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# *Neural and molecular mechanisms underlying the olfactory modulation of aggression in honeybees*

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**Abstract**

Although honeybees were domesticated over 7000 years ago, finding ways to manage their defensive responses against intruders, including humans, is still a current challenge. This is in part due to the complexity of this behaviour, which starts with the detection of the threat by a few specialized individuals and culminates into a generalized, collective attack triggered by the release of an alarm pheromone. Numerous studies have investigated honeybee aggression and stinging behaviour both in the laboratory and field, including the sensory triggers and the potential regulatory mechanisms. However the specific neural and molecular mechanisms regulating this behaviour are still unknown. In my PhD thesis, I investigated the role of olfactory signals and brain biogenic amines in modulating aggression in honeybees, integrating behavioural, physiological, and pharmacological experiments.

Using a novel assay to measure the stinging behaviour of individual bees under controlled conditions, I first explored whether a range of plant odours could modulate aggression, in particular by interacting with the alarm pheromone released by aroused bees. I identified two floral compounds, linalool and 2-phenylethanol, that block the recruitment elicited by the alarm pheromone. These odours do not prevent the bees from perceiving the alarm pheromone. Instead, this blocking effect appears to correlate with the appetitive value of these odours. This suggests that a complex sensory integration takes place when honeybees are faced with the decision of engaging or not into defensive tasks. Furthermore, a field test demonstrated that linalool could also be used to manage aggressive colonies, highlighting the practical application of these findings.

To gain a better understanding of the neuronal mechanism underlying this effect of floral odours on honeybee aggression, I next investigated how these floral compounds affect the representation of the alarm pheromone in the primary olfactory center of the honeybee brain, the antennal lobe, using *in vivo* calcium-imaging to monitor the activity of neurons in this area. The antennal lobes are structured into functional units called glomeruli, and an odour identity and concentration is encoded within the pattern and intensity of activated glomeruli. We expected that the representation of the mixture of an appetitive floral odour with the alarm pheromone may not be linearly obtained from the representation of each compound, thus

revealing the neuronal mechanisms at play during our previous aggression assays. However, analysis of the data suggests no such mechanism, which could be a clue that this processing is happening in higher brain centers.

Finally, I investigated the role of brain biogenic amines in honeybee aggression. Biogenic amines are important neuromodulators and have been implicated in the regulation of the aggressive behavior of a number of species. However, their potential role in regulating the honeybee's stinging behavior had not been investigated so far. My experiments showed that bees from aggressive colonies have higher serotonin levels in their central brain than bees from gentle colonies. In this region, bees exposed to the alarm pheromone during an aggression test also had more dopamine and serotonin than control bees. Serotonin levels were also higher in the optic lobes of aggressive bees, and in the subesophageal zone of bees responding to the alarm pheromone. Pharmacologically increasing serotonin and dopamine levels induced higher aggressiveness in bees, while decreasing them reduced aggressiveness. This confirms for the first time that serotonin and dopamine play a key role in regulating honeybee aggression.

This thesis is the first integrated study of the molecular and neural mechanisms underlying aggressive behