on GLMMs.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: P.d'E., M.G.; Methodology: N.R.; Formal analysis: N.R.; Investigation: N.R.; Resources: P.d'E., M.G.; Writing - original draft: N.R., M.G.; Writing - review & editing: N.R., P.d'E., M.G.; Supervision: P.d'E., M.G.; Project administration: P.d'E., M.G.; Funding acquisition: P.d'E., M.G.

Funding

This work was supported by the Agence Nationale de la Recherche (Project ID: ANR-14-CE18-0003, PHEROMOD), the IDEX initiative 'Chaire d'Attractivité' of the Université de Toulouse to Patrizia d'Ettorre, and by the Centre National de la Recherche Scientifique.

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(Z)-9-hexadecenal modulates sucrose responsiveness in the Argentine ant *Linepithema humile*

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Abstract

Pheromones are chemical substances released into the environment by an individual, which trigger stereotyped behaviors and/or physiological processes in individuals of the same species. Yet, a novel hypothesis has suggested that pheromones not only elicit innate responses but also contribute to behavioral plasticity by affecting the subjective evaluation of appetitive or aversive stimuli. To test this hypothesis, we exposed ants to a synthetic trail pheromonal component, (Z)-9-hexadecenal, which is used in a context of foraging to locate and connect a food resource to the nest. We then determined the effect of this exposure on the subjective evaluation of appetitive stimuli by quantifying the number of ants feeding on drops of different sucrose concentrations and their feeding time. We observed a general increase of feeding ants on drops of sugar for all concentrations when they had been exposed to (Z)-9-hexadecenal, and this was especially true at 1% sucrose concentration. Pheromone exposure increased the duration of feeding time, especially at the highest sucrose concentration (20%). Overall, our results demonstrate that (Z)-9-hexadecenal modulates the perception and/or evaluation of a food source, making it more attractive, irrespective of the quality of the food. In this way, the pheromone component would affect the motivation to engage in foraging behavior, thus acting as a modulator of behavioral plasticity.

KEY WORDS: Behavioral plasticity, Trail pheromone, Appetitive threshold, Chemical communication, Social insects

Introduction

Chemical communication is widespread in nature. Most of the chemical signals used in communication are pheromones, i.e. substances emitted by an individual that elicit behavioral or physiological responses in individuals of the same species (Karlson and Lüscher, 1959). Pheromones are used in various contexts, such as foraging, defense, or reproduction (Wyatt, 2014) and they are key factors in coordinating social interactions and maintaining the organization of societies, such as those of social insects. Pheromones typically elicit a stereotyped response that is species-specific and does not depend on experience (Karlson and Lüscher, 1959; Wyatt, 2014). However, recent findings indicate that the biological effects of pheromones are more complex than previously thought as they can also act as modulators of either experience-dependent behaviors (Urlacher et al., 2010; Vergoz et al., 2007a) or behaviors that are not explicitly related with the specific message they convey (Baracchi et al., 2017; Rossi et al., 2018b). The first scenario is well exemplified by the fact that the queen mandibular pheromone of the honey bee *Apis mellifera* blocks aversive learning in young bees but leaves intact their appetitive learning abilities (Vergoz et al., 2007a); also, isoamyl acetate, an alarm pheromone component, impairs appetitive learning of adult bees (Urlacher et al., 2010). The second scenario is illustrated by the modulation of reward (Baracchi et al., 2017) and noxious-stimulus sensitivity (Rossi et al., 2018b) by pheromones that are not directly related to these stimuli in honey bees, demonstrating that these substances can act on behaviors that are not related to their original message. Therefore, pheromones appear to be key players in behavioral plasticity, an aspect that is still underexplored.

Social insects are ideal model organisms to study the modulatory effects of pheromones because of the importance of these molecules in their chemical communication and their odor-based behaviors. In ants, for instance, almost all functional categories of communication responses (from alarm to recruitment, recognition, social interactions and sexual communication) are pervaded by chemical signals (Vander Meer et al., 1998). This chemical way of communicating may be an adaptation to their underground lifestyle making them rely heavily on olfaction. One of the more obvious and characteristic forms of behavior of some ant species is the scent-marking and sharing by many workers of a common path to and from a source of food. This behavior is typically the result of a chemical secretion (a trail pheromone) that leads members of a same colony towards a food source. It is very likely that the terrestrial lifestyle of ants led them to use trail pheromones intensively. Ants encode information about the quality of their environment (e.g., food sources) by dropping varying amounts of

pheromones on their trails and this information is then used by other individuals for directional choices (Wilson, 1962). As more ants choose the trail with a higher concentration, more ants deposit pheromones on that trail, increasing its pheromone concentration and generating a positive feedback loop. For instance, Goss et al. (1989) showed that *Linepithema humile* select the shortest path to a food source by depositing pheromones on their way to and from the food.

The Argentine ant *L. humile* is native to South America and has expanded towards Mediterranean and subtropical areas around the world. It is considered an invasive species in these areas where it has been introduced because it displaces native ants (Suarez et al., 1998) and other arthropods (Cole et al., 1992), disrupts mutualisms (Bond and Slingsby, 1984), and facilitate honeydew-producing hemipteran pests such as mealybugs (Holway et al., 2002). The spread and establishment of Argentine ant populations is considered the consequence of their marked aggressiveness toward other species and their capacity for mass recruitment (Holway et al., 1998). The trail pheromone of the Argentine ant has been the focus of numerous studies because of its significance in the species' mass recruitment behavior. Wilson and Pavan (1959) showed that the ventral gland of workers is the source of pheromones that elicit a trail-following response. Cavill et al. (1979) first isolated and characterized (Z)-9-hexadecenal from dissected ventral glands as being a component of the trail pheromone complex of the Argentine ant, but conservatively referred to it as ''a general aggregation factor''. Yet, recently, Choe et al. (2012) published results from Solid Phase Micro Extractions (SPME) that could not detect this molecule in the trail pheromones of *L. humile*. They proposed that two other compounds (dolichodial and iridomyrmecin) found in the trails and in the pygidial gland would instead constitute the pheromone. However, given the fact that von Thienen et al. (2014) did not find trail following behavior with pygidial gland extract but confirmed previous observations with Pavan's gland extract and (Z)-9-hexadecenal, we chose to use this molecule in our experiments as it remains the best candidate for the trail pheromone.

We asked if, besides eliciting stereotyped responses, (Z)-9-hexadecenal modulates the appetitive responsiveness of *L. humile* workers, in particular when the pheromone is no longer present and without direct contact with the pheromone. We exposed the ants to the pheromone and then measured their sucrose responsiveness. We hypothesized that the trail pheromone exerts an incremental modulatory effect on responsiveness given the consistency between the context signaled and the task. According to this view, pheromones (and their main components) would modulate the ants' subjective evaluation of appetitive stimuli, thus contributing to behavioral plasticity.

Material and methods

Experiments were performed in May 2017 using one queenright *L. humile* colony that had been collected in March 2017 from its native range in Argentina at the campus of the University of Buenos Aires (34°32″48.3′S; 58°26″21.0′W). Ants were kept under controlled conditions (26 ± 1°C, 56 ± 6% humidity, natural light-dark cycle) in artificial nests that consisted of large plastic boxes (30 × 50 × 30 cm) with Fluon-painted walls to prevent escapes. The floor was covered with plaster (Paris type), on which a stack of acrylic plates (12 x 8 cm) served as a refuge. The colony was fed daily with honey-water and three times a week with cockroaches (*Blaptica dubia*). Water was provided *ad libitum*.

Group feeding behavior

Ants were collected each day from the nest and separated in acrylic pots (2 cm Ø and 3 in height) in groups of four. As *L. humile* workers are monomorphic, all the experimental ants were of a similar size. After two hours of rest in darkness, ants in their acrylic pots were confined for 15 min in a bigger closed 216 ml plastic pot containing a filter paper (1 x 5 cm) soaked with either 0.2, 0.4, 0.8, 1.6, or 3.2 µl of (Z)-9-hexadecenal (Carbosynth) under air extraction (independent groups). Ants were therefore only exposed to the pheromone and not directly put in contact with it. Control ants were submitted to the same conditions but without the pheromone.

In order to establish an effective volume of pheromone on feeding behavior, the four-ant groups were given a 3 µl drop of 5% w/w sucrose solution at the center of the acrylic pot after exposure. Scanning of the number of ants feeding at the drop was then performed every 30 sec for 3 min. A total of 302 ants were recorded feeding on the sucrose drop (N_C=72, $N_{0.2}$=53, $N_{0.4}$=45, $N_{0.8}$=51, $N_{1.6}$=45, $N_{3.2}$=36), 49 of which did not touch the drop and were excluded from analyses (N_C=15, $N_{0.2}$=9, $N_{0.4}$=4, $N_{0.8}$=14, $N_{1.6}$=3, $N_{3.2}$=4, final sample sizes: N_C=57, $N_{0.2}$=44, $N_{0.4}$=41, $N_{0.8}$=37, $N_{1.6}$=42, $N_{3.2}$=32).

Individual feeding behavior

The collection process was the same as for the group feeding experiment. After one hour of rest in darkness, 108 ants (27 groups of four ants in their acrylic pots) were confined for 20 min in a bigger closed 216 ml plastic pot containing a filter paper (1 x 5 cm) soaked with 1.6 µl of (Z)-9-hexadecenal (experimental group) under air extraction. The other half of the ants (N=112, 28 groups of four ants) were submitted to the same conditions but without the pheromone (control group). This chosen volume of (Z)-9-hexadecenal was based on the group feeding behavior

results (Fig. 1A).

The protocol was based on Sola et al. (2013). On each trial, after pheromone exposure (or control treatment), one ant at a time was gently placed on a bridge (2 × 50 mm^2, at half length) that ended in a feeding arena containing a 3 µl drop of sucrose solution. As sucrose concentration is a crucial parameter for foragers as an estimator of food quality (Scheiner et al., 2004), ants were offered one of four different sucrose concentrations: 1, 5, 10, and 20% w/w (independent groups) in order to test for a potential pheromonal modulation of ants' evaluation of these stimuli. The order of sucrose concentrations was randomized for each four-ant group and individual ants were only used once. We registered individual responses to the sucrose concentrations as binomial responses (feeding or not). A total of 187 ants were recorded feeding on the sucrose solutions (95 control ants vs. 92 exposed to (Z)-9-hexadecenal). Thirty three ants (15% in total, 17 control and 16 pheromone-exposed ants) were excluded from analyses because data could not be obtained (ants felt from the bridge or did not touch the drop). Ants were filmed from a lateral view whilst they were drinking using a camera-fitted stereomicroscope (Leica MZ8 –25× magnification–with a Leica ICA camera). Feeding time (sec) was obtained from the videos and was defined as the time during which the ant's mandibles were in contact with the solution. A total of 177 ants were video-taped feeding on the sucrose solutions (89 control ants vs. 88 exposed to (Z)-9-hexadecenal). Ten additional ants (six control and four pheromone-exposed) were excluded from analyses because they were not well positioned to determine if they were feeding or not.

Data analysis

Proportions of ants exposed to the pheromone or not and feeding on the sucrose drop during the group feeding experiment were compared with χ^2 tests. Individual feeding responses of ants were examined using an additive generalized linear model (GLM) with "*Concentration*" (i.e. 1, 5, 10 and 20%) and "*Treatment*" (i.e. pheromone or nothing) as factors and a binomial error structure (logit-link function, *glm* function of R). Post-hoc comparisons were performed at each sucrose concentration to compare treatments. Feeding time was examined using an additive linear model (*lm* function of R) with "*Concentration*" (i.e. 1, 5, 10 and 20%) and "*Treatment*" (i.e. pheromone or nothing) as factors. Permutation tests were then performed to make sure that inferences were not biased by the non-normal distribution of the data (*PermTest* function of the *pgirmess* R package, 1000 permutations). Post-hoc LMs were then applied at each sucrose concentration to compare treatments. All statistical analyses were performed with R 3.4.2 (R Development Core Team, 2016) and the significance threshold was set at

0.05.

Results

The percentage of ants feeding on a drop of sucrose after exposure in a 216 ml closed pot to different volumes of (Z)-9-hexadecenal (0.2, 0.4, 0.8, 1.6, or 3.2 µl) or to nothing (control group) is shown in Fig. 1. Overall, there were more ants feeding on the sucrose drop when exposed to the pheromone but the difference with control ants was only significant when using 1.6 µl of (Z)-9-hexadecenal (χ^2=9.97, df=1, P=0.002; P>0.05 in all other cases).

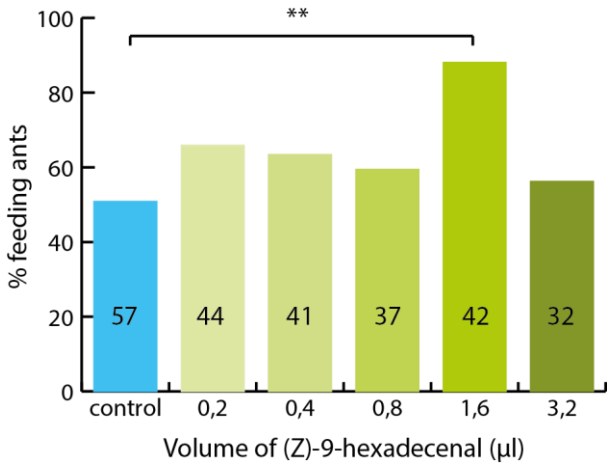

Figure 1. Volume-dependent feeding responses after pheromone exposure. Percentage of ants feeding on a 5% w/w sucrose drop (3 µl) after exposure in a 216 ml closed pot to different volumes of (Z)-9-hexadecenal or to nothing (control). There were more ants feeding on the sucrose drop when exposed to the pheromone but the difference with control ants was only significant when using 1.6 µl of (Z)-9-hexadecenal. Numbers inside bars indicate sample sizes. X^2 test, P=0.002.

The percentage of experimental and control ants that ingested sucrose when presented with different sucrose concentrations (1, 5, 10, and 20%) is shown in Fig. 2A. The percentage of ants that ingested sucrose increased with sucrose concentration, both for the experimental and control groups (GLM, *Concentration*: χ^2=30.74, df=3, P<0.001), but there were more pheromone-exposed ants that fed compared to control ants (GLM, *Treatment*: χ^2=12.25, df=1, P<0.001). In particular, significantly more pheromone-exposed ants ingested the 1% sucrose solution compared to control ants (Post-hoc GLM, *Treatment*: χ^2=4.62, df=1, P=0.032). A similar non-significant tendency was found for the 5% (Post-hoc GLM, *Treatment*: χ^2=3.31, df=1, P=0.069) and the 20% sucrose concentrations (Post-hoc GLM, *Treatment*: χ^2=2.71, df=1, P=0.099).

The time spent feeding on the sucrose drops of different concentrations for pheromone-exposed and control ants is shown in Fig. 2B. The duration of feeding was dependent on whether the ants had been exposed to the pheromone or not (LM, *Treatment*: χ^2=4.55, df=1, P=0.033), and on the sucrose concentration of the drop: the highest the concentration, the longer the feeding time (LM, *Concentration*: χ^2=12.18, df=3, P<0.001), a fact that is understandable in terms of collecting more food from more profitable food sources. Yet, a significant difference

between pheromone-exposed and non-exposed ants was found for the 20% sucrose concentration. In this case, pheromone-exposed ants spent significantly more time feeding on the sucrose drop than control ants (Post-hoc LM, *Treatment*: χ^2=5.25, df=1, P=0.026). There was also a non-significant tendency for pheromone-exposed ants to feed longer than control ones at the 1% sucrose concentration (Post-hoc LM, *Treatment*: χ^2=3.42, df=1, P=0.072).

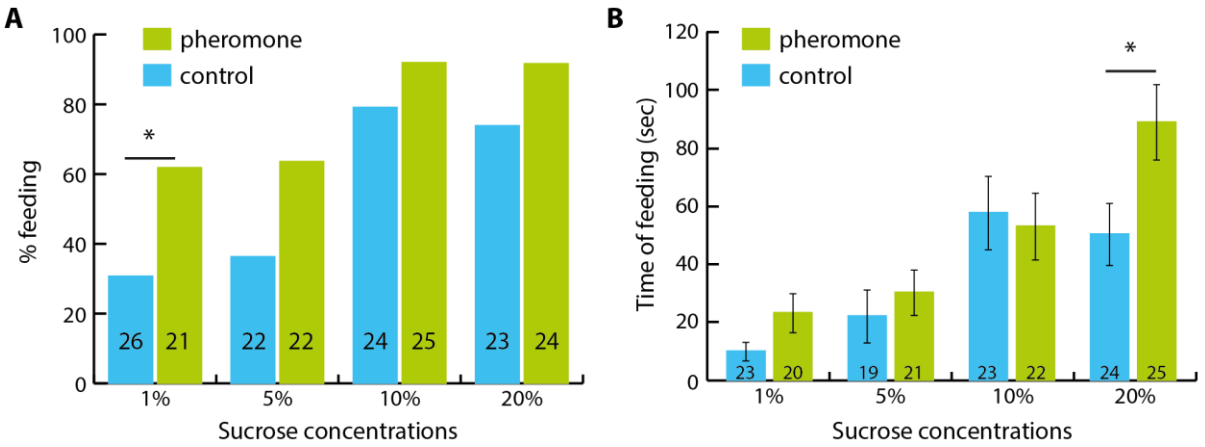

Figure 2. Effect of pheromone exposure on feeding responses to four sucrose concentrations. (A) Appetitive responsiveness was recorded as the number of ants that fed on a given drop of sucrose at four different concentrations (1, 5, 10, and 20%). Pheromone exposure increased the number of feeding ants for all sucrose concentrations but it was only significant at 1%. (B) Time ants spent feeding on drops of different sucrose concentrations. Pheromone exposure increased the time ants spent feeding on the 20% sucrose solution. Numbers inside bars indicate sample sizes. (*) P<0.05; (**) P<0.01.

Discussion

Our study aimed at investigating the role of a trail pheromone component as a modulator of the ants' subjective evaluation of appetitive stimuli and therefore as a key component of behavioral plasticity. To this end, we exposed Argentine ants to (Z)-9-hexadecenal, a substance characterized as a main component of their trail pheromone, and determined the effect of this exposure on their appetitive responsiveness. As responsiveness provides a reliable readout of the ants' subjective evaluation of reward (Scheiner et al., 2005a), changes in responsiveness following pheromone exposure indicate that pheromones are capable of neural and behavioral modulation.

(Z)-9-hexadecenal significantly modulated worker sucrose responsiveness, confirming the prediction that ants exposed to a trail pheromone would show higher responses, even when ants were only exposed to the pheromone and not put in direct contact with it. The prediction was based on previous results that (Z)-9-hexadecenal increased the number of ants being attracted to a 10% sucrose solution placed in the foraging areas of *L. humile* colonies (Greenberg and Klotz, 2000). In our experiment, however, the 10% sucrose solution is the only

concentration at which we could not detect a significant effect of the pheromone but this is probably due to a sampling effect as, overall, the pheromone appeared to increase the number of ants ingesting sucrose at every concentration. Two main effects of pheromone exposure were found in our work: 1) an increase in the number of ants accepting low sucrose concentrations, in particular a 1% sucrose solution, and 2) an increase of the time spent feeding on a drop of 20% sucrose solution.

In the first case, (Z)-9-hexadecenal would make a 1% sucrose solution more attractive, thereby changing the perception and/or evaluation of this food source by ants from "poor" to "valuable". Modulations of responsiveness by pheromone components are typically found for low concentrations/intensities (Baracchi et al. 2017; Rossi et al. 2018*a*,*b*). This can be understood assuming that low concentrations are the ones at which upward modulation is rendered more visible; for higher concentrations, closer to a ceiling response, the upward modulation would be less detectable. The neural mechanisms underlying the observed phenomenon remain unknown; nonetheless, biogenic amines could be involved in this modulation given their well-known role of modulators of several aspects of animal behavior, including appetitive responsiveness (Scheiner et al., 2002). Serotonin could be a good candidate as neuromodulator of feeding behavior and responsiveness to sucrose in ants. Falibene et al. (2012) showed that this amine decreased the volume of sucrose solution ingested per pump contraction in the ant *Camponotus mus*. On the other hand, Muscedere et al. (2012) found that *Pheidol dentata* workers in which the serotonin levels were experimentally lowered followed trails on significantly shorter distances than control workers, and responded less frequently to trails when encountered. Exposure to the trail pheromone could, therefore, modulate serotonin levels in the ant brain (among other biogenic amines), enhancing or depressing responsiveness to different kinds of stimuli according to their modality (gustatory or olfactory). Pheromone-exposed ants would therefore be more responsive to food and more accurate in following the trails at the same time. What appears counter adaptive is the fact that the trail pheromone would increase the motivation of a recruited ant to forage at her turn on a food source whatever the quality of the food. However, this mechanism could in fact be less costly and much faster for the individual than having to evaluate the food quality (and weight the gains and losses of energy in engaging in foraging behavior), which could explain the phenomenon of mass recruitment observed in Argentine ants and their invasive success. Congruently, Josens et al. (2016) showed that carpenter ants receiving social instructions through trophallaxis learning followed instructions even when presented with toxic food, suggesting that they did not evaluate

the food they were recruited for.

In the second case, ants exposed to the pheromone spent more time feeding on the more profitable 20% sucrose drop. In other words, the trail pheromone increased the feeding time at a valuable food source, thus resulting in an increase of the absolute quantity of food brought back to the nest. Sola and Josens (2016) found that, indeed, the crop load was the highest around 20% sucrose concentration and that the intake rate was also very high at this concentration. In order to test the hypothesis that the feeding time would be correlated with the absolute amount of food ingested, ants' volumes of ingestion should be measured in further analyses in order to see if the pheromone increases it or not.

Further studies should develop protocols to shed light on the biologically relevant concentrations of (Z)-9-hexadecenal that affect feeding behavior of Argentine ants and on the corresponding dose-effect responses. Indeed, current published studies using (Z)-9-hexadecenal lack homogenization in concentration ranges and units making the comparison between results difficult. This is particularly true in the case of (Z)-9-hexadecenal as it is commercially available as a liquid synthetized molecule, which should incite researchers to report absolute volumes used in addition to relative ones and/or concentrations.

The present study adds to the substantial literature on (Z)-9-hexadecenal as a control and management tool against the expansion of *L. humile* in introduced areas (e.g. as baits, Greenberg and Klotz 2000). Our results are in agreement with the conclusion of Sola and Josens (2016) regarding the optimum sucrose concentration to use as a bait. Indeed, they show that sucrose concentration affects feeding dynamics in this species and modulates decision making related to individual behavior and social interactions of foragers, and that 20% sucrose solution appears to be the most appropriate concentration for baits because it promotes rapid foraging cycles, a high crop load per individual, and a high degree of stimulation for recruitment. In our experiments, 20% corresponded to the sucrose concentration at which the trail pheromone increased feeding time the most. Therefore, toxic 20% sucrose baits (Sola et al., 2013) associated with the pheromone (Welzel and Choe, 2016), or 20% baits in pheromone dispensers positioned appropriately (e.g. in canopy, Westermann et al. 2016), could act as efficient control instruments against the invasive Argentine ant. Alternative methods use this pheromone to disrupt Argentine ant's trails instead of or in combination with baits (Suckling et al., 2011; Sunamura et al., 2011; Westermann et al., 2014).

As pheromones change the subjective perception of stimuli, they may also have an

impact on the ability to learn about these stimuli. Bees that exhibit high responsiveness to sucrose solutions of variable concentration are better learners in olfactory and tactile conditioning protocols that use sucrose solution as a reward (Scheiner et al. 2001*a*,*b*). Similarly in ants, foragers, which exhibit higher responsiveness to sucrose concentrations than other worker castes, are also the ones learning better in appetitive associative learning using sucrose as reward (Perez et al., 2013a). Therefore, the effect of pheromones might not only be restricted to responsiveness and motivation but could also affect learning and memory via the modulation of the salience of an unconditioned stimulus. Thus, besides conveying stereotyped messages, pheromones have an important role as modulators of behavioral plasticity.

Competing interests

No competing interests.

Author contributions

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

Funding

Supported by the Agence Nationale de la Recherche (ANR-14-CE18-0003, PHEROMOD), the Program of Scientific and Technological Cooperation between the Ministry of Science, Technology and Productive Innovation of the Argentine Republic (MINCYT,) and ECOS-Sud, France.

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CHAPTER 2: Pheromonal modulation of decision making

Pheromone-induced accuracy of nestmate recognition in carpenter ants: Simultaneous decrease of Type I and Type II errors

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Abstract

The ecological and evolutionary success of social insects relies on their ability to efficiently discriminate between group members and aliens. Nestmate recognition occurs by phenotype matching, the comparison of the referent (colony) phenotype to the one of an encountered individual. Based on the level of dissimilarity between the two, the discriminator accepts or rejects. The tolerated degree of mismatch is predicted by the acceptance threshold model, which assumes adaptive threshold shifts depending on the costs of discrimination errors. Inherent in the model is that rejection (Type I) and acceptance (Type II) errors are reciprocally related: if one type decreases, the other increases. Alarm pheromones might play a role in modulating the acceptance threshold. We exposed *Camponotus aethiops* ants to formic acid and subsequently measured aggression towards nestmates and non-nestmates. Formic-acid exposed ants consistently rejected more non-nestmates but at the same time accepted more nestmates than control ants. Formic acid thus improved nestmate discrimination accuracy by decreasing both types of errors. A shift in the acceptance threshold cannot explain the results. We propose that formic acid increases the amount of information available to the ants, thus decreasing the perceived phenotypic overlap between nestmate and non-nestmate recognition cues. This mechanism for improved discrimination has implications for the functioning of recognition systems in general.

KEYWORDS: Acceptance threshold model, Aggressive behaviors, Chemical communication, Formic acid, Social insects, *Camponotus aethiops*

Introduction

Social insects have evolved highly organized societies based on division of labor and defense of colony resources, which relies on the ability to discriminate between nestmates and non-nestmates. Nestmate recognition is indeed one of the conditions favoring the evolution and maintenance of sociality because it allows rejecting alien competitors (non-nestmate con- and hetero-specifics) while being tolerant towards group members, which are typically related (Hamilton, 1987). Apart from the rare exception of few social wasp species, where vision also plays a role in nestmate discrimination (Baracchi et al., 2015; Baracchi et al., 2016), the recognition of colony members in social insects is predominantly mediated by chemical cues (van Zweden and d'Ettorre, 2010). Insect cuticles are covered by complex mixtures of hydrocarbons forming a waterproof layer (Blomquist and Bagnères, 2010), whose original function is to prevent body dehydration but that acquired a prominent communication role in those insects living in groups (Blomquist and Bagnères, 2010). Cuticular hydrocarbons (CHCs) are low volatile compounds with long carbon chain lengths, c.a. C_{20} to C_{40} (van Zweden and d'Ettorre, 2010), that can be detected through antennal contact or at very short distances (Brandstaetter et al., 2008). Typically, CHCs vary qualitatively among species and quantitatively among colonies of the same species (vander Meer et al., 1989).

A long standing hypothesis is that information about an individual's own CHC profile (and therefore about nestmate CHC profiles) is represented as a neural template in the nervous system (Lenoir et al., 1999; Leonhardt et al., 2007). During encounters with other individuals, their chemical profile (label) would be compared to the stored template in a process described as phenotype matching (Lacy and Sherman, 1983). Aggressive behaviors would thus result from a mismatch between the label and the template, which is more likely to occur when facing a non-nestmate than a nestmate (Lacy and Sherman, 1983). As CHC profiles are influenced by environmental factors and vary with age, reproductive status, and caste (d'Ettorre and Lenoir, 2010), an adjustment of the template should occur in order to prevent rejection of nestmates with slightly different profiles. The formation and adjustment of the template are thought to require sensory adaptation or simple forms of learning (e.g. habituation, Guerrieri et al. 2009) and frequent interactions among colony members to unify the CHC label (Blomquist and Bagnères, 2010). Experiments in the honey bees also revealed that wax combs could change the template used by guards at hive entrances (Couvillon et al., 2007).

The study of recognition systems can be divided into three components: the expression, the perception, and the action (Liebert and Starks, 2004; Reeve, 1989; Sherman et al., 1997).

The expression component refers to the production of the cues used for recognition; they usually are any aspect of the phenotype that predicts group membership reliably. The perception component refers both to the recognition template and the matching algorithm between the perceived cues and this template. The action component refers to the decision rules derived from recognition, the actual behavioral response. The acceptance threshold model of Reeve (1989) predicts this action component, i.e. how the discriminator's action should vary in different recognition contexts. According to this model, the discriminator would have only two options when facing another individual: accept or reject. The acceptance threshold is the level of phenotype-template dissimilarity below which the actor will accept and above which it will reject conspecific. Because of inter-individual variation and overlap between nestmate and non-nestmate recognition cues, discrimination errors are inevitable. The model predicts the acceptance threshold that optimally balances the errors of rejecting nestmates (Type I) and accepting non-nestmates (Type II) in different contexts. Several studies have supported the acceptance threshold model with experimental data in different social insect species. It has been shown that the acceptance threshold varies as predicted by the model with, for instance, proximity to the nest (Buczkowski and Silverman, 2005; Starks et al., 1998), food availability (Downs and Ratnieks, 2000), robbing intensity (Couvillon et al., 2008), stage of colony cycle (Gamboa et al., 1991), and number of queens (Starks et al., 2010). However, there is little empirical evidence about the proximate mechanisms modulating the threshold.

Pheromones are substances emitted by an individual that elicit behavioral or physiological responses in individuals of the same species (Karlson and Lüscher, 1959). They are used in various contexts, such as foraging, defense, reproduction (Wyatt, 2014), and are key factors in coordinating social interactions and maintaining the organization of societies, such as those of social insects (Bonabeau et al., 1997). Pheromones typically elicit a stereotyped response that is species-specific and does not depend on experience (Karlson and Lüscher, 1959; Wyatt, 2014). However, recent findings indicate that the biological effects of pheromones are more complex than previously thought as they can also act as modulators of either experience-dependent behaviors (Urlacher et al., 2010; Vergoz et al., 2007a) or behaviors that are not explicitly related with the specific message they convey (Baracchi et al., 2017; Rossi et al., 2018b). Therefore, pheromones appear to be key players in behavioral plasticity, an aspect that is still underexplored.

It was hypothesized that alarm pheromones may shift the acceptance threshold towards a less permissive one, but experimental data in honey bees showed no significant effects of

alarm pheromones on the acceptance or rejection rate of nestmates or non-nestmates by guards (Couvillon et al., 2010). We tested the possible effect of formic acid (FA) on nestmate recognition; FA is an alarm pheromone released by most ants belonging to the Formicinae subfamily (O'Rourke, 1950). It is the main component of the secretion of the venom gland (Stumper, 1952) and its exposure results in increased spontaneous locomotor activity, which is part of a stereotyped alarm behavioral response (Löfqvist, 1976).

We asked the question of whether, besides eliciting stereotyped responses, FA modulates the nestmate discrimination process, in particular when the pheromone is no longer present. In a natural scenario, the situation would be that of an ant colony after an alarm. Does the nestmate discrimination behavior of workers change after an alert? Does FA shift the acceptance threshold of ants towards a less permissive one? Does FA modulate rejection (Type I) and acceptance (Type II) errors? To tackle these questions, we used the carpenter ant *Camponotus aethiops* as its workers use FA as alarm pheromone (Stumper, 1952) and are aggressive against non-nestmates (Stroeymeyt et al., 2010). Two experiments were designed to determine whether and how FA modulates nestmate discrimination in carpenter ants. The first one consisted in testing the effect of FA on ants' responsiveness to different concentrations of non-nestmate odor in order to mimic an odor gradient similar to the one an ant would perceive from a non-nestmate at different distances. The second experiment tested whether FA affects the level of discrimination between nestmate and alien odor cues. We hypothesized that FA should increase aggressiveness towards non-nestmates given the alarm nature of this pheromone and that it should modulate responsiveness at low concentrations given previous results in honey bees (Baracchi et al., 2017; Rossi et al., 2018b).

Material and Methods

Experiments were conducted in February/March 2017, at the Laboratory of Experimental and Comparative Ethology, Villetaneuse, France. We used five queen-right colonies of *C. aethiops* collected in 2016 at Pompertuzat (Midi-Pyrénées, France, latitude 43.5, longitude 1.5167) and kept in the laboratory under controlled conditions (25°C, light-dark cycle = 12:12, 40% humidity) each in two Fluon-coated plastic boxes connected by a tube. One box was provided with plaster floor and covered by cardboard (nest), the other was exposed to light and had sand on the floor (foraging area). Ants were fed twice a week with a mixture of honey and apples for carbohydrates and vitamins and pieces of crickets and flour worms for proteins; water was provided *ad libitum*.

Two experiments were conducted to determine the potential modulatory effect of FA on perception (of odor) and action components of the recognition system. Experiment 1 was conducted to determine whether FA modulates responsiveness to nestmate and non-nestmate cuticular extracts. We tested different concentrations of non-nestmate odor mimicking an approaching intruder from the distance. Perceiving an intruder from the distance would allow more efficient colony defense. Here, each ant was its own control as responsiveness was measured before and after pheromone exposure. Experiment 2 was a standard aggressive encounter protocol, which allowed determining whether FA modulates nestmate discrimination. In this experiment, one focal ant met two target ants, a nestmate *and* a non-nestmate, presented simultaneously and close to each other; this design was therefore more prone to errors (both Type I and Type II) than encountering a single ant and could allow observing subtle changes due to FA exposure. Target ants were previously killed by freezing so that their behavior would not influence the focal ant (*C. aethiops* ants are aggressive towards freshly killed non-nestmates, Stroeymeyt et al. 2010). Figure S1 summarizes the experimental procedure for all experiments.

Experiment 1: Responsiveness of ants to recognition cues measured with mandible opening response (MOR)

Medium size forager ants from two different colonies were used to study responsiveness to nestmate and non-nestmate odors (foragers are typically very aggressive in *C. aethiops,* Larsen et al. 2016). We used the mandible opening response (MOR) as a proxy for aggression (Guerrieri and d'Ettorre, 2008) (Fig. S2). Each ant was cold anesthetized until immobility (ca. 3 to 4 min) and harnessed within a small plastic holder using adhesive tape placed between the head and the thorax. Ants restrained in this way can freely move only their antennae and mouthparts. Once harnessed, the ants were kept in a dark and humid cardboard box (70%) at 25 ± 1 °C to recover from anesthesia and acclimatize to the harness. After the three-hour rest, ants were randomly allocated to two different groups (a control group and an experimental one) and tested for their responsiveness using MOR.

In the MOR assay, one ant at a time was placed under a stereomicroscope (Leica S8 APO, magnification 10 ×). Responsiveness to nestmate and non-nestmate odors was quantified by recording the MOR to different concentrations of cuticular extracts. Cuticular extracts were obtained by washing 20 nestmate or non-nestmate ants in 10 ml of solvent (pentane, HPLC grade, Sigma Aldrich) for 10 min; then, concentrations were obtained by serial dilutions (see Larsen et al. 2016). The amount of nestmate odor used was equivalent to that of a single ant,

while non-nestmate odor was used in six different concentrations (0.03, 0.06, 0.125, 0.25, 0.5 and 1 ant equivalents). An additional presentation of pure solvent (pentane) was performed as a control. The stimulus presentation was performed by placing a glass rod coated with the chemical extract next to the antennae, without touching them. Each ant was presented with the eight stimuli in a randomized order (pentane, nestmate extract and 6 non-nestmate extracts). Each stimulus was preceded by the presentation of a clear rod in order to familiarize the ants with the visual component of this stimulus. The occurrence of MOR (yes/no) to each stimulus presentation was recorded during the presentation.

Fifteen min later, ants were exposed either to FA (experimental group) or to the solvent alone (pure water, control group). Formic acid (Sigma-Aldrich) 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. Ants in their plastic holder were individually confined for 15 min in a 50 ml plastic flask containing a filter paper (1 x 5 cm) soaked with the pheromone (or water) placed under a hood. After the exposure, ants were kept resting for an additional 30 min and then tested again for responsiveness using the MOR assays (Fig. S1). A total of 244 ants were tested (124 ants exposed to FA and 120 ants exposed to water).

Experiment 2: one-to-two encounters

The arena used for aggressive encounters was a plastic cylinder (h = 5.3 cm, ∅ = 8 cm) with Fluon-coated walls placed on the floor of the foraging area of the focal colony the day before experiment in order for the ants to familiarize with it. We used three arenas, each placed in the foraging area of a colony (colonies D, F2 and F4), and used the foragers of each colony as the reciprocal nestmates and non-nestmates. The day of experiment, 10 ants from the focal colony and 10 ants from another colony were killed by freezing and then warmed up during 15 min at ambient temperature just before the experiment. One focal ant was collected from the foraging area of the focal colony and exposed either to FA (12% in water) or to pure water. Exposure lasted 15 min, each focal ant was placed inside a small glass vial (3.14 ml) placed in the middle of a 50 ml plastic flask containing the filter paper to avoid direct contact between the ant and FA/water. Trials were therefore spaced out of 15 min. We used 21 focal ants from colony D, 19 focal ants from colony F2, and 29 focal ants from colony F4.When focal ants from colony D were tested, we used non-nestmates from colony F4, when focal ants from colony F2 were tested, we used non-nestmates from colony D, and when focal ants from colony F4 were tested, we used non-nestmates from colony F2.

After exposure, the focal ant was placed in a Fluon-coated ring (h = 3 cm, ∅ = 2.2 cm) inside the arena. Two target dead ants (a nestmate and a non-nestmate), presented simultaneously and interspaced of 1 cm, were placed at one extremity of the arena and the focal ant was released from the opposite extremity after 3 min of acclimatization. The respective positions of the nestmate and the non-nestmate were randomized over trials. All trials were videotaped. Target ants were marked with a small paint dot on the thorax for identification during video analysis. Trials lasted 3 min during which duration and occurrence of mandible opening, biting, and gaster flexing were recorded (ascending order of aggression, from low to high, Stroeymeyt et al. 2010). All behaviors were exclusive, meaning that only the most aggressive behavior was recorded when two behaviors occurred at the same time. Video analysis was done blindly (video names did not indicate which treatment the focal ant had been exposed to) using BORIS software (Friard and Gamba, 2016). A total of 69 ants were tested (35 ants exposed to FA and 34 ants exposed to water).

Data analysis

Experiment 1. Ants exhibiting inconsistent responses to non-nestmate odors (i.e. responding to a low but not to a higher concentration, 31 (ca. 25%) of FA-exposed ants and 34 (ca. 28%) of water-exposed ants, were discarded because the lack of response to the highest concentrations may be due to an uncontrolled motor problem and not to odor sensitivity itself. The percentage of inconsistent ants did not differ between the two treatments (χ^2 test, water vs. FA: $\chi^2 = 0.35$, df = 1, p = 0.55). For each ant retained in the analysis (ants exposed to FA: n = 93; ants exposed to water: n = 86), an individual MOR score (MORS) was calculated as the number of non-nestmate odor stimulations eliciting MOR. MORS ranged from 0 to 6, i.e. ants with a MORS of 0 did not respond to any stimulation while ants with a MORS of 6 responded to all non-nestmate odor stimulations. Delta scores were calculated subtracting the MORS measured after exposure from the MORS measured before exposure to FA (or control) and compared with a Z-test since the distribution was approximately normal and the sample size was large (n > 30).

Stimulus responses (MOR: 1 or 0) of individual ants 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). In all models, colony of origin was entered as random factor and when appropriate (i.e., repeated measures) ant individual identity was entered as a random factor nested inside colony of origin.

We first analyzed the effect of the treatment (‘*Treatment time’*, i.e. before/after exposure

to either water or FA) on ants' response to non-nestmate odor concentrations ('*Stimulus concentration*', i.e., non-nestmate ant equivalents), using independent models for ants exposed to water and ants exposed to FA as these groups were independent. In a second analysis, we tested the effect of the exposure to FA or water on ants' MOR to pentane (solvent only), nestmate and non-nestmate odors both with a concentration of one ant equivalent ('*Stimulus type*'). Again, independent models were run for ants exposed to water and ants exposed to FA. Then, we classified the responses of ants to one ant equivalent of nestmate and non-nestmate odors as 'improved' or 'worsened' according to their change of response before and after exposure to water or FA and run χ^2 tests to compare these proportions. Post-hoc χ^2 tests were applied within and between treatments.

For experiment 2, we used the mandible opening, biting and gaster flexing as aggressive behaviors and we analyzed the occurrence and duration of the sum of these three behaviors. Occurrences of aggressive behaviors were examined using GLMMs with a Poisson distribution for count data (link = 'log') (*glmer* function of the *lme4* R package) while duration of aggressive behaviors was examined using linear models (*lme* function of the *nlme* R package) with permutation tests to make sure that inferences were not biased by the non-normal distribution of the data (*PermTest* function of the *pgirmess* R package, 1000 permutations). For both '*Occurrence'* and '*Duration'* responses, the full models were retained, i.e., with the interaction between '*Treatment'* (FA/water) and '*Target ant*' (nestmate/non-nestmate). We added '*ID'* as a random factor nested in '*Colony of origin*' to account for repeated measurements. As the interaction between '*Treatment*' and '*Target ant*' was significant for both response variables, we proceeded with GLMMs on nestmate and non-nestmate subsets separately.

In all analyses, we retained the significant model with the highest explanatory power (i.e. the lowest AIC value). When we used post-hoc tests, we applied the Bonferroni correction to correct for the familywise error rate (*p.adjust* function from R package *stats*, R Core Team, 2017). All statistical analyses were performed with R 3.4.2 (R Development Core Team, 2016) and the significance threshold was set at 0.05.

Results

Experiment 1: Mandible opening response (MOR) assays

The MOR of harnessed ants depended strongly on non-nestmate concentrations (0.03, 0.06, 0.125, 0.25, 0.5 and 1 ant equivalents) in both groups (water and FA): the higher the concentration, the higher the percentage of ants displaying MOR (GLMM, control group:

Stimulus concentration: $\chi^2 = 114.66$, df = 5, n = 86, $p < 0.001$; experimental group: *Stimulus concentration:* $\chi^2 = 125.33$, df = 5, n = 93, $p < 0.001$; Fig. 1). The non-nestmate concentration was the only factor that had a significant effect for the control group and was consequently the only predictor retained in the model. It therefore appears that control ants' responses were similar before and after water exposure and that this treatment did not affect the MOR to non-nestmate odors (Fig. 1B). In contrast, ants were more likely to respond aggressively (MOR) to non-nestmate odors after being exposed to FA (GLMM, *treatment time:* $\chi^2 = 24.10$, df = 1, $p < 0.001$, Fig. 1A). In particular, a higher proportion of ants responded to the two lowest concentrations of non-nestmate extracts after FA exposure (GLMM, S*timulus concentration * Treatment time*: $\chi^2 = 10.73$, df = 5, p = 0.057; *post-hoc GLMM with Bonferroni correction*: 0.03 ant equivalent: $p < 0.001$; 0.06 ant equivalent: p = 0.002).

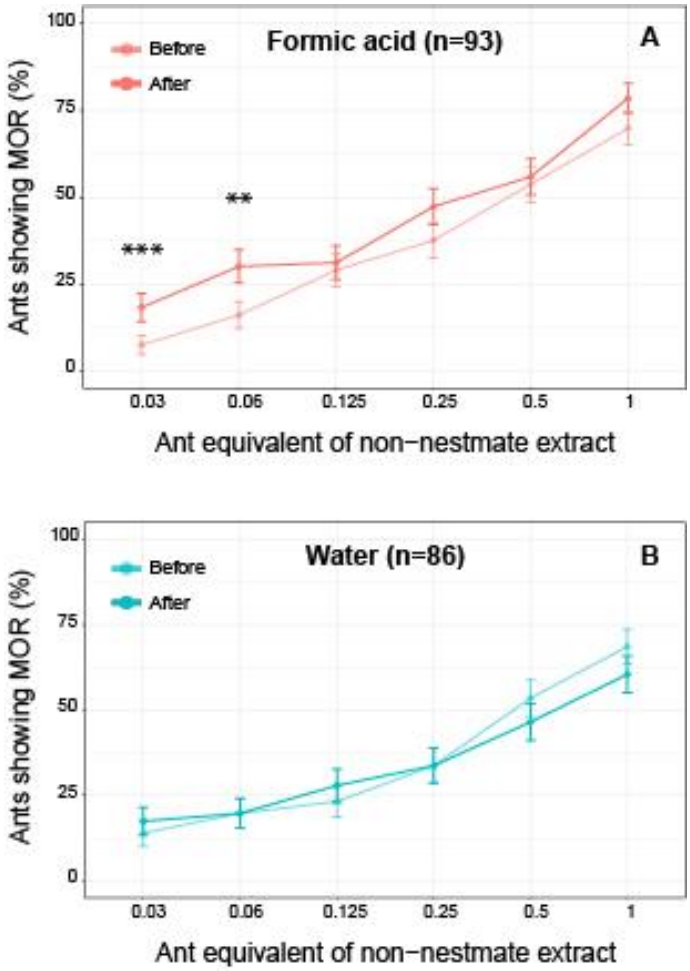

Figure 1. Pheromone exposure affects responsiveness to non-nestmate odors. Means represented with their standard errors. Percentage of ants showing MOR when presented with 0.03, 0.06, 0.125, 0.25, 0.5 and 1 ant equivalents of non-nestmate extracts. The more concentrated was the extract, the more ants displayed MOR, independently of the treatment. In the experimental group (**A**), ants displayed more aggressiveness (MOR) after exposure to formic acid than before but it was not the case in the control group (**B**). This was especially true at 0.03 and 0.06 ant equivalents. Post-hoc GLMM with Bonferroni correction, (**) p = 0.002, (***) $p < 0.001$

At the individual level, the analysis of the MOR delta scores confirmed that FA affects the aggressive response of ants towards non-nestmate odors. In particular, ants exposed to the pheromone (n = 93) had higher delta scores than ants exposed to water (n = 86), (Z-test, Z =

2.80, p = 0.005), meaning that they had a higher responsiveness to non-nestmate odors after FA exposure than before (Fig. 2A).

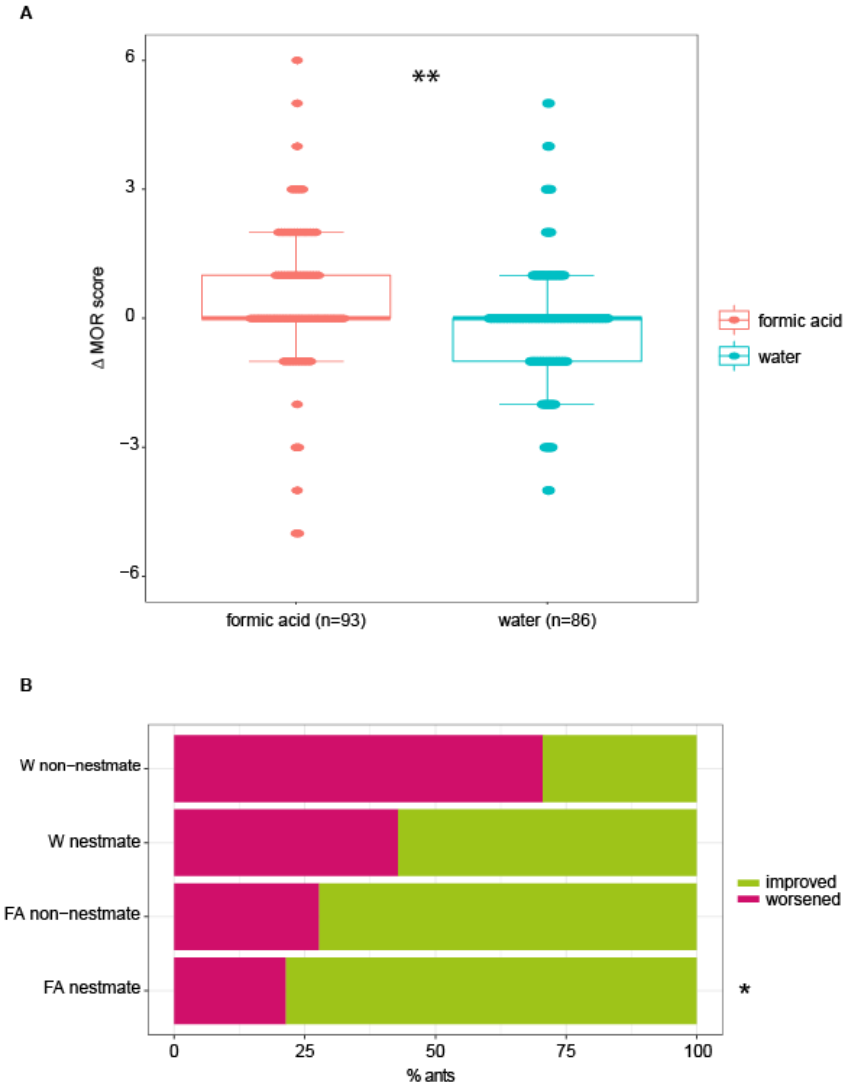

Figure 2. Pheromone exposure increases mandible opening responses (MOR) towards non-nestmate odors (A) and decreases it towards nestmate odors (B). (**A**) Delta scores of ants for the six non-nestmate stimuli, ranging between six (ants responding to all concentrations) and zero (ants responding to none). Delta scores were calculated subtracting the scores measured after exposure from those measured before exposure to formic acid (FA) or water. Box plots represent median, quartiles, 10th and 90th percentiles (lower and upper whiskers), dots represent individual ants. Formic-acid exposed ants displayed more MOR to non-nestmate extracts than water-exposed ants. Z-test, (**) p = 0.005. (**B**) MOR of ants to one ant equivalent of nestmate and non-nestmate extract was evaluated before and after water or FA exposure. Based on their change of responses to nestmate and non-nestmate odors, ants' behaviors were classified as 'appropriately changed' when displaying MOR towards non-nestmate but not towards nestmate extracts, or 'inappropriately changed' when displaying MOR towards nestmate but not towards non-nestmate extracts. When stimulated with the nestmate extract, the percentage of ants exposed to FA that changed their response appropriately was significantly higher than the percentage of ants that changed their response inappropriately. Post-hoc χ^2 test with Bonferroni correction, (*) p = 0.021. W: water; FA: formic acid

We then focused on the analysis of the MOR response to one ant equivalent of nestmate and non-nestmate odors and to pentane (solvent) to represent the potential encounter with one enemy or nestmate. Pheromone exposure induced a change in the ant discrimination ability between nestmate and non-nestmate odors. In particular, FA exposure decreased the MOR towards nestmates (GLMM, *Stimulus type * Treatment time*: χ^2 =17, df = 2, p < 0.001; *post-hoc GLMM with Bonferroni correction*: pentane: p = 0.128; non-nestmate p = 0.134; nestmate p = 0.032). On the contrary, water exposure did not affect ant responsiveness as the '*Treatment time*' factor (before/after exposure) was not retained in the model. The only predictor retained was the kind of stimulus presented (nestmate, non-nestmate or pentane) (GLMM, *Stimulus type*: χ^2 = 77.05, df = 2, p < 0.001). This effect was also present in the group exposed to FA (GLMM, *Stimulus type*: χ^2 = 56.92, df = 2, p < 0.001). In general, ants responded more to both nestmate and non-nestmate odors than to pentane, and more to non-nestmate odors than to nestmate odors (*post-hoc GLMM with Bonferroni correction*: in all cases p < 0.001).

Table 1: Number of ants that did or did not change their response to nestmate and non-nestmate extracts (one ant equivalent) after treatment

	Did not change	Changed		Total changed
		Appropriately	Inappropriately	
FORMIC ACID				
Nestmate	65 (70%)	22 (24%)	6 (6%)	28 (30%)
Non-nestmate	75 (81%)	13 (14%)	5 (5%)	18 (19%)
WATER				
Nestmate	58 (67%)	16 (19%)	12 (14%)	28 (33%)
Non-nestmate	69 (80%)	5 (6%)	12 (14%)	17 (20%)

Pheromone exposure also affected nestmate recognition accuracy. Of the 93 ants exposed to FA and the 86 ants exposed to water, 38% and 25% changed their response appropriately after treatment when stimulated with nestmate and non-nestmate extracts (1 ant equivalent) (i.e. exhibited MOR only towards the non-nestmate extract and not towards the nestmate extract); and respectively 11% and 28% changed it incorrectly (Table 1). The number of ants that improved their discriminative performance was higher in the experimental group than in the control group (*post-hoc χ^2 test with Bonferroni correction*: χ^2 = 7.00, df = 1, p = 0.033). We then subdivided the data into changes of responses towards nestmate and non-nestmate extracts separately to see if this treatment effect was dependent on the identity of the

targeted ant (Fig. 2B). We found that it was the case (χ^2 = 13.42, df = 3, p = 0.004): when stimulated with the nestmate extract, the percentage of ants that changed their response appropriately (i.e. stopped responding with MOR) after FA exposure was higher than the percentage of ants that changed their response inappropriately (i.e. started responding with MOR) (*post-hoc χ^2 test with Bonferroni correction*: χ^2 = 9.83, df = 1, p = 0.021). This was neither the case after exposure to 1 ant equivalent of non-nestmate extract (*post-hoc χ^2 test with Bonferroni correction*: χ^2 = 3.82, df = 1, p = 0.607), nor after exposure to water (*post-hoc χ^2 test with Bonferroni correction*: nestmate: χ^2 = 0.66, df = 1, p = 1; non-nestmate: χ^2 = 3.35, df = 1, p = 0.806).

Experiment 2: one-to-two encounters

Ants showed more aggressive behaviors towards non-nestmates than towards nestmates (GLMM, χ^2 = 87.89, df = 1, p < 0.001) but their responses depended on whether they had been exposed to FA or water (GLMM, χ^2 = 6.08, df = 1, p = 0.014; Fig. 3A). In particular, the effect of FA depended on whether the target ant was a nestmate or a non-nestmate (GLMM, χ^2 = 19.68, df = 1, p < 0.001). Formic acid-exposed ants were less aggressive towards nestmates than water-exposed ants (GLMM, χ^2 = 9.14, df = 1, p = 0.002) but they were more aggressive towards non-nestmates than control ants (GLMM, χ^2 = 12.73, df = 1, p < 0.001). Regarding the duration of aggressive behaviors, the analysis revealed very similar results (Fig. 3B). Thus, compared to water exposure, FA exposure increases differentiation between nestmates and non-nestmates, triggering in each case the appropriate response (non-aggression and aggression, respectively).

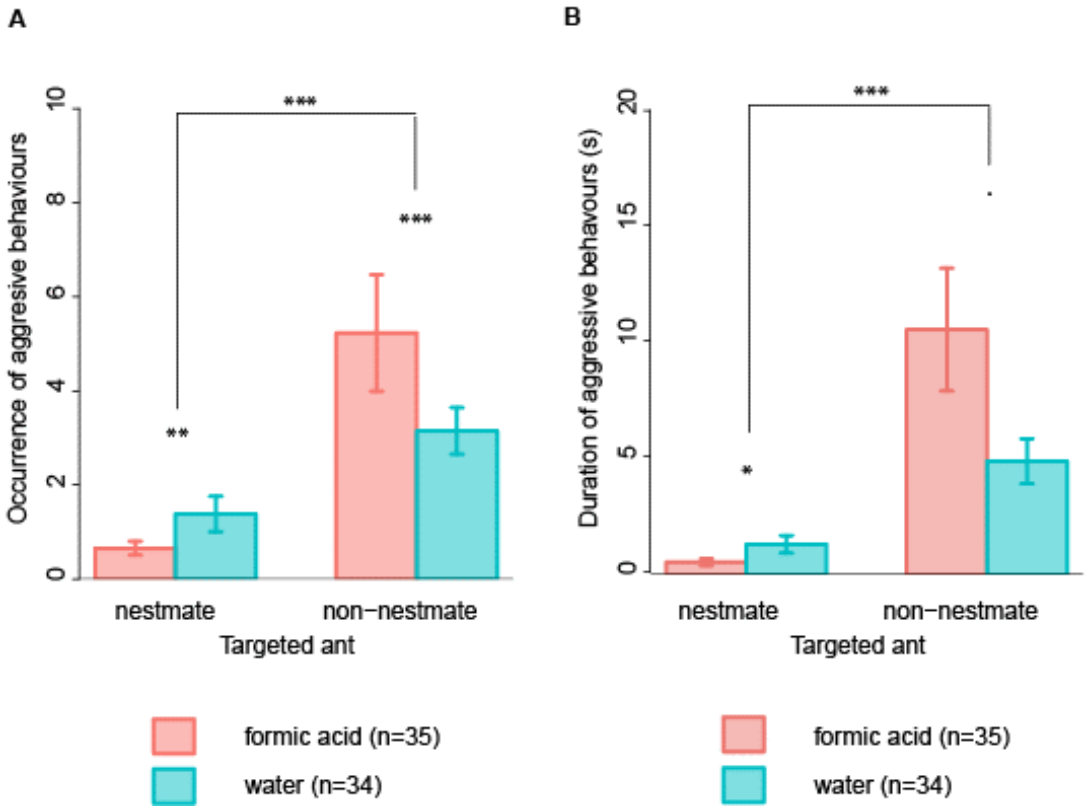

Figure 3. Formic acid increases the occurrence of aggressive behaviors towards non-nestmates and decreases aggressive behaviors towards nestmates. Means represented with their standard errors. (**A**) In one-to-two encounters, formic-acid (FA) exposed ants displayed aggressive behaviors more frequently towards non-nestmates and less frequently towards nestmates than water-exposed ants. GLMM, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$. (**B**) Ants exposed to FA spent less time in aggressive interactions with nestmates than control ants (LMM with permutation test, $\chi^2 = 4.06$, df = 1, $p = 0.045$) but showed a tendency to spend more time in aggressive interactions with non-nestmates (LMM with permutation test, $\chi^2 = 3.49$, df = 1, $p = 0.065$). LMM with permutation test, (.) $p < 0.1$, (*) $p < 0.05$, (***) $p < 0.001$

Discussion

Our study aimed at investigating the role of FA as possible modulator of nestmate discrimination in the ant *C. aethiops*. To this end, we exposed ants to FA and we determined its effect on the ability to discriminate nestmates and non-nestmates when the pheromone was no longer present. We therefore assessed response modulation rather than reflexive responses to FA in two independent experiments. The first experiment relied on a protocol for harnessed ants, which also allowed testing for responsiveness to different concentrations of non-nestmate odor using MOR as a proxy for aggression. We found that FA increased the aggressive response of ants and in particular improved responsiveness to low concentrations of non-nestmate odor. The second experiment relied on a protocol for free-walking ants, which allowed testing ants in their foraging arena in one-to-two encounters. When ants encountered a nestmate and a non-

nestmate at the same time, we observed both an increase of aggressive behaviors towards non-nestmates and a decrease of aggressive behaviors against nestmates, compared to control. This unforeseen effect of FA was also found in the first experiment, where ants decreased recognition errors towards cuticular extracts of nestmates. Formic acid thus increases accuracy in the process of nestmate discrimination. Such a modulatory effect of a pheromone on conspecific recognition has not been described before and it is highly relevant in the context of colony defense where nestmates and non-nestmates are encountered at the same time around the nest.

What are the mechanisms underlying the observed effect of FA? According to the "pre-filter" hypothesis for nestmate recognition, antennal sensilla would convey information about non-nestmates but not about nestmates due to sensory adaptation to self-CHCs (and therefore nestmate-CHCs) (Ozaki and Hefetz, 2014). Our results do not support this hypothesis as the observed effect of FA affected response to both non-nestmate and nestmate, thus implying that nestmate-CHCs are also processed (see Brandstaetter et al. 2011). However, a modified version of the pre-filter hypothesis states that the pre-filter acts imperfectly and that some nestmate information passes through the chemosensory filter without inducing aggressive behavior. Aggressiveness would be triggered only when a certain amount of neural inputs comes through the chemosensory filter (Ozaki and Hefetz, 2014). Our results suggest that FA affects the sensitivity to CHCs by modulating either CHC-receptor activity at the sensilla level in a top-down manner or CHC processing in the ant brain, thereby allowing the ants to detect more compounds in the CHC profile (Fig. 4A). This modulation could occur via the action of biogenic amines, which affect receptor sensitivity to key odorants (see Pophof 2000). In this scenario, FA exposure would result in specific increases/decreases of certain biogenic amines at several stages of the olfactory processing circuits. Such an effect has been shown in the honey bee upon exposure to the alarm pheromone component isopentyl acetate (Nouvian et al., 2018). This pheromone component enhances the levels of serotonin and dopamine in specific regions of the bee brain. If FA has a similar effect, biogenic amines could affect in a top-down manner olfactory receptor activity, but also odor processing of CHCs at the central level. In the first experiment, we found that the higher the concentration of non-nestmate extracts, the higher the percentage of ants displaying MOR. Exposure to FA increased aggression against non-nestmate odors, particularly at low concentrations of cuticular extracts. The ant cuticular signature consists of dozens of CHCs (c.a. 40 in *C. aethiops*, van Zweden and d'Ettorre 2010), but at very low concentrations (e.g., when the target ant is at some distance) only the most abundant CHCs

would be detected. The modulatory effect of FA would result in an improvement of CHC detection and/or perception, and thus in an increase of the amount of information (i.e. the number of detected CHCs) available to the ant. As a result, discrimination would be improved (Fig. 4).

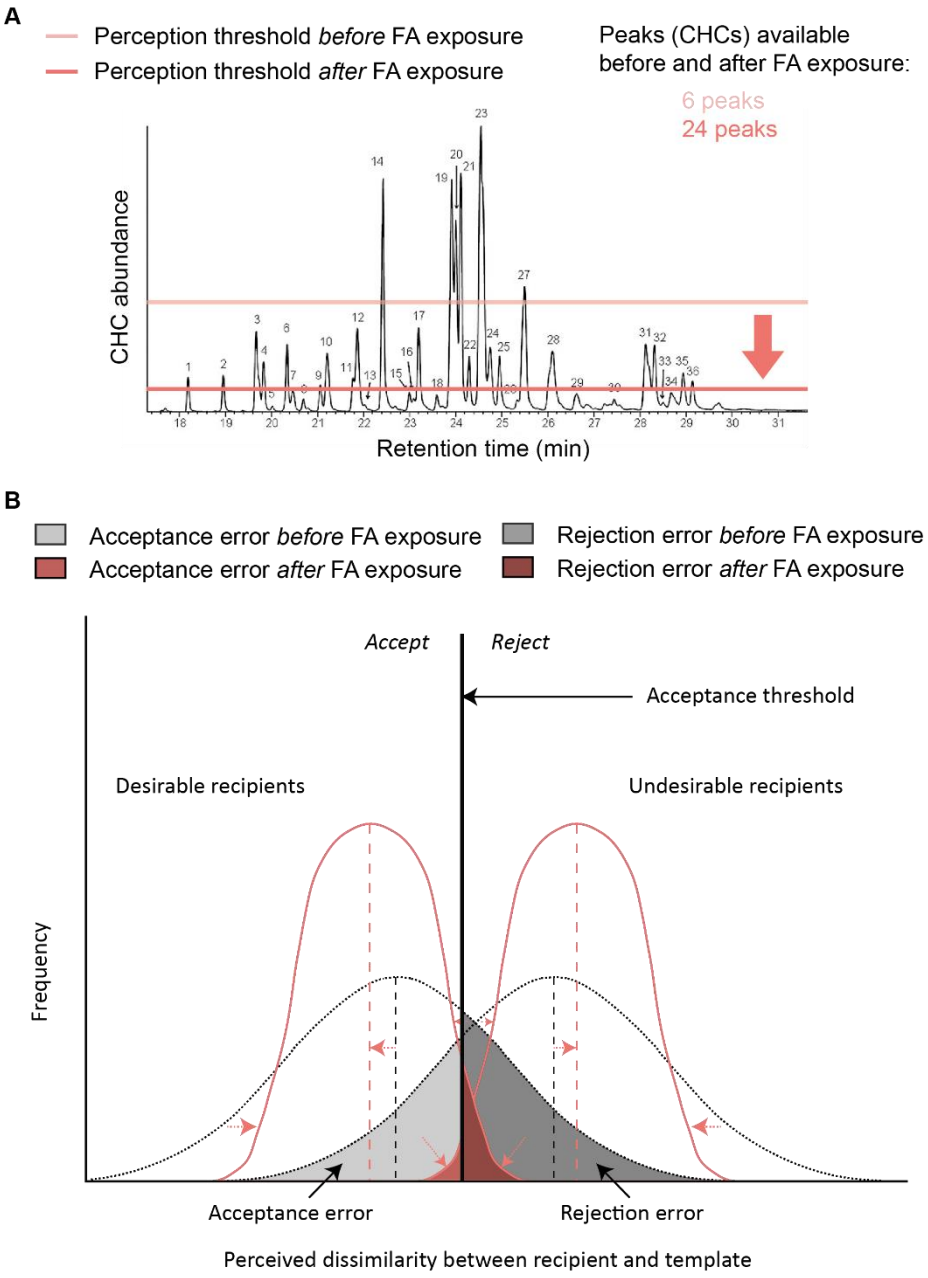

Figure 4. Putative modulation of an olfactory perception threshold by formic acid. (**A**) Example of a cuticular profile (adapted from van Zweden et al. 2009): the x-axis represents the retention time of different components of the cuticular profile (CHC) in a gas chromatograph and the y-axis represents their abundance. After exposure to formic acid (FA), the olfactory perception threshold of an ant would be lowered, allowing the detection of a higher number of components. (**B**) Frequency of interaction between desirable or undesirable recipients and the resulting distributions of perceived dissimilarity between the discriminator's template and the recipient's recognition cues for nestmates and non-nestmates (adapted from van Zweden and d'Ettorre 2010). By lowering the olfactory

detection threshold, FA would allow ants to access more cues, thereby decreasing the perceived variance and accentuate the dissimilarity between nestmate and non-nestmate CHCs. This would result in the decrease of acceptance and rejection errors.

The improved discrimination between nestmates and non-nestmates could be due to the action of FA at different levels, from olfactory receptors to higher brain centers. Information from olfactory receptors is conveyed to the antennal lobes (ALs) where a local network reshapes the olfactory message, and from there, it is passed via projection neurons to higher-order brain centers (e.g. mushroom bodies, MBs, and lateral horns, LHs). One possibility is that discrimination between nestmate and non-nestmate odor occurs at the level of the ALs (Guerrieri et al., 2009); local inhibitory circuits in the ALs provide a mechanism to reduce the signal-to-noise ratio from olfactory-receptor information by selectively inhibiting glomeruli with overlapping response profile, and thus to enhance odor discriminability (Sachse and Galizia, 2002). Formic acid could affect these circuits or act also at the level of higher-order brain centers such as the MBs or the LHs. The latter, in particular, has been associated with responses to signals with intrinsic biological value such as pheromones (Jefferis et al., 2007). In *Camponotus* ants, FA information is processed in a set of specific glomeruli in the ALs (Mizunami et al., 2010) and has specific projection neurons (uni-glomerular projection neurons) conveying information to integration areas (MBs and LHs) and premotor areas, which are directly linked to motor centers. It has been proposed that this specific FA pathway mediates aggressive behaviors, which are triggered by CHCs (Mizunami et al., 2010). In this case, FA would modulate the motivation to be aggressive. Our behavioral results do not allow identifying the precise mechanism of action of FA and neural analyses are necessary to segregate between possible circuits of pheromone modulation.

Our results allow examining the way in which recognition systems operates. The acceptance threshold model (Reeve, 1989) states that, when the cue-distribution of nestmates and non-nestmates overlap, there is a risk of error. The behavior of the discriminating individual is an all-or-none response with a threshold above which all recipients are rejected. If the threshold is too restrictive, the discriminator runs a risk of erroneously rejecting desirable recipients (Type I errors); if the threshold is too permissive, the discriminator runs a risk of erroneously accepting undesirable recipients (Type II errors). According to this model, no matter in which direction this threshold moves, in no case it is possible to obtain both an increase of rejection frequency of non-nestmates *and* a decrease of rejection frequency of nestmates: if rejection errors increase, acceptance errors decrease, and *vice versa*. However, our

results show that FA induces a decrease of both types of error. Hence, it seems unlikely that FA simply shifts this threshold.

This apparent incompatibility between the model predictions and the observed alarm pheromone modulation of nestmate recognition can be reconciled by focusing on a sentence in Reeve (1989, p. 409): "Discrimination errors are inevitable whenever phenotype matching is based on a *finite set of cues*". We propose that FA actually acts on this set of cues by increasing the amount of information available to the ants to perform discrimination. This would result in a decrease of variance and possibly a shift of the dissimilarity mean values for nestmates and non-nestmates, thereby decreasing the perceived overlap between the two (Fig. 4B). This allows decreasing at the same time both acceptance and rejection errors. This hypothesis is supported by a model by Lehmann and Perrin (2002) showing that the distributions of similarities between a recipient and the discriminator depend on the number of recognition traits sampled: a high number of traits (and a high similarity among nestmates) decrease overlap and thus increase discrimination ability. According to Sherman et al. (1997), the changes in magnitudes of and balance between acceptance and rejection errors are either the results of changes in the recognition cues (production component), or changes in the recognition template or matching algorithm (perception component). Formic-acid induced changes in the production of CHCs seem highly unlikely due to the rapid action of the pheromone in our protocols. Therefore, the most likely scenario is that FA acts at the perception level as we argued above.

Our results bring suggestions about how nestmate discrimination could be modulated at the perception level. To our knowledge, few works have discussed nestmate recognition models from a perceptual perspective because accessing the insect brain upon an inter-individual recognition task is difficult. Our study shows that behavior can sometimes provide clues about mechanisms that are not necessarily related to the action component. This is especially true when studying pheromones that are suspected to act on the motivation of an animal that perceives them (Baracchi et al., 2017; Nouvian et al., 2015; Rossi et al., 2018b). In our study, FA did not simply elicit an enhanced stereotyped aggressive response but acted as a modulator of adaptive behavioral plasticity by promoting appropriate responses to nestmates and non-nestmates. Attacking enemies and not related nestmates would indeed increase the fitness of the colony and therefore the inclusive fitness of the individual belonging to this colony. Our study suggests that the amount of perceptual information available for decision making is not necessarily fixed and that it can be modulated by "priming", i.e., exposure to one stimulus that influences a response to a subsequent stimulus. This opens new perspectives for a deeper

understanding of recognition systems and decision making in general.

Competing interests

No competing interests.

Funding

Supported by the Agence Nationale de la Recherche (ANR-14-CE18-0003, PHEROMOD), IDEX initiative ‘Chaire d’Attractivite’, University Toulouse to PdE, Centre National de la Recherche Scientifique.

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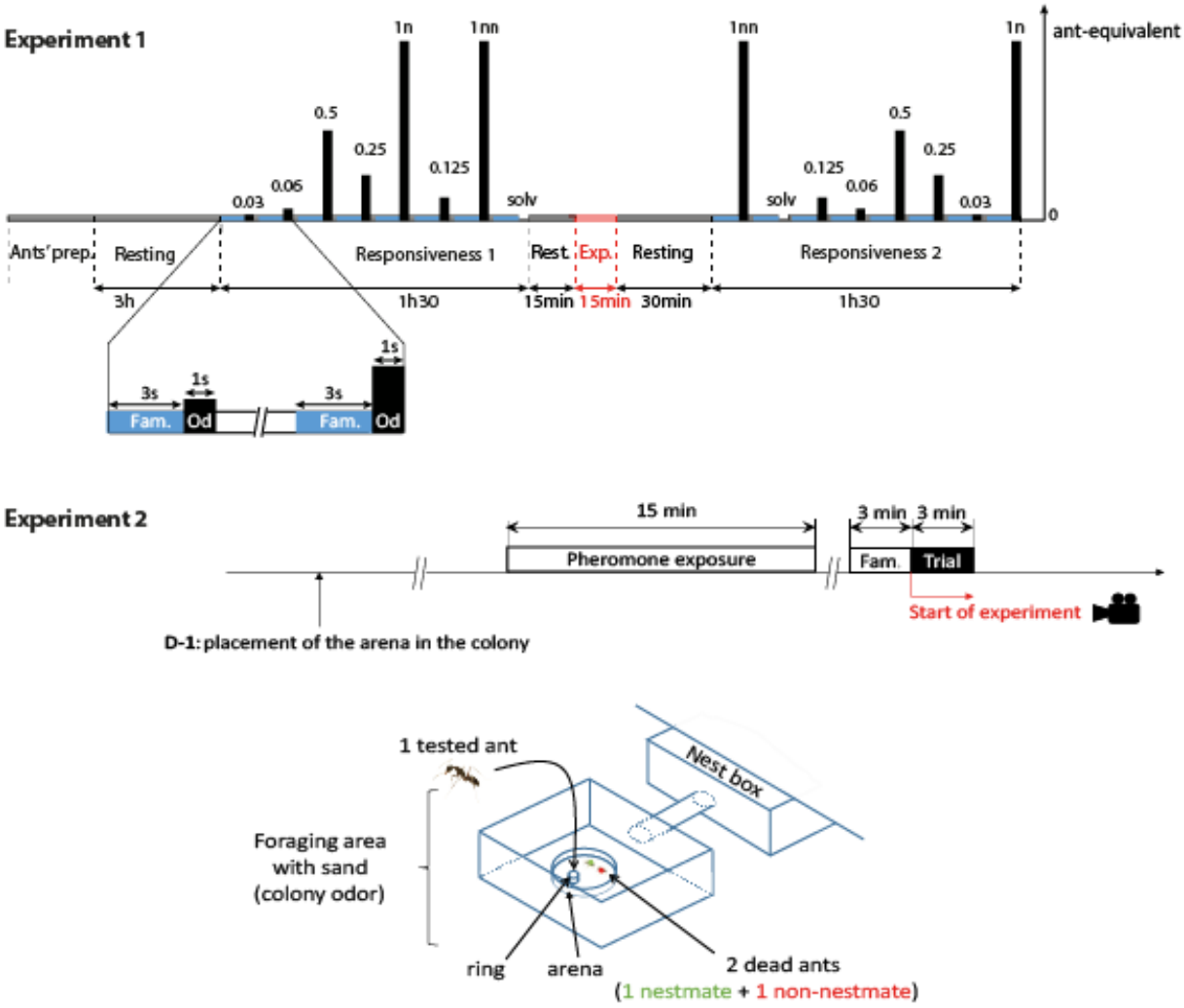

Figure S1. Protocols of experiments 1 and 2. In experiment 1, ants were harnessed and their responsiveness to cuticular hydrocarbons (CHCs) extracts was assessed twice, before and after exposure to formic acid (FA) or water. Presentation of chemical extracts was randomized. Here an example of two randomized sequences is represented. In experiment 2, ants were exposed either to FA or water before introduction in an arena. The tested ant faced both a dead nestmate and a dead non-nestmate at the same time. Ant's prep.: preparation of the ants; Fam.: familiarization; Od: odor (CHCs extracts); ITI: inter-trial interval; 1n: one nestmate equivalent; 1nn: one non-nestmate equivalent; solv: solvent; Rest: resting; Exp.: exposure (to FA in the experimental group or to water in the control group); D-1: Day-1.

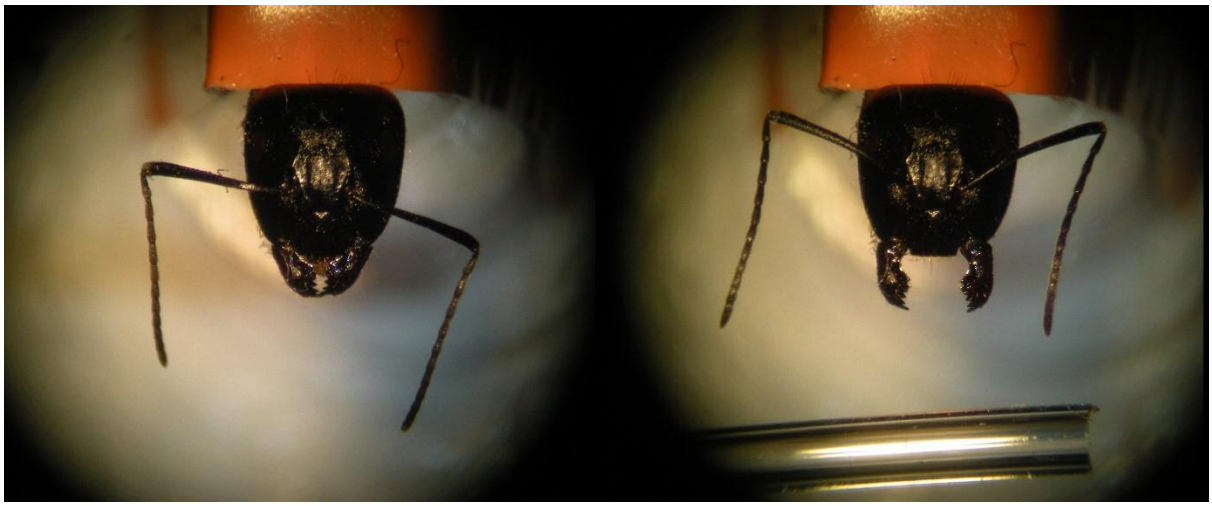

Figure S2. Mandible opening response (MOR) of harnessed *Camponotus aethiops* ants. On the left, a harnessed ant before the stimulus presentation. On the right, the ant displaying the MOR when presented with a glass rod coated with a non-nestmate odor without touching the antennae (magnification 10 ×).

CHAPTER 3: Pheromonal modulation of learning

An alarm pheromone modulates learning and the evaluation of odor similarity in carpenter ants

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Abstract

Pheromones are chemical substances released into the environment by an individual, which trigger stereotyped behaviors and/or physiological processes in individuals of the same species. Yet, pheromones exert modulations that exceed the original message they convey. In particular, olfactory perception is modulated by pheromones in mammals and insects. The modulation of responsiveness and experience-dependent behaviors has been mostly studied in honey bees, whereas the subject remains unexplored in ants, despite their high reliance on pheromones for intra-specific communication. We therefore tested the effect of formic acid, an alarm pheromone, on aversive and appetitive olfactory differential learning in *Camponotus aethiops*. We subsequently focused on appetitive learning and evaluated the generalization gradients resulting from conditioning and the effect of formic acid on them. We found that formic acid enhanced discrimination in the aversive differential conditioning. In the appetitive conditioning, formic acid facilitated the association between a non-preferred odor and the reward, and reversely impeded the association between a preferred odor and the reward. We suggest that these results are explained by the congruence or incongruence between the alarm signal, the hedonic value of the conditioned odors, and the valence of reinforcements. Eventually, we found that formic-acid exposed ants consistently generalized to heptanal, the only odor lying between the rewarded and the punished odor. We suggest that formic acid triggers asymmetries around the positively reinforced odor, a phenomenon called area shift. Formic acid therefore acts on the perception and/or evaluation of odors and their similarity, thus being a modulator of behavioral plasticity.

KEY WORDS: Pheromone modulation – Appetitive olfactory conditioning – Aversive olfactory conditioning – Generalization – Social insects – *Camponotus aethiops*

Introduction

A critical task for survival is to differentiate between stimuli that constitute a reward or positive experience, and stimuli that represent punishment or a negative experience. Learning and memory endow the animal with the capacity to establish stable predictions about positive and negative outcomes associated with specific stimuli. Moreover, when facing a new stimulus, animals can rely on generalization processes to establish adaptive responses despite stimulus novelty. Generalization allows treating unknown stimuli as equivalent of those that have been learned based on their similarity, and thus transferring outcome predictions to them (Ghirlanda and Enquist, 2003). This is particularly relevant in an environment that is continuously changing, as for instance the volatiles emitted by a flower that quantitatively and qualitatively vary in time and space (Dudareva et al., 2004) without necessarily implying a change in nectar quality. Therefore, responding differently to stimuli that differ only slightly is not necessarily advantageous.

Perceptual similarity may be influenced by the perceiver's experience. Indeed, discrimination abilities between similar stimuli vary depending on how an animal learns them. Absolute conditioning, which consists of training a single stimulus paired with reinforcement (i.e. A+), induces higher generalization than differential conditioning, which implies training to discriminate a reinforced from a non-reinforced stimulus (i.e. A+ vs B-) (Matsumoto et al., 2012). The ability to improve discrimination between similar stimuli depending on the conditioning procedure has been shown in several species and sensory modalities such as vision and olfaction (Barth et al., 2014; Chen et al., 2011; Cleland et al., 2002; Dyer and Chittka, 2004; Perez et al., 2016). In all cases, differential conditioning improved discrimination between stimuli and decreased generalization towards novel stimuli, compared to absolute conditioning.

In olfaction, characteristics such as the functional chemical group or the carbon-chain length of a chemical substance influence the perception of similarity (Bos et al., 2013; Guerrieri et al., 2005; Laska and Teubner, 1998; Perez et al., 2015b). The degree of similarity along one of these perceptual dimensions therefore determines the degree of generalization across stimuli (Shepard, 1987). Testing an individual along a given dimension after having trained him to a particular stimulus allows determining a generalization gradient which typically shows stronger responses to stimuli that are similar to the trained one and a progressive decrease in response with decreased similarity (Ghirlanda and Enquist, 2003). For instance, along the carbon-chain length gradient, the smaller the difference between odors, the stronger the generalization (Perez et al., 2016).

Pheromones are substances emitted by an individual that elicit behavioral or physiological responses in individuals of the same species (Karlson and Lüscher, 1959). They are key factors in coordinating social interactions and play a central role in maintaining the organization of societies, such as social insect colonies. Recent findings indicate that pheromones can also act as modulators of learning and therefore change the internal representation of the learned odors. For instance, exposure of young worker bees to the queen pheromone blocks their capacity to learn aversive associations while leaving intact their capacity to learn appetitive associations (Vergoz et al., 2007a). Still in honey bees, exposure of foragers to an alarm pheromone impairs their appetitive learning performances (Urlacher et al., 2010). These examples show that, besides triggering stereotyped responses, pheromones can act on behaviors that can be modified through individual experience by affecting their intensity, success or probability of occurrence. As such, they have a role as modulators of behavioral plasticity.

Ants are ideal model organisms to study the effects of pheromones on associative learning because of the importance of these molecules in their chemical communication and their developed odor-based behaviors (Hölldobler and Wilson, 1990; Vander Meer et al., 1998). At the same time, several laboratory protocols have been developed in the recent years, showing that ants can learn different sensory cues, in particular olfactory ones. Using *Camponotus aethiops* as a model, Guerrieri and d'Ettorre (2010) developed an appetitive conditioning paradigm similar to the conditioning of the proboscis extension response in honey bees. When the antennae of a harnessed ant are stimulated with sucrose solution, the ant extends its *maxilla-labium* to absorb the sucrose. This ''*maxilla-labium* extension response'' (MaLER) could be conditioned by forward pairing an odor (conditioned stimulus) with sucrose (unconditioned stimulus). On the other hand, Desmedt et al. (2017) developed the aversive learning protocol for harnessed ants by using the mandible opening response (MOR) as a proxy for aggression. This MOR could be conditioned by forward pairing an odor (conditioned stimulus) with heat (unconditioned stimulus).

Here we studied several aspects of pheromone modulation of associative olfactory learning in ants, by focusing on the carpenter ant *Camponotus aethiops*, given its well-documented learning abilities (Perez et al., 2013b; Perez et al., 2015b), and on formic acid (FA), an alarm pheromone used by this species to communicate the presence of danger to nestmates (Stumper, 1952). Formic acid is the main component of the secretion of the venom gland (Stumper, 1952). It triggers an increased spontaneous locomotor activity in the minute

following exposure, which is part of a stereotyped alarm behavioral sequence consisting of: (1) stopped locomotion and antennae movements, (2) mandible opening, (3) slow attraction towards the odor source, (4) increased locomotion, (5) potential attack, (6) cleaning of the antennae and abdominal tip (Ayre and Blum, 1971; Löfqvist, 1976). Our previous study on the effect of FA on nestmate discrimination showed that this substance improved discrimination abilities in *C. aethiops* (Rossi et al., 2018a). We thus asked whether FA improves discrimination accuracy in the context of a differential learning of odorants. Two independent experiments were addressed to test whether and how FA modulates aversive and appetitive olfactory differential learning and whether it affects the evaluation of new stimuli after training. Given that an increase in discrimination performances is correlated with a decrease in generalization (Ghirlanda and Enquist, 2003), we predicted that FA-exposed ants should generalize less not only between trained odors, but also towards novel odorants.

Material and methods

Study Organism

Experiment 1 (aversive differential conditioning) was conducted in February 2017, while *experiment 2* (appetitive differential conditioning) was conducted in March to May 2018, at the Laboratory of Experimental and Compared Ethology, Villetaneuse, France. We used five queen-right colonies of *Camponotus aethiops* collected in 2014 and 2016 at Pompertuzat (Midi-Pyrénées, France, latitude 43.5, longitude 1.5167) and kept in the laboratory under controlled conditions (25°C, light-dark cycle = 12:12, 36% humidity) in two Fluon-coated plastic boxes connected by a tube. One box was provided with plaster floor and covered by cardboard (nest), the other was exposed to light and had sand on the floor (foraging area). Ants were fed twice a week with a mixture of honey and apples for carbohydrates and vitamins, and with pieces of crickets for proteins; water was provided *ad libitum*. In *experiment 2*, two weeks prior to the onset of the experiment, the ants' diet was changed to crickets and water *ad libitum* but no carbohydrates were provided to increase the insects' motivation for sucrose used as reward during conditioning.

Conditioning and test procedures

(a) Individual handling

Medium-sized workers were collected because this category usually forages for food (Dupuy et al., 2006). They were subsequently anaesthetized on ice for harnessing in individual holders as previously described (*experiment 1*: appetitive conditioning, Perez et al. 2016; *experiment 2*:

aversive conditioning, Desmedt et al. 2017). In *experiment 1*, fixed ants could only move their antennae and mouthparts, while in *experiment 2*, they could move all their appendages, thus limiting the stress associated with the harnessing, which might affect the aversive response. Ants were then left in a dark and humid box during 3 h to recover from anesthesia and accustom to harnessing conditions.

(b) Pheromone exposure

Formic acid (Sigma-Aldrich) was diluted to 12% (3 µl pheromone + 22 µl water, equivalent to one third of the content of one poison gland, Stumper 1952). Ants were exposed either to FA (experimental group) or to 25 µl of pure water (control group). Ants harnessed in their holders were individually confined for 15 min in a 50 ml plastic flask containing a filter paper (1 x 5 cm) soaked with the pheromone (or water) placed under a hood. After exposure, ants were directly transported to another hood for conditioning.

(c) Stimuli

In *experiment 1*, ants were subjected to an aversive differential conditioning using a thermal stimulation of 75°C (unconditioned stimulus, US) applied to the hind legs as aversive reinforcement. Ants had to learn to discriminate octanal as punished odor CS+, and 1-hexanol as unreinforced odor CS- (Sigma Aldrich, France). 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). Its temperature was adjusted via a laboratory power supply (Velleman HQ-power, PS1503), and was measured with a contact thermometer (Voltcraft VC-150-1) at the beginning and end of each series of tested ants. Before each training phase, five microliters of pure odorant were applied onto a 1-cm^2 piece of filter paper, which was then inserted in a 20 ml plastic syringe for delivery. During memory tests, the same odors were used without reinforcement (Desmedt et al., 2017).

In *experiment 2*, ants were subjected to a differential conditioning in which an appetitive 2.32 M sucrose solution (50% w/w) and an aversive 3 M NaCl solution (purity 99.5%, Sigma Aldrich, France) were used as reinforcements. Ants had to learn to discriminate octanal and hexanal that varied in two carbon-chain length (Sigma Aldrich, France) and that were used as conditioned stimuli, either as a CS+ paired with sucrose or as punished CS- paired with NaCl. Before each training phase, two microliters of pure odorant were applied onto a 1 cm^2 piece of filter paper, which was then inserted in a 10 ml plastic syringe to allow odor delivery. During memory tests, besides assessing responses to the CS+ and the CS- in the absence of

reinforcement, we also evaluated generalization towards novel odorants, and the impact of FA on it. Five aldehydes were used that varied along the carbon-chain length gradient, two of which were the trained odorants (hexanal and octanal) and the three others were the novel odorants (heptanal nonanal, and decanal).

(d) Conditioning and test procedures

In *experiment 1*, to test whether aversive learning was influenced by pheromone exposure, ants were exposed to pheromone or water and then subjected to differential conditioning of the mandible opening response (MOR), an aversive reaction to thermal stimulation (Desmedt et al., 2017). Ten replicates of 12 ants were performed, leading to 120 conditioned ants (60 exposed to FA and 60 exposed to water).

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. The sequence started with the punished odor CS+ for half of the individuals and with the unreinforced odor CS- for the other half. Each trial lasted 50 s. Twenty-three seconds after placing the ant under a binocular, a CS was presented during 4 s to the ant's head by blowing an air puff with the syringe placed at 2 cm. Three seconds after the onset of odor presentation, the thermal stimulation (US) was delivered for one second (eliciting MOR) in the CS+ trials. Thus, the overlap between odor and reinforcement was always 1 s. During CS- trials, ants only received the odor (no US) using the same method, thus ensuring that the odor puff could not act as a predictor of reinforcement *per se*. The ant was then left in the conditioning place during 23 s in order to impede a predictive, forward association between context and reinforcement. The entire protocol took place under a hood in order to remove remaining odor stimulations. The average inter-trial interval between two CS+ trials was 20 min. 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 (unconditioned response). Individuals that did not respond at least three times to the punishment were discarded (18 out of 120 ants, 15% in total) as they were considered unresponsive to thermal stimulations (Desmedt et al., 2017).

During memory test, ants were presented with the same two odors in a randomized order one hour after the last conditioning trial. As for training, each test lasted 50 s. Within each test, odors were presented during 4 s without reinforcement. After the end of the test phase, the thermal stimulation was presented again to each ant to verify whether MOR was still elicited by the aversive US. Ants that did not respond to the US (3 out of 120 ants, 2.5% in total) were

not included in the statistical analyses because the absence of response could reflect a lack of motivation or poor physical condition. One ant (0.83%) for which measurements could not be obtained because she continuously displayed MOR during the all process was also discarded from analyses. The mortality rate during experiments was 5.83% (7 out of 120 ants). The number of ants retained in the analyses was therefore 91 (44 FA-exposed ants and 47 control ants).

In *experiment 2*, to test whether olfactory generalization gradients were influenced by pheromone exposure, ants were exposed to pheromone or water and then subjected to differential conditioning of the *maxilla-labium* extension response (MaLER), an appetitive reaction to sucrose stimulation (Perez et al., 2013a). Separate groups of ants were trained to discriminate two odorant combinations: octanal+/hexanal-, and hexanal+/octanal-; where '+' indicates the presence of reward and '-' that of punishment. Eight replicates of 12 ants were performed for each subgroup within an odor pair (e.g. octanal+/hexanal-, and hexanal+/octanal-), leading to a total of 192 ants (96 exposed to FA and 96 exposed to water).

Training consisted of six CS+ and six CS- trials that followed a pseudo-random sequence as for aversive differential conditioning (see above). For half of the individuals of a group, the sequence started with the CS+ while for the other half, it started with the CS-. Each trial lasted 1 min. Twenty-five seconds after placing the ant under a binocular microscope, a CS was presented during 5 s to the ant's head by blowing an air puff with the syringe placed at 2 cm. Three seconds after the onset of odor presentation the ant's *maxilla-labium* was stimulated during 5 s with sucrose in the CS+ trials; NaCl was used in the CS- trials. Thus, the overlap between odor and reinforcement was always 2 s. The ant was then left in the conditioning place during 27 s in order to impede a predictive, forward association between context and reinforcement. The mechanical stimulation of the air puff was common to both CS+ and CS- trials so that it could not act as predictor of appetitive reinforcement. The entire protocol took place under a hood in order to remove remaining odor stimulations. The average inter-trial interval between two CS+ trials was 30 min. Individuals that did not respond at least three times to the sucrose reward were discarded (Perez et al. 2015, 9 out of 192 ants, 4.69% in total) to prevent confounding effects of a low motivation for the appetitive US on acquisition rates of the CS+.

During memory test, ants were presented with the five aldehydes in a randomized order ten minutes after the last conditioning trial. As for training, each test lasted 1 min. Within each test, odors were presented during 5 s without reinforcement. After the end of the test phase (all

five odorants tested, ca. 1 h), a droplet of sucrose was presented to each ant to verify the presence of MaLER to the appetitive US. Seven out of 192 ants (3,65%) for which measurements could not be obtained were discarded from analyses; the mortality rate during experiments was of 1.04% (2 out of 192 ants). The numbers of ants retained in the analyses were 89 for the octanal+/hexanal- condition (46 FA-exposed ants and 43 control ants), and 85 for the hexanal+/octanal- condition (42 FA-exposed ants and 43 control ants).

Data analysis

In *experiment 1*, the ants' responses during conditioning and tests were scored as 1 when MOR occurred during odor presentation and 0 otherwise. Generalized linear mixed models (GLMM, package *lme4*; Bates et al. 2011) with a binomial error structure (logit-link) were used to analyze the acquisition data. In all GLMMs, the ants' response was used as response variable. Moreover, we included the ants' identity as a random effect to account for the repeated measurements performed, and nested it into the colonies of origin to account for the fact that ants tested within a given colony were more likely to behave similarly due to genetic similarity. We analyzed each treatment separately as treatments required independent groups. The GLMM retained for FA contained the interaction between the CSs and trials, while the GLMM retained for water only contained the CSs as fixed factor. For the analysis of the memory test, the full model was retained, i.e. with the interaction between "*Treatment"* (FA/water) and "*CS*" (CS+/CS-). Post-hoc tests were performed using least-squares means (LSM, *lsmeans* function from R package *lsmeans*; Lenth, 2016). Responses during memory tests were also classified in four categories according to the ants' responses, i.e. "correct" (CS+:1/CS-:0), "generalization" (CS+:1/CS-:1), "no response" (CS+:0/CS-:0), or "reverse" (CS+:0/CS-:1). Differences in the proportions of ants of these categories according to their treatments were analyzed using χ^2 tests.

In *experiment 2*, the ants' responses during conditioning and tests were scored as 1 when MaLER occurred during odor presentation and 0 otherwise. GLMMs with a binomial error structure (logit-link) were again used to analyze the acquisition data, with the identity of the ants nested in the colonies of origin. To test for a potential effect of the rewarded odor on acquisition, we run a GLMM for each treatment separately with "*CS+*" (octanal or hexanal), "*CS*" (CS+ or CS-), and "*Trial*" (one to six) as fixed factors. The GLMM retained for the experimental group also contained the interaction between "*CS+*" and "*CS*" while the model retained for the control group contained the interaction between "*CS+*" and "*CS*" as well as the interaction between "*CS+*" and "*Trial*".

We then analyzed the acquisition for each condition separately (octanal+/hexanal- and

hexanal+/octanal-). For the octanal+/hexanal- condition, the GLMM retained contained "*Treatment*" (FA/water), "*CS*", and "*Trial*" as fixed factors with the interactions between "*Treatment*" and "*Trial*", and "*CS*" and "*Trial*". The models retained for the acquisition analyses of each treatment included the interaction between CSs and trials. The GLMM retained for the analysis of the memory test contained "*Treatment*" and "*Odor*" (the five aldehydes) as fixed factors, and the GLMMs for FA and water only contained "*Odor*" as fixed factor. For the hexanal+/octanal- condition, the GLMM retained contained "*Treatment*", "*CS*", and "*Trial*" as fixed factors with the interaction between the CSs and trials. The models retained for the acquisition analyses of each treatment included the interaction between "*CS*" and "*Trial*". For the memory test, the GLMM retained contained "*Treatment*" and "*Odor*" as fixed factors, and the GLMMs for FA and water only contained "*Odor*" as fixed factor.

In all analyses, we retained the significant model with the highest explanatory power (i.e. the lowest AIC value). All statistical analyses were performed with R 3.4.2 (R Development Core Team, 2016) and the significance threshold was set at 0.05.

Results

Experiment 1: Aversive differential conditioning

Formic-acid exposed ants learnt to differentiate the CS+ from the CS-, as indicated by a significant interaction between trials and CSs (GLMM, *CS* * *Trial*: $\chi^2 = 11.45$, df = 5, P = 0.043, Fig. 1A). The analysis of the responses of control ants, however, did not reveal any interaction between trials and the CSs but there was a difference of responses between the CS+ and the CS- (GLMM, *CS*: $\chi^2 = 17.13$, df = 1, P < 0.001, Fig. 1B). This result indicates that more ants displayed MOR to the punished odor than to the unreinforced odor but that the dynamics along trials did not significantly differ.

The analysis of the proportions of ants displaying MOR during the memory tests revealed a marginally non-significant tendency to respond differently to the CSs according to their treatments (GLMM, *Treatment* * *CS*: $\chi^2 = 3.06$, df = 1, P = 0.080, Fig. 1C). Specifically, FA-exposed ants responded more to the CS+ than to the CS- (LSM *post-hoc*, P = 0.002), while this was not the case for control ants (LSM *post-hoc*, P = 0.221). Moreover, FA-exposed and control ants responded similarly to the CS+ (LSM *post-hoc*, P = 0.982), and to the CS- (LSM *post-hoc*, P = 0.289).

The proportion of ants that responded correctly during the memory test was higher in the FA-exposed group compared to the water-exposed group ($\chi^2 = 4.27$, df = 1, P = 0.039).

Inversely, the proportion of ants that generalized during the memory test was lower in the FA-exposed group compared to control (χ^2 = 4.92, df = 1, P = 0.027). There was no effect of treatment for the other two categories of responses (i.e. "no response" and "reverse", P > 0.05 in both cases; Fig. 1D).

Taken together, the results of *experiment 1* indicate that FA exposure improved odor discrimination during aversive differential conditioning compared to water exposure.

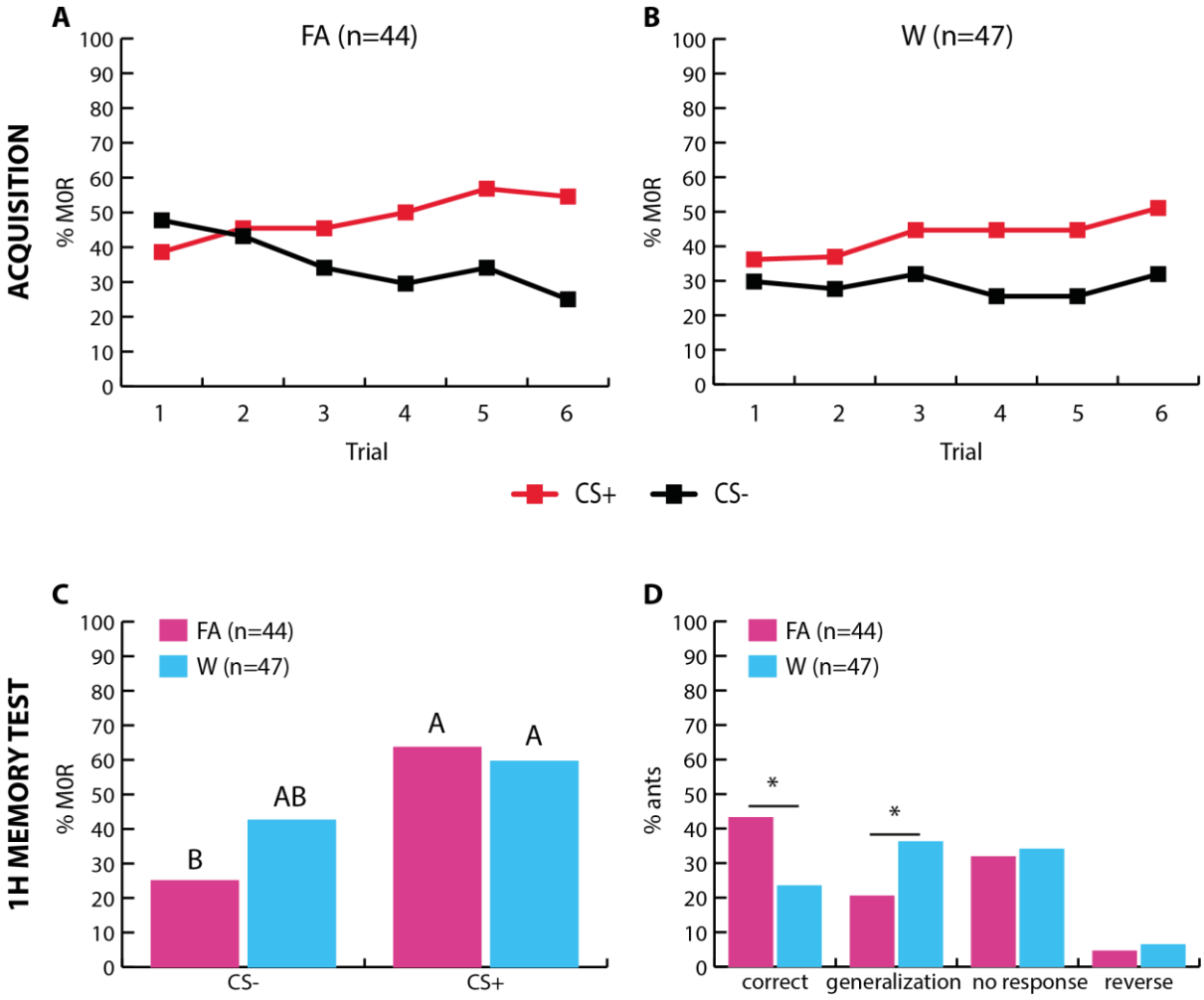

Figure 1. Formic acid exposure improves discrimination. (A) Acquisition curves of formic-acid (FA) exposed ants. The percentages of mandible opening responses (%MOR) to the punished conditioned stimulus (CS+) and to the unreinforced conditioned stimulus (CS-) are represented along trials. Ants exposed to FA learned to discriminate between the two odors (significant interaction between the CSs and trials). (B) Acquisition curves of water (W)-exposed ants. Control ants discriminated the two odors but did not show evidence of learning (no interaction between the CSs and trials). (C) Aversive responses of ants one hour after conditioning (memory test). Formic-acid exposed ants discriminated between CS+ and CS- but control ants did not. (D) Proportions of ants that responded either correctly (CS+:1/CS-:0), generalized (CS+:1/CS-:1), did not respond (CS+:0/CS-:0), or responded reversely (CS+:0/CS-:1) during the memory test. A higher proportion of ants exposed to FA responded correctly and a lower proportion of them generalized compared to control ants.

All groups learnt to differentiate the CS+ from the CS-, as indicated by a significant interaction between trials and CSs (GLMM, octanal+/hexanal-, FA: $\chi^2 = 36.75$, df = 5, $P < 0.001$, Fig. 2A; octanal+/hexanal-, water: $\chi^2 = 14.26$, df = 5, P = 0.014, Fig. 2B; hexanal+/octanal-, FA: $\chi^2 = 20.66$, df = 5, $P < 0.001$, Fig. 2D; hexanal+/octanal-, water: $\chi^2 = 18.27$, df = 5, P = 0.003, Fig. 2E).Overall, ants responded more to the CS+ (69% in average) than to the CS- (25% in average) in the last trial. Moreover, all groups showed memory, as indicated by a significant difference between the CSs (LSM *post-hoc*, $P < 0.001$ in all cases), with ants responding more to the CS+ (69% in average) than to the CS- (25% in average) and at the same level as during the 6th trial of the acquisition.

When ants were conditioned with octanal as CS+ and hexanal as CS-, acquisition dynamics did not differ between treatments despite the marginally non-significant p-value found (GLMM, *Treatment* * *Trial*: $\chi^2 = 10.13$, df = 5, P = 0.072). When odors were reversed (hexanal+/octanal-), the acquisition curves differed significantly between FA-exposed and control ants (GLMM, *Treatment*: $\chi^2 = 4.01$, df = 1, P = 0.045). Moreover, control ants did not behave similarly during acquisition, depending on whether octanal and hexanal were rewarded or punished (GLMM, *CS+* * *CS*: $\chi^2 = 4.46$, df = 1, P = 0.035; *CS+* * *Trial*: $\chi^2 = 13.96$, df = 5, P = 0.016). Ants responded more with hexanal than with octanal as the CS+ (77% vs. 58% at the 6th trial respectively). Pheromone-exposed ants also differed in their acquisition depending on whether octanal and hexanal were rewarded or punished (GLMM, *CS+* * *CS*: $\chi^2 = 6.64$, df = 1, P = 0.010). Formic-acid exposed ants responded more with octanal than with hexanal as the CS+ (76% vs. 64% at the 6th trial respectively); the responses to the CS- were similar (22% vs. 19% at the 6th trial respectively).

When conditioned in the octanal+/hexanal- condition, ants exposed to FA and to water behaved differently during the memory and generalization tests, with FA-exposed ants responding generally more than control ants (GLMM, *Treatment*: $\chi^2 = 7.04$, df = 1, P = 0.008, Fig. 2C). Moreover, irrespective of the treatment, ants responded differently to the different aldehydes (GLMM, *Odor*: $\chi^2 = 48.04$, df = 4, $P < 0.001$). When analyzing each treatment separately, the analyses showed that FA-exposed ants generalized their appetitive response to all three novel odorants (pairwise comparisons between octanal and the three new aldehydes, LSM *post-hoc*, $P > 0.05$ in all cases). It should be noted that these aldehydes were closer to the CS+ in terms of carbon-chain length than to the CS-, except for heptanal which corresponds to the exact intermediate between CS+ (hexanal) and CS- (octanal). Water-exposed ants, however,

did not generalize (LSM *post-hoc*, octanal – heptanal: P = 0.077; octanal – nonanal: P = 0.040; octanal – decanal: P = 0.010; hexanal – heptanal: P= 0.058; hexanal – nonanal: P = 0.110; hexanal – decanal: P = 0.328).

In the case of the hexanal+/octanal- condition, there was only an effect of the tested aldehydes on the ant's responses during the memory/generalization tests (GLMM, *Odor*: χ^2 = 41.13, df = 4, P < 0.001, Fig. 2F). When analyzing each treatment separately, the analyses showed that FA-exposed ants generalized their appetitive response to heptanal only, i.e. to the aldehyde lying between the CS+ (hexanal) and the CS- (octanal) in terms of carbon-chain length. Generalization did not occur to the other two new aldehydes, which were more similar to the CS- (octanal) in terms of carbon-chain length, and contained three to four supplementary carbons compared to hexanal (LSM *post-hoc*, hexanal – heptanal: P = 0.296; hexanal – nonanal: P = 0.056; hexanal – decanal: P = 0.028). Water-exposed ants, however, did not generalize their appetitive response to heptanal (LSM *post-hoc,* hexanal – heptanal: P = 0.037), and responded similarly to the CS- and to the new aldehydes (LSM *post-hoc*; octanal – heptanal: P 0.160; octanal – nonanal: P = 0.764; octanal – decanal: P = 0.974).

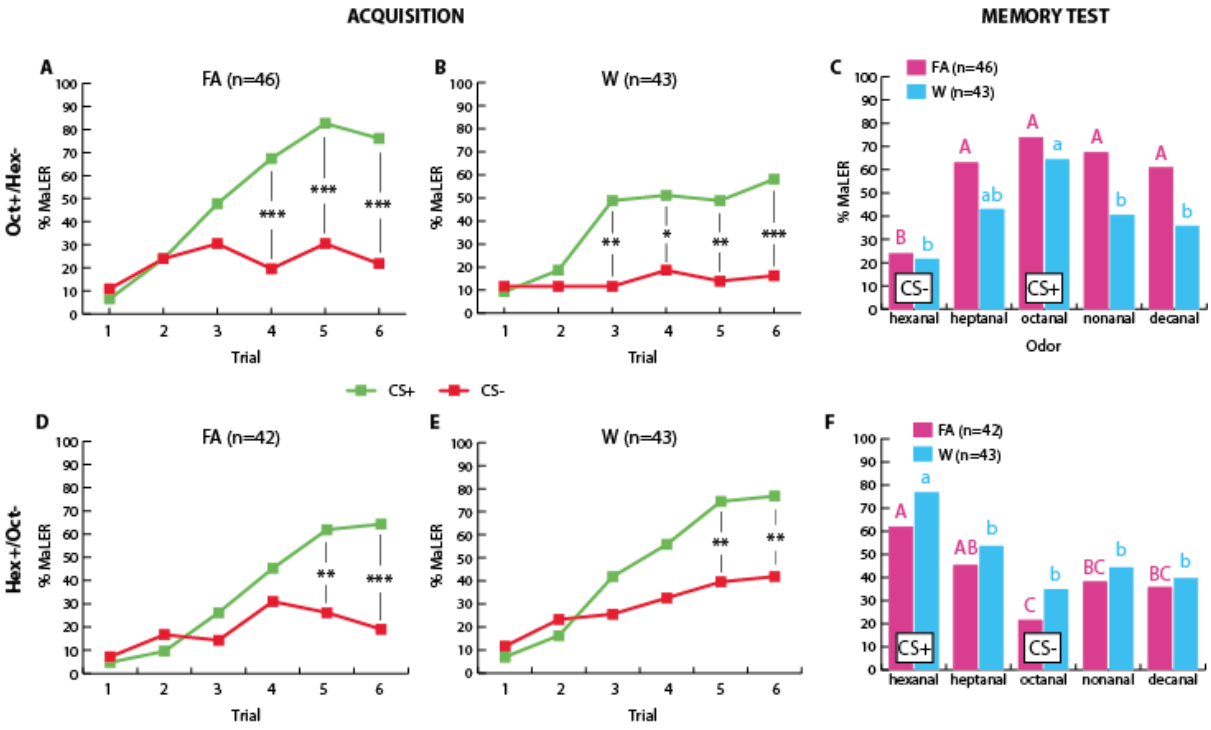

Figure 2. Formic acid modulates both acquisition and the evaluation of novel stimuli during appetitive differential conditioning. (A,B,C) octanal+/hexanal- conditioning. (A) Acquisition curves of formic-acid (FA) exposed ants. The percentages of *maxilla-labium* extension responses (%MaLER) to the rewarded conditioned stimulus (CS+) and to the punished conditioned stimulus (CS-) are represented along trials. (B) Acquisition curves of water (W)-exposed ants. Formic-acid exposed and control ants differed in their acquisition dynamics. (C) Appetitive responses of ants during the memory test to the conditioned stimulus and three new aldehydes. Formic-acid exposed ants generalized their response to the CS+ to all the new

aldehydes, while control ants did not. (D,E,F) hexanal+/octanal- conditioning. (D) Acquisition curves of formic-acid (FA) exposed ants. (E) Acquisition curves of water (W)-exposed ants. Formic-acid exposed and control ants differed in their acquisition dynamics. (C) Appetitive responses of ants during the memory test to the conditioned stimulus and three new aldehydes. Formic-acid exposed ants generalized their response to the CS+ to all three new aldehydes, while control ants did not. LSM *post-hoc*. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$. Different letters indicate significant differences in the levels of responses to test odors for ants exposed to water (blue lowercase) and to FA (pink uppercase).

Taken together, the results of *experiment 2* indicate that FA modulated the acquisition dynamics of the appetitive differential conditioning. Moreover, this experiment showed that discrimination learning of control ants depended on whether octanal or hexanal was rewarded, and that FA modulated it accordingly. Control ants associated hexanal with the reward more easily than octanal, and FA facilitated the discrimination with the non-preferred odor octanal but not with the preferred odor hexanal. Formic-acid exposed ants also consistently generalized to heptanal, which lays between the CS+ and the CS- in terms of carbon-chain length.

Discussion

Our study aimed at investigating the role of an alarm pheromone as a modulator of olfactory learning and generalization in ants. To this end, we exposed carpenter ants *C. aethiops* to FA, a substance characterized as an alarm pheromone in Formicine ants, and determined the effect of this exposure on both aversive differential conditioning of the MOR, and appetitive differential conditioning of the MaLER. In this last experiment, we also evaluated the effect of pheromone exposure on odor generalization following conditioning. Our study shows that pheromones modulate learning and generalization, thereby contributing to behavioral plasticity.

Three main results were found in our work: 1) FA exposure induced better discriminative performances in the context of aversive differential conditioning of the MOR; 2) FA modulated the acquisition dynamics of appetitive differential conditioning of the MaLER in an odor-dependent way. In particular, it seemed easier for FA-exposed ants to learn a discrimination with a non-preferred rewarded odor (octanal) than with a preferred odor (hexanal); 3) FA exposure induced generalization of appetitive responses to heptanal, the odorant that lays between the CS+ and the CS- (octanal and hexanal) in terms of carbon-chain length, regardless of which odorant was rewarded or punished.

The effect of FA in an aversive context

Our results reveal that the alarm pheromone component FA has the capacity to modulate aversive olfactory differential learning. Exposure to this substance increased discriminative performances during acquisition, an effect that translated into memory retention performances

one hour after conditioning. Indeed, FA-exposed ants were more likely to respond correctly during the memory test, and conversely less likely to generalize, compared to control ants exposed to water. Formic acid thus increased discrimination accuracy, a phenomenon already observed in the process of nestmate discrimination where FA-exposed ants were more likely to be aggressive towards non-nestmates, and less likely to display aggressiveness towards nestmates at the same time (Rossi et al., 2018a). We suggested that FA would lower a hypothetical threshold of olfactory perception, thereby increasing the amount of information available to the ant (both qualitatively and quantitatively) to perform discrimination. Such a process may rely on biogenic amines acting at peripheral and/or central levels in the nervous system and whose action may be modulated by FA. This hypothesis revolves around the established role of certain biogenic amines such as dopamine (DA) in selective attention in insects. Selective attention describes the experience-dependent stimulus suppression dynamics that allow an animal to make adaptive choices at the right time (Van Swinderen and Andretic, 2011). Several studies in the fruit fly, *Drosophila melanogaster*, suggest that one of the central roles played by DA may involve perceptual suppression, a necessary component of selective attention (Andretic et al., 2005; Zhang et al., 2007). Supposing that selective attention is the process underlying discrimination accuracy, FA could act on DA levels, thus improving discrimination abilities of ants in the olfactory domain. To test this hypothesis, it would be important to quantify DA levels in the ant's brain (e.g. via High-Performance Liquid Chromatography quantification) or DA receptor expression following FA exposure. Additionally manipulating DA levels and establishing how this procedure affects the accuracy of olfactory discrimination is of fundamental importance. Furthermore, it cannot be excluded that other biogenic amines such as octopamine (OA) or serotonin (5HT) play a similar role as the one described for DA.

It should be noted that our control ants did not learn properly the olfactory discrimination, contrary to what was reported by Desmedt et al. (2017) who used the same conditioning protocol and in the same ant species. In our case, ants did not show signs of acquisition although they discriminated both odors as suggested by the significant effect of the conditioned stimuli. Differences in results could be attributed to multiple factors including experimenter effects, colonies of origin, season, abiotic factors, conditioning sequence effect etc.

Formic acid modulation of acquisition

Discrimination learning of control ants depended of the odorants used as CS+ and CS-. A higher percentage of ants learned the association with the reward when the CS+ was hexanal and the CS- octanal than in the reversed situation (already observed in Perez et al., 2016). A possible explanation for this odor asymmetry is that ants had some naïve preference or prior positive experience with hexanal that favored its association with sucrose reward. As hexanal is found in flowers of several plant genus and notably one, the *Ophrys* genus (Knudsen et al., 1993), which overlaps with the distribution range of *C. aetiops*, it might be that this odor already had an appetitive value for them. However, we did not perform a botanical survey when we collected ant colonies and cannot infer about the presence or not of *Ophrys* species at this exact location. In order to test the hypothesis of a preference for hexanal in carpenter ants, one possible experiment to perform could be to place ants of the colonies used in our experiments in a Y-maze with hexanal in one branch and other odors in the second branch and record the choice they make.

The FA modulation of appetitive learning was also dependent on the nature of the odorants used as CS+ and CS-. Contrary to what we observed for control ants, discrimination was favored in FA-exposed ants when octanal was the CS+ and hexanal the CS-. In other words, FA favored odor discrimination in the direction opposite to the bias exhibited by control ants exposed to water. We propose that the lack of congruence between the alarm signal and the learning context produces asymmetries in odor learning. In other words, as control ants seem to have a predisposition to learn hexanal as a rewarded odorant, the effect of FA would be to revert this bias in the opposite direction, i.e. favoring the learning of the odor that was not naturally preferred. An inconsistency between signal and learning valence has already been shown to induce deterrent effects of alarm pheromones on appetitive learning in honey bees. Baracchi et al. (2018) showed that geraniol (an appetitive pheromone in honey bees) enhanced appetitive olfactory differential learning, while 2-heptanone (an alarm pheromone in honey bees) exerted a decremental effect on learning. Similarly, Urlacher et al. (2010) found that isopentyl acetate (another alarm pheromone in honey bees) impaired appetitive olfactory absolute learning. Moreover, Guiraud et al. (2018) found that bees conditioned with a non-preferred sweetener associated with an electric shock and a preferred sweetener with the absence of shock discriminated better than when inversing this contingency. In other words, making a non-preferred taste even more aversive due to its association with shock facilitated

discrimination from a preferred sweetener; on the contrary, punishing a preferred taste induced an impairment of discrimination due to the inconsistency of the information provided. A similar effect could occur here. While hexanal appears to be a preferred odorant, FA, an alarm signal, would induce a change of context favoring the learning of the non-preferred odorant, octanal. Therefore, we suggest that it would be easier to learn a discrimination with a non-preferred rewarded odor in the presence of FA than with a preferred odor in which case incoherence between the hedonic value of CS+ and the alarm signal would be higher.

Formic acid modulation of odor generalization

One consistent result in *experiment* 2 is the enhancement of appetitive responses to heptanal by FA in the generalization tests performed after conditioning, irrespective of the role (CS+ or CS-) of octanal and hexanal. From the new odorants presented in the generalization tests, heptanal was the only one with a carbon-chain length that laid between those of the CS+ and the CS-. Formic-acid exposed ants therefore consistently evaluated heptanal as being more similar to the CS+ than to the CS-. Given the asymmetry of the generalization gradient following the octanal+/hexanal- conditioning when ants were exposed to FA, we suggest that FA provokes an area shift towards the CS+ (ten Cate and Rowe, 2007). It would be coherent in the octanal+/hexanal- condition that FA facilitated the association between octanal and the reward and that this facilitation transferred into generalization around the octanal and away from the CS-. Regarding the hexanal+/octanal- condition, the generalization gradient used could not allow us to infer about an asymmetry around the CS+ because we did not test novel odorants in this direction. In order to evaluate for a consistent asymmetry in the generalization gradients, new odors lying away from octanal as the CS- and closer to hexanal as the CS+ (i.e., pentanal and butanal) should be tested after appetitive conditioning. Studies in bees have shown that the shape of the generalization gradient obtained after differential conditioning (and in these cases, the magnitude of the area shift) could vary according to some stimulus characteristics, such as the relative difference between the positive and the negative reinforcements (Wright et al., 2009). We therefore hypothesize that the potential area shift observed in our experiment results from an imbalance in intensity between the reward and punishment, which would be accentuated by FA. In order to test for the actual presence of an area shift, modelling of the "excitatory" and "inhibitory" generalization gradients mediated by the CS+ and the CS-, respectively, would be required (see Perez et al., 2016).

In conclusion, pheromones change the way insects perceive their environment, depending on the context signaled, the one they are subsequently submitted to, and the nature

of the perceived stimuli (whether they are unconditioned stimuli prompting reflex responses, see Baracchi et al., 2017; Rossi et al., 2018b, or conditioned ones). However, as shown by the complicated results of this study, further experiments are required to decipher all the parameters in action here and reconcile those with the function of FA as an alarm pheromone.

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GENERAL DISCUSSION

The studies presented in this thesis allowed us to expand our knowledge about the way pheromones modulate the perception of unconditioned stimuli (US) depending on their valence in honey bees and ants. The results obtained also brought essential information to understand the factors implicated in the modulation of olfactory learning and odor perception and their consequences in decision making in free-walking conditions that are more similar to natural situations (e.g. nestmate discrimination).

The pheromonal modulation of responsiveness

Our experiments aimed at investigating the role of pheromones as modulators of insects' subjective evaluation of US and thus at uncovering a non-canonical function of pheromones as key components of behavioral plasticity. As aversive and appetitive responsiveness provide a reliable readout of the insects' subjective evaluation of punishment and reward, respectively (Roussel et al., 2009; Scheiner et al., 2005b; Tedjakumala et al., 2013; Tedjakumala et al., 2014), changes in responsiveness following pheromone exposure show that pheromones are capable of behavioral modulation beyond the specific context in which they are released.

The effect of appetitive pheromones on responsiveness

We refer to appetitive pheromones as pheromone components signaling valuable resources, triggering attraction and relating to appetitive searching behavior motivation. Our results revealed the novel finding that an appetitive pheromone component (geraniol) has the capacity to modulate the subjective evaluation of aversive stimuli in honey bees (**Chapter 1**). Exposure to this substance decreased aversive responsiveness to electric shock, thus showing that it diminished the perceptual impact of shock in bees. Nouvian et al. (2015) also found that innate appetitive floral odors (linalool and 2-phenylethanol) diminish defensive responses (attack of a moving dummy) of honey bees. We propose that the conflict between an appetitive signal (attractive floral odors, geraniol) and an aversive signal or context (enemy, electric shock) would be responsible for downregulating aversive responsiveness in honey bees. In the carpenter ant *Camponotus aethiops*, Desmedt (2016) found no effect of the supposedly appetitive pheromone mellein on thermic aversive responsiveness (Fig. 1). This component has been shown to be a trail pheromone in other *Camponotus* species (Kohl et al., 2003) but no trail pheromone has been identified in *C. aethiops*. Moreover, the results of Desmedt (2016) showing similar responses between control and mellein-exposed ants, it is possible that mellein would not be perceived as an appetitive pheromone in these ants.

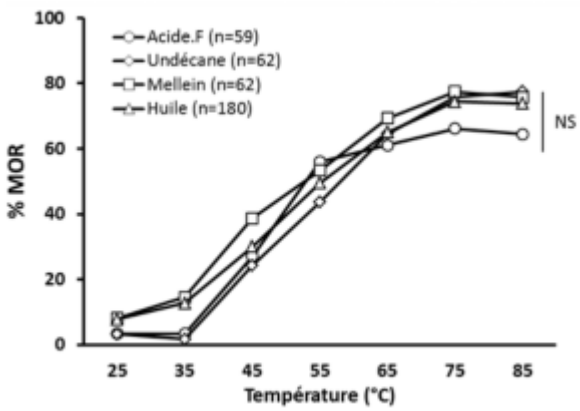

Figure 1. Effect of pheromones on thermic responsiveness in carpenter ants. Percentage of ants which responded with mandible opening responses (MOR) to ascending thermic stimulations after exposure to two alarm pheromone components (formic acid and undecane), one appetitive pheromone component (mellein), and oil. Pheromone exposure did not affect thermic responsiveness (NS: not significant).

In honey bees, Baracchi et al. (2017) found that geraniol enhanced sucrose responsiveness, notably at the lowest sucrose concentrations. In Argentine ants, (Z)-9-hexadecenal (a synthetic trail pheromone) also enhanced sucrose responsiveness (**Chapter 1**). When ants were presented with sucrose drops of various concentrations, the pheromone increased the number of ants accepting low sucrose concentrations. Appetitive pheromones would therefore make low sucrose concentrations more attractive, thereby changing the perception and/or evaluation of this food source by honey bees and ants from "poor" to "valuable". The fact that appetitive pheromones would prompt insect foragers to look for food whatever the quality of it could be less costly and much faster for individuals than having to weight the gains and losses of energy in engaging in foraging behavior. Social instructions would therefore be stronger than evaluating food quality. Argentine ants exposed to the synthetic pheromone also spent more time feeding on the more profitable sucrose drop presented (**Chapter 1**). This could result in an increase of the absolute quantity of valuable food brought back to the nest, which would be particularly advantageous for the colony. In this sense, pheromonal modulation of responsiveness would contribute to adaptive behavioral plasticity.

The effect of alarm pheromones on responsiveness

Alarm pheromones are defined as chemicals eliciting dispersion in the presence of a predator in aggregating non-eusocial species, or aggression in the case of nest disturbance in eusocial insects (Blum, 1985). We found that one alarm pheromone (isopentyl acetate) on two (+ 2-heptanone) modulated aversive responsiveness in honey bees (**Chapter 1**). We propose that isopentyl acetate (IPA) provides a relevant alarm context enhancing aversive responsiveness, while 2-heptanone (2H), because of the multiplicity of its roles (repellent agent, Shearer and Boch, 1965; negative scent marker, Giurfa, 1993; Vallet et al., 1991; paralyzing agent, Papachristoforou et al., 2012), does not act as a "true" alarm pheromone and is not involved in

stinging responses. Conversely, in the carpenter ant *C. aethiops*, Desmedt (2016) found that alarm pheromones did not affect thermic aversive responsiveness (Fig. 1). However, when assessing responsiveness to different concentrations of non-nestmate cuticular extracts, we found that formic acid (FA) increased aggressive responses, particularly at low concentrations of non-nestmate extracts (**Chapter 2**). This can be understood assuming that low concentrations of US are the ones at which upward pheromonal modulation is rendered more visible; for higher concentrations, closer to a ceiling response, the upward modulation would be less detectable.

In the appetitive context, Baracchi et al. (2017) found that IPA and 2H induced a significant decrease of sucrose responsiveness as ca. 50% of the exposed bees did not respond to any sucrose concentration. In bees that responded to sucrose, 2H, but not IPA, decreased sucrose responsiveness. This decremental effect of 2H is consistent with its role as a deterrent scent used to mark recently visited and depleted flowers in the appetitive context of food search (Giurfa, 1993; Vallet et al., 1991). Both pheromones could also activate the endogenous opioid system (Núñez et al., 1998), explaining the lack of response to sucrose for 50% of the exposed honey bees. It was proposed that alarm pheromones decrease the responsiveness to a nociceptive stimulus through the activation of an opioid analgesia, which would increase the individual defensive efficiency for the colony because the probability of withdrawal when facing an enemy would be reduced (Núñez et al., 1998). Given that 2H has also been reported as being a paralyzing agent towards wax moth larvae and *Varroa* mites (Papachristoforou et al., 2012), we propose that its anesthetic effect is even stronger than the one of IPA, thus explaining the further modulation of appetitive responsiveness. In *C. aethiops*, Desmedt (2016) similarly found that FA increased the percentage of ants not responding to sucrose.

Reconciliation of the results

Taken together, our results and those of Baracchi et al. (2017) in honey bees show that IPA increases shock responsiveness and suppresses sucrose responsiveness. In contrast, geraniol enhances sucrose responsiveness and decreases aversive responsiveness. These results demonstrate that the same pheromone, at the same concentration, can have different effects according to the context (i.e. appetitive or aversive) in which it is released. The case of 2H seems more complex because of the possible multiple roles of this pheromone (see above): Baracchi et al. (2017) found that 2H exerted a decremental effect on appetitive responsiveness; in our case, no effect on aversive responsiveness was detected. In carpenter ants, results showed that FA exerted a decremental effect on sucrose responsiveness, no effect on thermic responsiveness, but an increase of non-nestmate aggressive responsiveness. Given the non-

identification of a trail pheromone in this species, we used Argentine ants to study the effect of appetitive pheromones on sucrose responsiveness. We showed that (Z)-9-hexadecenal enhanced sucrose responsiveness. In carpenter ants, mellein had no effect on thermic responsiveness but it could be due to the fact that this component is not a pheromone in C. aethiops.

Based on the effect of alarm pheromones on appetitive responsiveness in honey bees and ants, especially regarding the fact that IPA, 2H and FA increased the number of individuals not responding at all to sucrose stimuli, results support the hypothesis of Núñez et al. (1998) stating that alarm pheromones induce stress-related analgesia through the activation of the opioid system. They emphasized that this mechanism might be shared across phyla as analgesic effects of alarm pheromones have also been reported in rats (Abel, 1991; Fanselow, 1985).

Our original hypothesis stated that, given the consistency between the context signaled and the context individuals are subsequently submitted to, appetitive pheromones should exert an incremental effect in appetitive contexts and alarm pheromones should exert an incremental effect in aversive contexts. Reversely, given the inconsistency between contexts, appetitive pheromones should exert a decremental effect in aversive contexts and alarm pheromones should exert a decremental effect in appetitive contexts. This hypothesis was globally validated in honey bees and for the appetitive context in ants. However, further studies are required to disentangle the effect of pheromones in the aversive context in ants (Table 1). Notably, it might worth test the effect of FA again on thermic responsiveness by using a 12% (w/w) concentration of pheromone (Rossi et al., 2018a) instead of a 24% (w/w) (Desmedt, 2016) as the latter induced mortality in aversive conditioning (ca. 25%, personal observations) and might be to noxious for the ants to be able to see any modulation by it.

Table 1: Summary of the pheromonal modulation of responsiveness according to the valence of the pheromone and that of the unconditioned stimulus (US).

	HONEY BEES		**ANTS**	
US / ***Pheromone***	**Aversive**	**Appetitive**	**Aversive**	**Appetitive**
Appetitive	↙	↗	No effect?	↗
Alarm	↗	↙	Ambiguous	↙

The modulatory effect of pheromones might be based on the action of these chemicals on different aminergic circuits modulating behavior. Such modulation could take place at two basic levels: the perceptual one, thus affecting the evaluation of US, and/or the motor-output one, thus affecting the production of appetitive or defensive responses. Distinguishing between these alternatives is difficult based on behavioral evidence; neural analyses would be necessary to determine whether and how their corresponding neural circuits are affected by pheromone exposure.

In the honey bee, several studies have shown that octopamine (OA) acts as a crucial neuromodulator of appetitive responses (Hammer, 1993; Scheiner et al., 2002). Regarding defensive responses, Nouvian et al. (2018) found that the stinging attacks of bees towards a rotating dummy were triggered by IPA, which is consistent with the enhancement of sting extension responses (SER) found in our work. They quantified the levels of biogenic amines in the brain of stinging bees exposed to IPA and found that serotonin (5-HT) and dopamine (DA), but not OA, were increased upon IPA exposure. As these two biogenic amines have been related to aggression and attentional processes (Tedjakumala et al., 2013; Tedjakumala et al., 2014), this finding can be linked to a modulatory effect of IPA on noxious-stimulus perception. Recent studies in the bee have cast doubt about the validity of such a clear separation between OA and DA in appetitive and aversive reinforcement signaling, respectively (Klappenbach et al., 2013).

At the motor-output level, analyses performed on isolated terminal abdominal ganglia of bees have shown that OA is a crucial modulator of SER (Burrell and Smith, 1994). This ganglion receives innervation from dorsal and ventral unpaired neurons, which are major releasers of OA (Stevenson and Spörhase-Eichmann, 1995). Not surprisingly, therefore, OA modulates several motor components of SER (Burrell and Smith, 1994). The fact that IPA exposure does not affect brain levels of OA (Nouvian et al., 2018) seems to favor the hypothesis that the modulatory effect of pheromones found in our work occurs at the perceptual rather than the motor level. Alternatively, the two levels could be affected sequentially with extremely short delays. Whether and how the increase in 5-HT and DA found upon IPA exposure translates into a major release of OA for motor control of SER remains to be determined.

In ants, 5-HT could be a good candidate as neuromodulator of feeding behavior and responsiveness to sucrose. Falibene et al. (2012) showed that this amine decreased the volume of sucrose solution ingested per pump contraction in the ant *Camponotus mus*. On the other

hand, Muscedere et al. (2012) found that *Pheidol dentata* workers in which the 5-HT levels were experimentally lowered followed trails on significantly shorter distances than control workers, and responded less frequently to trails when encountered. Exposure to the trail pheromone could, therefore, modulate 5-HT levels in the ant brain (among other biogenic amines), enhancing or depressing responsiveness to different kinds of stimuli according to their modality (gustatory or olfactory). Pheromone-exposed ants would therefore be more responsive to food and more accurate in following the trails at the same time.

Regarding defensive responses, Aonuma and Watanabe (2012) showed that the ratio of OA to N-acetyloctopamine in the brain of *Formic japonica* foragers was significantly higher than queens, which corresponded to higher aggression levels after mechanical tactile stimulus or interspecific predation. Similarly, in the fire ant *Solenopsis invicta*, Vander Meer et al. (2008) demonstrated that queenless workers exhibit reduced brain OA levels and reduced aggression levels in nestmate discrimination bioassays; however, feeding queenless workers OA restored both. In *Formic rufa*, Kostowski et al. (1975) found that concentrations of both 5-HT and adrenaline were higher in ants that displayed aggressiveness (interspecific and intrageneric) while concentrations of noradrenaline was decreased.

Irrespective of a clear separation between biogenic amine actions, pheromones could regulate the balance of the biogenic amines contained in the insect brain, enhancing or depressing responsiveness to different kinds of stimuli according to their valence and context of release.

The pheromonal modulation of decision making

Foraging vs. defensive behaviors

The pheromonal modulation of noxious-stimulus perception (**Chapter 1**) is consistent with a new model describing the decision making process underlying the defensive response of bees (Nouvian et al., 2015). In this model, an individual defensive score resulting from the integration of stimuli related to a potential threat would be weighed against an internal threshold influenced by intrinsic (e.g. genetic traits, caste, age, etc.) and extrinsic (e.g. weather, season, available resources, etc.) factors to determine whether the bee engages in colony defense (Fig. 2A). We suggest that pheromones change this threshold, and that this change depends on pheromone valence. Alarm pheromones, associated with aversive, dangerous events, would lower defensive thresholds, making individuals more likely to engage in defensive behaviors. When neurons associated with threat stimuli would fire enough, this "defense score" would

overcome the threshold, thereby eliciting defensive responses. For a same amount of firing by threat-associated neurons, appetitive pheromones would increase the defensive threshold, making bees less likely to engage in defensive behaviors.

In the appetitive scenario, an individual appetitive score resulting from the integration of stimuli related to a potential food would be weighed against an internal threshold influenced by intrinsic (e.g. genetic traits, caste, age, etc.) and extrinsic (e.g. weather, season, available resources, etc.) factors to determine whether the bee engages in foraging (Fig. 2B). We suggest that pheromones change this threshold, and that this change depends on pheromone valence. Appetitive pheromones, associated with food searching, would lower appetitive thresholds, making individuals more likely to engage in foraging behaviors. When neurons associated with food stimuli would fire enough, this "appetitive score" would overcome the threshold, thereby eliciting foraging responses. For a same amount of firing by food-associated neurons, alarm pheromones would increase the appetitive threshold, making bees less likely to engage in foraging behaviors.

Although the study of pheromone valence on appetitive and defensive behaviors has only been shown in honey bees so far, it is possible that this model would extend to other species using appetitive and alarm pheromones (i.e. eusocial species).

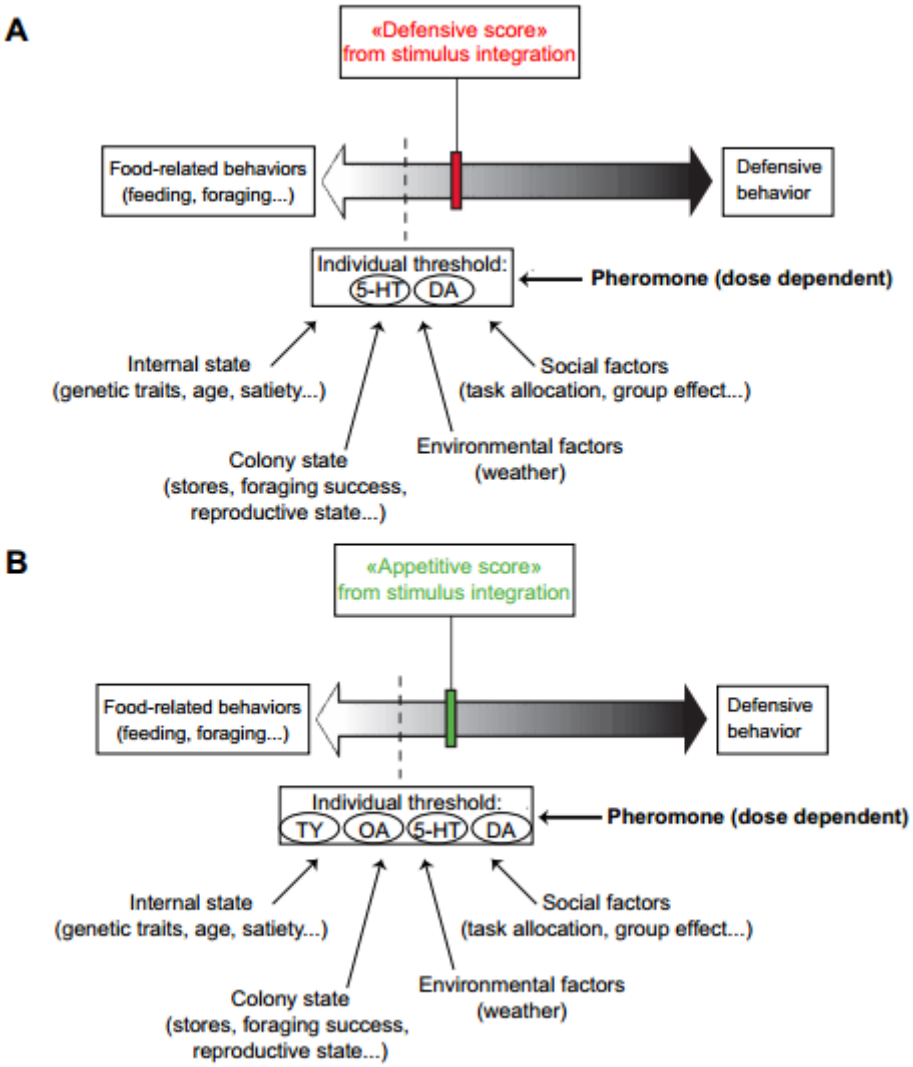

Figure 2. A model accounting for the modulatory effect of pheromones on decision making in honey bees. The model (adapted from Nouvian et al., 2015) postulates that each individual is characterized by a defensive and an appetitive threshold, which are determined by extrinsic and intrinsic factors. Pheromones may act on this threshold, moving it away from or towards a score resulting from the integration of surrounding stimuli. In order to elicit a specific behavior, the score needs to overcome the threshold. (A) Defensive score and its relationship with a defensive-response threshold. Pheromones modify the threshold depending on their valence. Positive, appetitive pheromones move it away from the score (shaded arrow and red bar), thus decreasing the probability of a defensive response. Negative, aversive pheromones have the opposite effect, moving the threshold towards the score and thus increasing the probability of a defensive response. (B) Appetitive score and its relationship with an appetitive-response threshold. Pheromones modify the threshold depending on their valence. Positive, appetitive pheromones move the threshold towards the score (shaded arrow and green bar), thus increasing the probability of an appetitive response. Negative, aversive pheromones have the opposite effect, moving the threshold away from the score, thus decreasing the probability of an appetitive response. 5-HT, serotonin; DA, dopamine; TY, tyramine; OA, octopamine.

Recognition systems

Focusing on the modulation of defensive behaviors by an alarm pheromone, we then studied the pheromonal modulation of recognition in the frame of nestmate discrimination. When

testing the effect of an alarm pheromone on nestmate discrimination with free-walking ants, we found that FA increased aggressive behaviors towards non-nestmates and decreased aggressive behaviors against nestmates at the same time (**Chapter 2**). Formic acid thus increases accuracy in the process of nestmate discrimination. Such a modulatory effect of a pheromone on conspecific recognition has not been described before and is highly relevant in the context of colony defense where nestmates and non-nestmates are encountered at the same time around the nest.

Our results allow examining the way in which recognition systems operates. The acceptance threshold model (Reeve, 1989) states that, when the cue-distribution of nestmates and non-nestmates overlap, there is a risk of discrimination error. According to this model, no matter in which direction this threshold moves, in no case it is possible to obtain both an increase of rejection frequency of non-nestmates *and* a decrease of rejection frequency of nestmates. However, our results show that FA induces both. Hence, it seems unlikely that FA simply shifts a theoretical acceptance threshold.

We propose that FA increases the amount of olfactory information available to the ants to perform discrimination (Fig. 3A). By acting on this set of olfactory cues, FA would decrease the variance associated to the cues and shift the dissimilarity mean values for nestmates and non-nestmates, thereby decreasing the perceived overlap between the two (Fig. 3B). This allows decreasing at the same time both acceptance and rejection errors. This hypothesis is supported by a model by Lehmann and Perrin (2002) showing that the distributions of similarities between a recipient and the discriminator depend on the number of recognition cues sampled: a high number of cues (and a high similarity among nestmates) decrease overlap and thus increase discrimination ability.

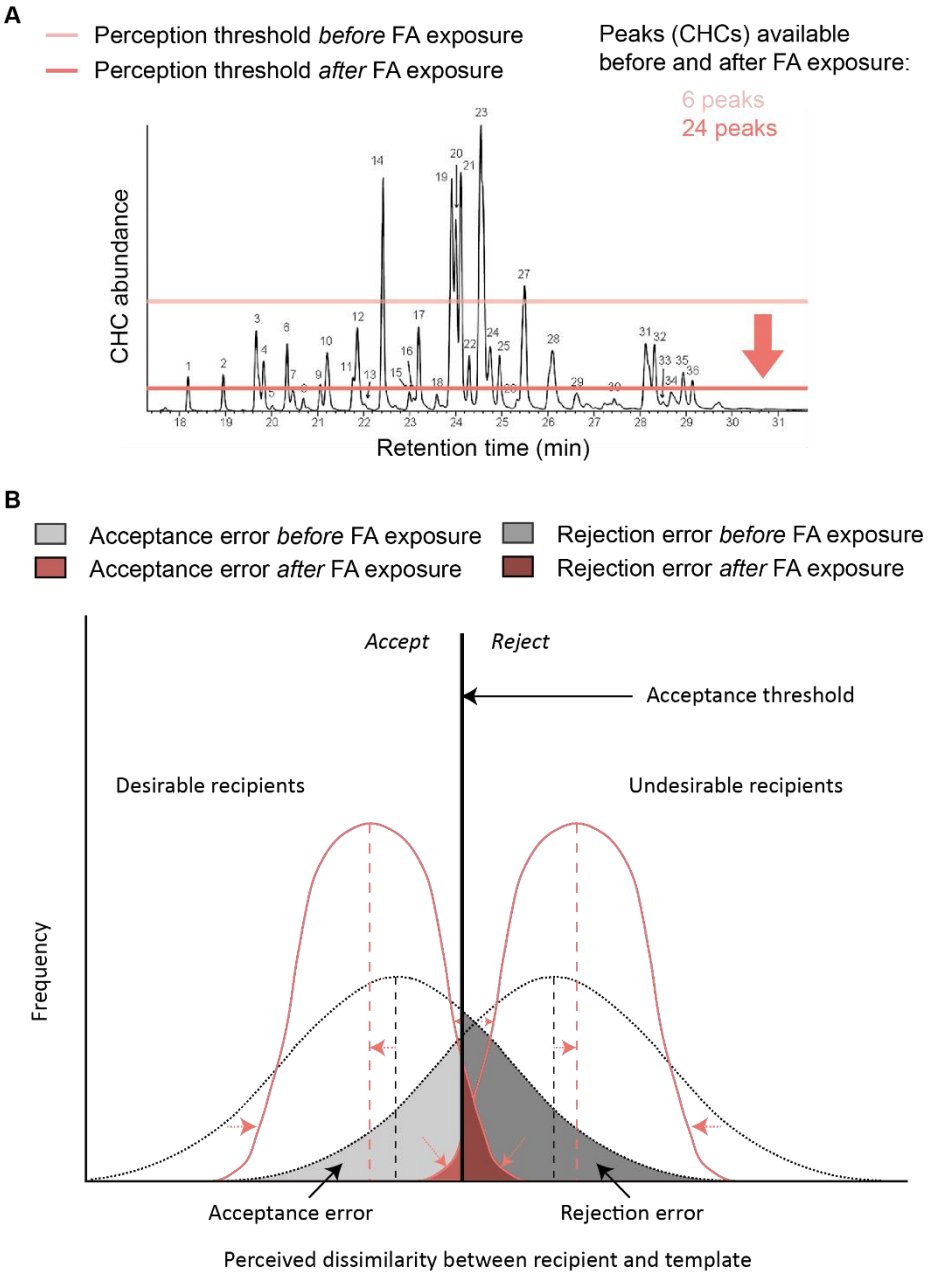

Figure 3. Putative modulation of an olfactory perception threshold by formic acid. (**A**) Example of a cuticular profile (adapted from van Zweden et al. 2009): the x-axis represents the retention time of different components of the cuticular profile (CHC) in a gas chromatograph and the y-axis represents their abundance. After exposure to formic acid (FA), the olfactory perception threshold of an ant would be lowered, allowing the detection of a higher number of components. (**B**) Frequency of interaction between desirable or undesirable recipients and the resulting distributions of perceived dissimilarity between the discriminator's template and the recipient's recognition cues for nestmates and non-nestmates (adapted from van Zweden and d'Ettorre 2010). By lowering the olfactory detection threshold, FA would allow ants to access more cues, thereby decreasing the perceived variance and accentuate the dissimilarity between nestmate and non-nestmate CHCs. This would result in the decrease of acceptance and rejection errors.

Our results bring suggestions about how nestmate discrimination could be modulated at the perception level. To our knowledge, few works have discussed nestmate recognition models from a perceptual perspective because accessing the insect brain upon an inter-individual

recognition task is difficult. Our studies show that behavior can sometimes provide clues about mechanisms that are not necessarily related to the action component. This is especially true when studying pheromones that are suspected to act on the motivation of an animal that perceives them (Baracchi et al., 2017; Nouvian et al., 2015; Rossi et al., 2018b). In our study, FA did not simply elicit an enhanced stereotyped aggressive response but acted as a modulator of adaptive behavioral plasticity by promoting appropriate responses to nestmates and non-nestmates. Attacking enemies and not nestmates would indeed increase the colony fitness and therefore the inclusive fitness of the worker performing the discrimination. Our study suggests that the amount of perceptual information available for decision making is not necessarily fixed and that it can be modulated by "priming", i.e., exposure to one stimulus that influences a response to a subsequent stimulus. This opens new perspectives for a deeper understanding of recognition systems and decision making in general.

The pheromonal modulation of learning

Chapter 3 aimed at investigating the role of an alarm pheromone as a modulator of ants' olfactory learning and subjective evaluation of new odor stimuli. To this end, we exposed carpenter ants to FA and determined the effect of this exposure on two paradigms in *C. aethiops*: the aversive differential conditioning of the mandible opening response (MOR), and the appetitive differential conditioning of the *maxilla-labium* extension response (MaLER). In this last experiment, we also presented the ants with three new odor stimuli during the memory tests to assess if and how pheromones modulate generalization. This study provides evidences that pheromones can modulate learning and generalization, thereby contributing to behavioral plasticity by producing different behavioral phenotypes in response to distinct environmental conditions.

The effect of appetitive pheromones on learning

The effect of appetitive pheromones on learning has only been studied in an appetitive context and not in an aversive one. In honey bees, Baracchi et al. (2018) found that geraniol (an appetitive pheromone component) improved their learning performance and long term memory retention (Fig. 4). This result is congruent with findings in newborn rabbits showing that the mammary pheromone promotes learning of neutral odorants in the context of food searching (Coureaud et al., 2006). The aversive part of the project in honey bees could not be successfully conducted because in a series of preliminary experiments not reported in this thesis I had difficulties in conditioning bees to aversively respond to one odor and not to another; in both

cases, bees nearly did not display SER to the conditioned odors. This was due to technical problems, which would have required some time to be solved. Given the short time window of a PhD thesis, I decided to concentrate my experimental efforts on other parts of the project. In *C. aethiops*, as previously mentioned, no trail pheromone has been identified. Conditioning procedures should therefore be performed in Argentine ants.

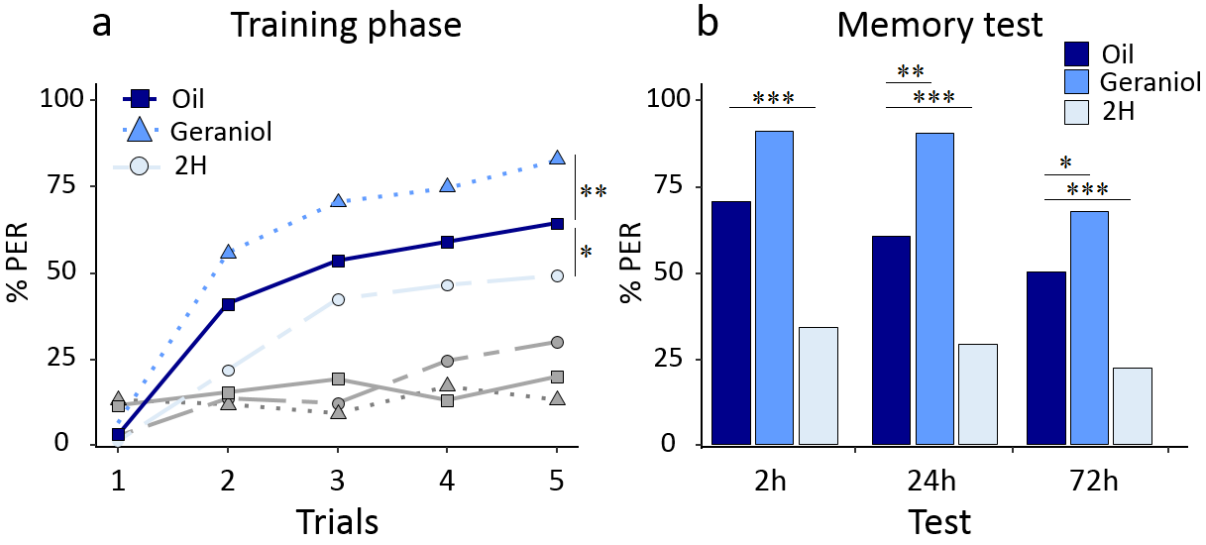

Figure 4. Pheromones affect olfactory appetitive learning performance. Associative olfactory conditioning of the proboscis extension response (PER) in honeybees previously exposed to either 25 µl 24% pheromone (geraniol: n = 75, 2H: n = 73) or 25 µl pure mineral oil (n = 129) as control. (**A**) PER of bees trained in an appetitive olfactory differential conditioning where an odorant was paired with a reward (colored lines) and another odorant with no reward (grey lines) during 5 trials. (**B**) Olfactory appetitive memory test performed 2h, 24h, and 72h after conditioning. Bees exposed to geraniol improved their learning performance and memory retention compare to control bees exposed to mineral oil. 2H inhibited appetitive learning and memory retention with respect to controls. (*) $p < 0.05$; (**) $p < 0.001$; (***) $p < 0.0001$ (Baracchi et al. 2018).

The effect of alarm pheromones on learning

In carpenter ants, our results reveal that FA has the capacity to modulate aversive olfactory differential learning (**Chapter 3**). Exposure to this pheromone increased discriminative performances during acquisition, which remained visible during the memory test one hour later. Indeed, FA-exposed ants were more likely to respond correctly during the memory test, and conversely less likely to generalize, compared to control ants. Formic acid thus increased discrimination accuracy, a phenomenon already observed in the process of nestmate discrimination where FA-exposed ants were more likely to be aggressive towards non-nestmates, and less likely to display aggressiveness towards nestmates at the same time (Rossi et al., 2018a, **Chapter 2**). In the appetitive context, Baracchi et al. (2018) found that 2H exerted a decremental effect on appetitive differential learning in bees (Fig. 4), so as Urlacher et al. (2010) with IPA. In ants, FA did not impair or enhance appetitive learning but did modulate the

acquisition dynamics depending on the rewarded odor. During the memory tests, FA-exposed ants also appeared to generalize to the new presented odor laying between CS+ and CS- in terms of carbon-chain length, regardless of the rewarded odor (**Chapter 3**).

Our working hypothesis stated that, given the consistency between the context signaled and the context individuals are submitted to, appetitive pheromones should exert an incremental effect on appetitive learning (association with reward) and alarm pheromones should exert an incremental effect on aversive learning (association with punishment). Reversely, given the inconsistency between contexts, appetitive pheromones should exert a decremental effect on aversive learning and alarm pheromones should exert a decremental effect on appetitive learning. Pheromones would thus provide contextual information rendering subsequent learning more or less relevant for the context signaled. This hypothesis was validated for the appetitive learning of honey bees but further studies are required regarding all other cases (Table 2). Indeed, in the case of pheromonal modulation of aversive learning in ants, we found an incremental effect of FA on thermic olfactory differential learning but our control group did not show evidences of learning (**Chapter 3**). Replications of this experiment should therefore be performed in order to confirm these results. In honey bees, further attempts to condition the SER should be achieved, if not by the use of electric shocks as US, by the use of thermic stimulations (see Junca et al., 2014). Eventually, regarding the modulation of appetitive learning by alarm pheromones, results showed that FA did not exert an incremental nor a decremental effect but rather affected the perception of conditioned stimuli and new odors (**Chapter 3**). This suggests that the roles and mechanisms underlying the effect of alarm pheromones in honey bees and ants are quite different, at least regarding the modulation of appetitive learning. It should be noted though that the differences observed could be due to the specific action mechanisms of FA and that other alarm pheromones might act differently. Further experiments using other alarm pheromones in ants are therefore required.

Table 2: Summary of the pheromonal modulation of learning according to the valence of the pheromone and that of the unconditioned stimuli (US).

	HONEY BEES		**ANTS**	
US / ***Pheromone***	**Aversive**	**Appetitive**	**Aversive**	**Appetitive**
Appetitive	?	↗	?	?
Alarm	?	↙	↗	Acquisition dynamics

Reconciliation of the responsiveness and learning pheromonal modulation results

From the results obtained in an appetitive context in honey bees regarding the pheromonal modulation of responsiveness and learning, it seems that pheromones exert a consistent modulation according to their valence (Baracchi et al., 2017; Baracchi et al., 2018). Appetitive pheromones (i.e. geraniol) enhanced both sucrose responsiveness and appetitive olfactory differential conditioning of the proboscis extension response (PER), while alarm pheromones (i.e. 2H) exerted the opposite effect (i.e. a decrease of both appetitive responsiveness and learning) (Table 3). These results suggest that pheromones act on the reinforcing signaling (i.e. sucrose reward) shared in responsiveness and learning assessments, either increasing or decreasing it depending on the pheromone valence.

In ants, the story seems to be more complicated. Regarding the effect of alarm pheromones, results showed that although FA did not have an effect on thermic responsiveness (Desmedt, 2016; Fig. 1), it did increase responsiveness towards non-nestmate extracts and similarly increased both nestmate discrimination (Rossi et al., 2018a, **Chapter 2**) and odorant discrimination in an aversive olfactory differential conditioning using heat as punishment (**Chapter 3**). These results suggest that FA could also act consistently on responsiveness and learning but further experiments are required to infer about the action of the pheromone on the reinforcing signaling by testing the effect of 12% (w/w) FA on thermic responsiveness again. On the other hand, although FA increased the number of ants not responding to sucrose (Desmedt, 2016), thereby exerting a decremental effect on appetitive responsiveness, it did not impair appetitive olfactory differential learning (**Chapter 3**). Again, further experiments should test the effect of 12% (w/w) FA on sucrose responsiveness to be consistent with the concentration used on conditioning. However, it seems that in this case, the effect of FA is more complex than a modulation of reinforcing signals and that the alarm pheromone also impacts the evaluation/perception of the conditioned stimuli depending on their hedonic values for the exposed individuals.

Table 3: Summary of the pheromonal modulation of responsiveness and learning according to the valence of the pheromone and that of the unconditioned stimuli (US).

	HONEY BEES				ANTS			
US / *Phero*	Aversive		Appetitive		Aversive		Appetitive	
	Resp.	Learn.	Resp.	Learn.	Resp.	Learn.	Resp.	Learn.
Appetitive	↙	?	↗	↗	No effect?	?	↗	?
Alarm	↗	?	↙	↙	Ambig.	↗	↙	Ambig.

Resp. = responsiveness; Learn. = learning; Ambig. = ambiguous. Green arrows indicate consistent modulation by appetitive pheromones between responsiveness and learning. Red arrows indicate consistent modulation by alarm pheromones between responsiveness and learning.

The neural mechanisms underlying the pheromonal modulation of learning

Olfactory representation in the AL is plastic and subjected to modifications resulting from associative learning. In honey bees, results show that odor representations in the AL are dynamic and subjected to changes depending on learning and memory consolidation. The basis of the learning-dependent changes in the AL is not simply an increase in activity in the neural network representing an odorant (Faber et al., 1999). Rather, learning produces a restructuring of spatial and temporal components of network responses to odors in the AL (Rath et al., 2011). In ants, no study has focused so far on possible variations of olfactory representations of conditioned odors since the development of a protocol for olfactory conditioning in harnessed animals is very recent (Desmedt et al., 2017; Guerrieri and d'Ettorre, 2010).

As shown in **chapter 3**, the modulation of appetitive olfactory differential conditioning in *C. aethiops* depended on which odor was rewarded (octanal or hexanal). Based on these results, we postulate that, similar to experience-dependent changes in odorant representation, pheromone exposure exerts a significant modification of subsequent odor encoding, modulating odor salience either at the spatial or temporal level. We suggest that pheromone valence has an impact on odor coding, with appetitive pheromones inducing different changes compared to aversive pheromones. Testing this hypothesis would require studying neural coding of neutral odorants in naïve (i.e. non-conditioned) animals after appetitive (e.g. geraniol) or aversive (e.g. IPA) pheromone exposure by *in vivo* calcium imaging. As controls, animals would be exposed to a neutral, non-pheromonal, odorant to account for possible changes of odor coding induced by mere olfactory exposure, irrespective of the significance of the odorant. Comparing calcium imaging recordings at the level of the AL before and after pheromone exposure, for the same set of neutral odorants would allow detecting potential changes induced by this treatment in the

intensity of calcium activation patterns, or in their temporal dynamics.

Simultaneously, as aminergic neurons serve as a value system in associative learning phenomena, i.e. as a system allowing ordering, prioritizing and assigning ‘good’ or ‘bad’ labels to odorants (Giurfa, 2006), pheromones could act on it as well. The multiple functions of biogenic amines in behavioral modulation make them suitable candidates for the study of pheromonal modulation in insects. On one hand, pheromones may act on aminergic global-gain systems inducing behavioral resting or behavioral excitation (e.g. Giurfa, 2006; Scheiner et al., 2006; Schulz et al., 2002; Tedjakumala et al., 2014), which may prone or not an insect to learn specific events. On the other hand, pheromones may modulate the instructive aminergic circuits themselves, and thus affect reinforcing signaling, either increasing or decreasing it. Indeed, biogenic amines have been shown to substitute for the reinforcement function in associative learning (Giurfa, 2006; Perry and Barron, 2013; Waddell, 2013). In various insect species, biogenic amines such as DA and OA act as a value system (Giurfa, 2006) as they instruct the nervous system about the relevance of external events (Aso et al., 2010; Hammer, 1993; Schwaerzel et al., 2003). In the honey bee, the activity of a single, identified octopaminergic neuron (VUMmx1, the "ventral unpaired median neuron of the maxillary neuromere 1") substitutes for the reinforcing function of sucrose in olfactory PER conditioning (Hammer, 1993). Similarly, *Drosophila* mutant flies that have the biosynthetic pathway to OA blocked cannot learn to associate an odor with a sugar reward but can learn an aversive olfactory discrimination (Schwaerzel et al., 2003). Similar results were found in crickets (Mizunami et al., 2009). Thus, OA seems necessary for appetitive olfactory learning. This is in congruence with the results of Baracchi et al. (2018) who found that OA did not improve the performances of bees exposed to geraniol (Fig. 5), which already had good performances due to the pheromone action, but it was sufficient to restore those of bees exposed to 2H (Fig. 6), which had compromised learning and memory. On the contrary, the blockade of octopaminergic neurons inhibited appetitive memory formation in bees previously exposed to geraniol but it did not in those exposed to 2H. Most likely, a floor effect might have prevented the OA antagonist to further inhibit the already abolished performance of the 2H-exposed bees. It is therefore likely that both 2H and geraniol exert their action by modulating the firing activity of the VUMmx1 neuron and the consequent releasing of OA in the main neuropils of the bee brain involved in olfactory learning (i.e. the ALs, LH, and MBs). In particular, Baracchi et al. (2018) hypothesize that geraniol increases the firing activity of the VUMmx1 neuron and OA titers in the hemolymph and the brain, while 2H has the opposite effect, decreasing its firing activity.

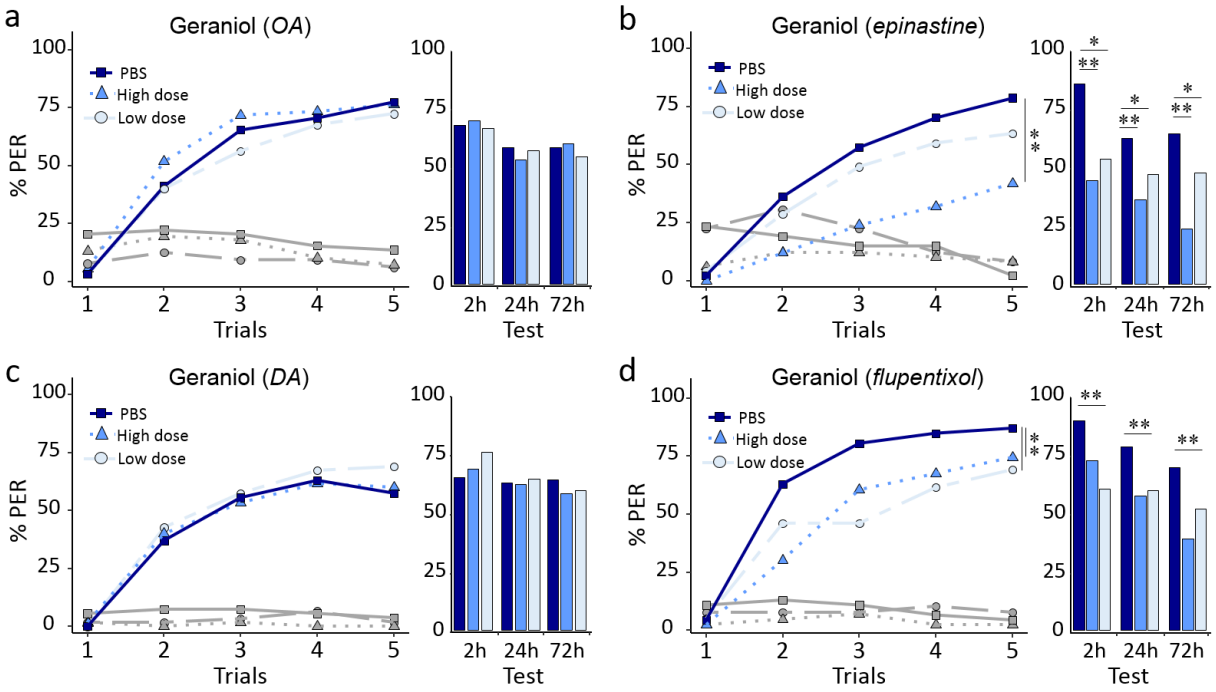

Figure 5. Pharmacological study in bees exposed to geraniol. The effect of octopamine (*OA*), dopamine (*DA*) and the octopaminergic and dopaminergic receptor antagonists (*epinastine hydrochloride* and *cis-(Z)-flupentixol dihydrochloride* respectively) on appetitive olfactory conditioning of the proboscis extension response (PER) of honey bees previously exposed to the appetitive pheromone geraniol. The left side of each panel reports the PER to the odor paired with a reward (colored lines) and to the non-reinforced odor (CS-, grey lines) of bees trained in a five-trial differential conditioning. The right side of each panel reports the memory retention performance of bees tested 2h, 24h, and 72h after conditioning. For each drug (either agonist or antagonist), three groups of bees (low-dose, high-dose and PBS-injected bees) were exposed, treated, conditioned and tested in parallel. (*) $p < 0.05$; (**) $p < 0.001$ (Baracchi et al. 2018).

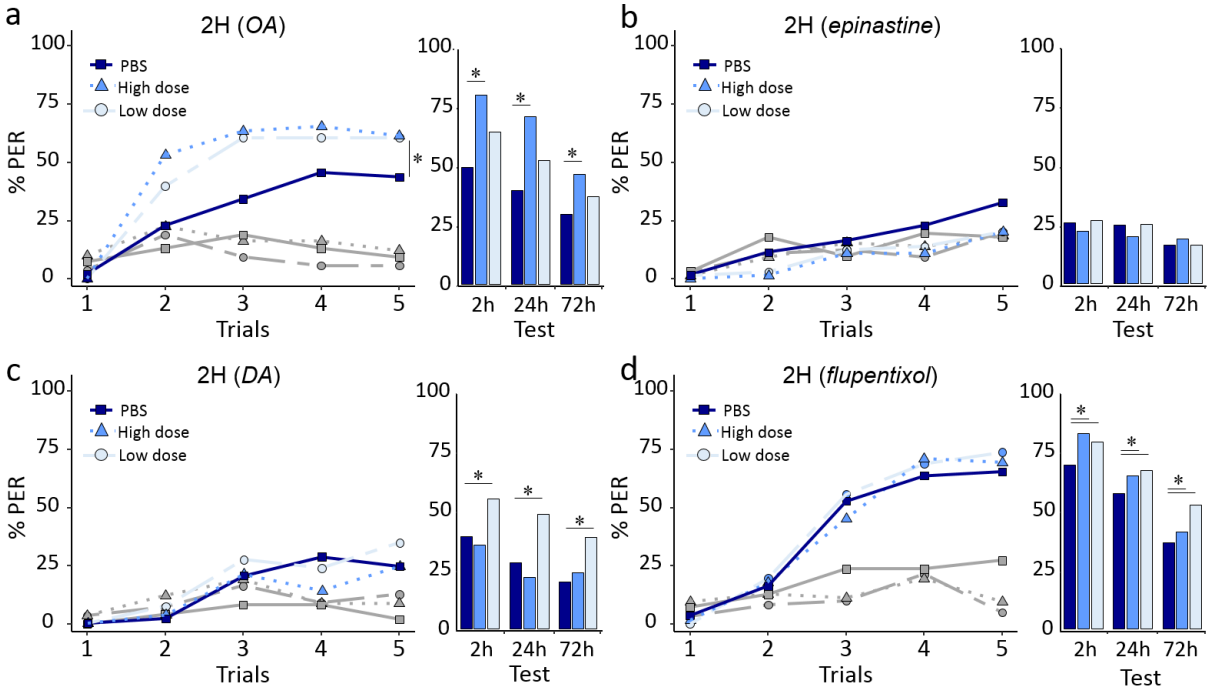

Figure 6. Pharmacological study in bees exposed to 2-Heptanone (2H). The effect of octopamine (*OA*), dopamine (*DA*) and the octopaminergic and dopaminergic receptor antagonists (*epinastine hydrochloride* and *cis-(Z)-flupentixol dihydrochloride*

respectively) on appetitive olfactory conditioning of the proboscis extension response (PER) of honey bees previously exposed to the aversive pheromone 2H. The left side of each panel reports the PER to the odor paired with a reward (colored lines) and to the non-reinforced odor (CS-, grey lines) of bees trained in a five-trial differential conditioning. The right side of each panel reports the memory retention performance of bees tested 2h, 24h, and 72h after conditioning. For each drug (either agonist or antagonist), three groups of bees (low-dose, high-dose and PBS-injected bees) were exposed, treated, conditioned and tested in parallel. (*) $p < 0.05$; (**) $p < 0.001$ (Baracchi et al. 2018).

Besides the role of the octopaminergic system, Baracchi et al. (2018) showed that at least another aminergic system (i.e. the dopaminergic pathways) is involved in the process of learning modulation by pheromones (Fig. 5-6). Indeed, while they found that DA did not affect the learning performances of bees exposed to both pheromones, the authors also found that this amine weakly enhanced memory recall in bees whose performance was previously compromised by 2H. Moreover, the blockade of neurons sensitive to DA with an antagonist counterbalanced the enhancing effect induced by geraniol, so that the learning was compromised in respect to that of control bees. These results contradict previous works in honey bees, flies, and crickets which suggested a clear separation between the octopaminergic and dopaminergic circuits, each supposed to underlie appetitive and aversive learning, respectively (Mizunami et al., 2009; Schwaerzel et al., 2003; Vergoz et al., 2007b). On the contrary, this study suggests that both circuits are required in appetitive learning, which is congruent with recent studies in the fruit fly which have shown that appetitive (sucrose) reinforcement is in fact mediated by a combination of successive peripheral octopaminergic and central dopaminergic signaling (Burke et al., 2012; Liu et al., 2012). Honey bees might therefore follow a similar neural organization.

Given the modulation consistency of geraniol and 2H on appetitive responsiveness and learning (Table 3), and the fact that OA has also been shown to be a crucial neuromodulator of appetitive responsiveness (Scheiner et al., 2006), we propose that pheromones acted on the appetitive reinforcing signal in both cases, by modulating the firing activity of the octopaminergic VUMmx1 neuron. Moreover, as DA was also involved in appetitive learning modulation and that different classes of dopaminergic neurons exist (AmDOP1, Blenau et al., 2002; AmDOP2, Humphries et al., 2003; and AmDOP3, Beggs et al., 2005), we join the suggestion of Tedjakumala et al. (2014) regarding the implication of one class as reinforcing signals (either appetitive or aversive) and a gain-control class inducing behavioral resting or behavioral excitation. Supposing that selective attention is the process underlying discrimination accuracy, and since the results reported here always came from differential conditioning procedures, DA could also be involved in the experience-dependent stimulus suppression dynamics that allow an animal to make adaptive choices at the right time (Van

Swinderen and Andretic, 2011). Several studies in the fruit fly, *Drosophila melanogaster*, suggest that one of the central roles played by DA may involve perceptual suppression, a necessary component of selective attention (Andretic et al., 2005; Zhang et al., 2007). This action of DA could be part of the gain-control system.

In ants, the role of biogenic amines has been mainly investigated in the context of social organization, namely reproductive dominance, colony foundation and task specialization (Kamhi and Traniello, 2013). Several biogenic amines such as OA, DA and 5-HT have been shown to modulate various components of social behaviors in different castes and species. Yet, the involvement of these amines in associative learning has not been studied until now, probably because -as for imaging studies - a controlled learning protocol in immobilized ants is only available since 2010 (Guerrieri and d'Ettorre, 2010). Pharmacological experiments are therefore required to fill this gap.

In conclusion, the modulatory effect of pheromones on learning would be possible through either mechanism: the action on aminergic global-gain systems inducing behavioral resting or behavioral excitation (which may prone or not an insect to learn specific events), or through the instructive aminergic circuits themselves which affect reinforcing signaling (either increasing or decreasing it). Such an effect would have an obvious impact on associative learning through a modulation of the US function.

Conclusion

Towards a redefinition of pheromones

Since Karlson and Lüscher (1959), pheromones are defined as "*substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behavior or a developmental process*". The present work now confirms that the role of pheromones extends beyond the trigger of stereotyped behaviors (primer pheromones) and physiological changes (releaser pheromones), by modulating reflex and experience-dependent behaviors. In this context, a pheromone, as a modulator, affects the intensity or the probability of behaviors.

Results showed that pheromones affected the evaluation/perception of US eliciting aversive or appetitive reflex responses depending on the congruence between the valence of the pheromone and that of the reinforcement (**Chapter 1**). This modulation tended to be seen for low to intermediate concentrations/intensities of the US. In particular, our results reveal the novel finding that geraniol diminished the perceptual impact of shock in bees. Through this

non-canonical action (in the sense of not being associated directly with the response modulated, like the effect of geraniol on SER or of 2H on PER), pheromones would act on an animal's motivation to engage in a given behavior.

Pheromones act in a dose-dependent manner as previously shown in Africanized honey bees with the modulation of shock responsiveness by alarm pheromones (Balderrama et al., 2002; Núñez et al., 1998), and as our results regarding the modulation of sucrose responsiveness in Argentine ants by a synthetic trail pheromone suggest as well (**Chapter 1**).

We also showed that pheromones could act on recognition systems in an unexpected way, i.e. not by shifting Reeve's conspecific acceptance threshold (1989), but by increasing the amount of information available to the individual, thereby facilitating discrimination (**Chapter 2**).

Moreover, as pheromones change the subjective perception of stimuli, being attractive (sucrose) or aversive (electric shock), they may also have an impact on the capacity to learn about these stimuli. Therefore, the effect of pheromones might not only be restricted to responsiveness and motivation but could also affect learning and memory via the modulation of the salience of a US. Indeed, results seem to confirm that this is the case (Baracchi et al., 2018; Urlacher et al., 2010; Vergoz et al., 2007a). **Chapter 3** showed that besides modulating the perception of the US, pheromones also acted on the evaluation of the CSs depending on the hedonic values of the odors used. In addition, pheromones impacted the evaluation of odor similarity and generalization.

As such, pheromones have an important role as modulators of behavioral plasticity.

Pheromone modulation contributes to behavioral plasticity

Phenotypic plasticity is the property of a given genotype to produce different phenotypes in response to distinct environmental conditions (Pigliucci, 2001). This includes changes in all sets of observable traits of an organism, from morphology, to development, physiology, life history, and behavior. Included under the heading of behavioral plasticity are adjustment, learning, memory and changes in adult behavior as a result of experience during development (Binder et al., 2008).

Our results showed that pheromones modulated responsiveness, learning and memory, generalization, recognition, and therefore decision making in a general way. Moreover, other studies (Baracchi et al., 2018) have now shown that this action was achieved through neuromodulation, i.e. the physiological process by which a given neuron uses one or more

chemicals (biogenic amines) to regulate diverse populations of neurons. This process allows dramatic but reversible reconfiguration of a sensory circuit without changing the 'hard-wiring'. As such, we suggest that pheromone modulation is a drive of activational behavioral plasticity (Snell-Rood, 2013), which elicits fast and reversible responses particularly suited to changing environments during a lifespan (e.g; non-migratory, temperate species that are subjected to seasons).

The illustration of this process is given Fig. 2. Indeed, according to this model, one given pheromone, at a given concentration, would adjust the individual response according to the environmental condition in which it is placed: an appetitive pheromone would increase the probability of engaging in foraging behavior and decrease the probability of engaging in defensive behavior. Reversely, an alarm pheromone would increase the probability of engaging in defensive behavior and decrease the probability of engaging in foraging behavior (**Chapter 2**).

Limitations

The major limitation of this work was the absence of determination of dose-dependency and biologically relevant concentrations of the pheromones used. Indeed, all the experiments presented here were performed using only one concentration of the pheromones (except for the study of sucrose responsiveness modulation in Argentine ants), and this concentration was elected based on the literature in honey bees (Hunt et al., 2003; Urlacher et al., 2010). However, quantifying the concentration of volatile chemicals emitted by an individual is not trivial since, in nature, pheromones are released in the air, which is volume-less, therefore, any measurement of pheromone emission would depend on the distance between the emitter and receptor individuals, the time of release, the intensity of the threat (in the case of alarm pheromones) or the quality of the food (in the case of appetitive pheromones), abiotic factors (wind, temperature…), etc. This quantification could have been done by evaluating all these parameters prior to Solid-Phase Micro-Extraction but it would have been too time-consuming for the time window of my PhD thesis. Instead, we opted for the strategy of starting with high concentrations, mimicking massive emissions of pheromones, which proved to have an effect in honey bees in previous works, so as to be able to observe any modulation of the studied behaviors.

Another major limitation was the impossibility of conditioning the SER in honey bees, which prevents us to establish a full model of pheromone modulation in honey bees. On the

other hand, the time we did not invest in resolving this issue allowed us to obtain preliminary results of learning pheromonal modulation in ants. Other aversive paradigms in honey bees could be considered as an alternative since the SER conditioning does not appear to be that trivial. Thermic conditioning for example could be performed in harnessed bees. In free-walking bees, fear conditioning protocols could be used.

Given that behavioral experiments could not yet be performed in each condition (i.e. aversive and appetitive) and for each taxa (honey bees and ants), neural mechanisms underlying such pheromonal modulation can only be hypothesized at this stage. It is primordial that future works focus on studying these mechanisms and the possible implication of biogenic amines by using a combination of behavioral and pharmacological approaches. As mentioned above, *in vivo* calcium imaging of the AL should also be performed to detect potential modulation of odor coding by pheromones.

The lack of results regarding the pheromonal modulation of aversive behaviors in honey bees and the preliminary results obtained in ants do not allow us determining the mechanisms that are either conserved across species or species-specific, as it is not feasible at this stage. Based on the different effects of alarm pheromones on appetitive learning in honey bees and ants, we can only assume that the mechanisms would be different. However, since the effect of pheromones on the hedonic values of conditioned odors has not been assessed in honey bees, this hypothesis is highly speculative.

Applications

The aim of this research project was to achieve a comprehensive knowledge on how pheromones influence learning performances and perception of social and environmental stimuli in two paradigmatic insect taxa that are models for fundamental and applied biological research. Focusing on behavioral modulation by pheromones opens valuable perspectives for insect control, which is particularly important for agricultural pests. This is indirectly the case of *L. humile*, which favors agricultural pests targeting various plants of economic importance (e.g. pomes, citrus, grape, drupes, fruits: Lester et al., 2003), besides being an invasive species in Western countries (Holway et al., 2002; Suarez et al., 1998). Olfaction plays an essential role in many of their behaviors. A better understanding of the olfactory modulation of these behaviors via pheromones is important in fighting this pest without relying exclusively on pesticides (**Chapter 1**). This project could lead to the improvement of the design of specific lures through a better understanding of the plasticity of ants' responses to synthetic attractants.

Understanding the multifaceted role of pheromones in ant societies may help finding appropriate solutions for pest management. Finally, the results of appetitive pheromones and floral odors detracting bees from aggressive behaviors (**Chapter 1**) could be useful for bee management by beekeepers or in greenhouse productions to prevent bees from stinging humans. Moreover, the effect of geraniol on appetitive behaviors could lead to applications in agriculture by attracting bees to specific crops for pollination (for instance organic crops instead of crops submitted to pesticides).

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SUMMARY

Pheromones are chemical substances released into the environment by an individual, which trigger stereotyped behaviors and/or physiological processes in individuals of the same species. Yet, a novel hypothesis has suggested that pheromones not only elicit innate responses but also contribute to behavioral plasticity by acting as "modulators" of cognitive phenomena.

We studied the modulator effect of pheromones on reflex responses, decision making and learning in three insect species that are emblematic models for fundamental and applied research: the honeybee *Apis mellifera*, and the ants *Camponotus aethiops* and *Linepithema humile*.

In the first study, we found that an appetitive pheromone decreased aversive responsiveness, while an alarm pheromone increased aversive responsiveness in honey bees. In *L. humile*, a synthetic trail pheromone increased sucrose responsiveness and feeding time. Overall, our results demonstrate that certain pheromones modulate the salience of aversive and appetitive stimuli according to their valence. In this way, they would affect the motivation to engage in aversive or appetitive responses, thus acting as modulators of behavioral plasticity.

We then determined the effect of an alarm pheromone (formic acid) on decision making and recognition systems in the frame of nestmate discrimination in carpenter ants. We found that the alarm pheromone improved discrimination by increasing aggressiveness towards non-nestmates and decreasing aggressiveness towards nestmates at the same time. These results challenge the established model of nestmate recognition. We therefore propose a revised version of this model.

Eventually, we tested the effect of formic acid on learning and generalization. Formic acid increased discrimination in aversive olfactory differential conditioning. In appetitive olfactory differential conditioning, formic acid modulated the acquisition dynamics and perceived odor similarity. We suggest that pheromones affect the perception of conditioned odors and reinforcements depending on the nature of the odorants and their intrinsic values for the individual, as well as the valence of the reinforcements.

This thesis presents the first integrated analyses of pheromone modulation in two insect taxa: honey bees and ants. The presented results allow us to understand some modes of action of pheromones and pave the way for future studies to understand the underlying mechanisms of this modulator effect of pheromones.

RESUME

Les phéromones sont des substances chimiques relâchées dans l'environnement par un individu qui déclenchent des comportements stéréotypés et/ou des processus physiologiques chez des individus de la même espèce. Cependant, une nouvelle hypothèse suggère que les phéromones non seulement suscitent des réponses innées mais contribuent également à la plasticité comportementale en agissant en « modulateurs » de phénomènes cognitifs.

Nous avons étudié l'effet modulateur des phéromones sur les réponses réflexes, la prise de décision, et l'apprentissage chez trois espèces d'insectes qui sont des modèles emblématiques en recherche fondamentale et appliquée : l'abeille *Apis mellifera*, et les fourmis *Camponotus aethiops* and *Linepithema humile*.

Dans une première étude, nous avons trouvé qu'une phéromone appétitive diminuait la sensibilité aversive, tandis qu'une phéromone d'alarme augmentait la sensibilité aversive chez l'abeille. Chez *L. humile*, une phéromone de piste synthétique augmentait la sensibilité au sucre et le temps de nourrissage. Globalement, nos résultats démontrent que certaines phéromones modulent la prépondérance des stimuli aversif et appétitif selon leur valence. De cette manière, elles affecteraient la motivation à s'engager dans des réponses aversives ou appétitives, agissant ainsi comme modulateurs de la plasticité comportementale.

Nous avons ensuite déterminé l'effet d'une phéromone d'alarme (l'acide formique) sur la prise de décision and les systèmes de reconnaissance dans le cadre de la discrimination de congénères chez des fourmis charpentières. Nous avons trouvé que la phéromone d'alarme améliorait la discrimination en augmentant l'agressivité envers les non congénères et en la diminuant envers les congénères en même temps. Ces résultats remettent en question le modèle établi de reconnaissance de congénères. Nous proposons donc une version révisée de ce modèle.

Enfin, nous avons testé l'effet de l'acide formique sur l'apprentissage et la généralisation. L'acide formique augmentait la discrimination en conditionnement différentiel olfactif aversif. En conditionnement différentiel olfactif appétitif, l'acide formique modulait les dynamiques d'acquisition et la perception de la similarité des odeurs. Nous suggérons que les phéromones affectent la perception des odeurs conditionnées et des renforcements selon la nature des odeurs et leurs valeurs intrinsèques pour l'individu, ainsi que la valence des renforcements.

Cette thèse présente les premières analyses intégrées de la modulation phéromonale chez deux taxa : les abeilles et les fourmis. Les résultats présentés nous permettent de comprendre une partie des modes d'action des phéromones et ouvrent la voie à de futures études afin de comprendre les mécanismes qui sous-tendent l'effet modulateur des phéromones.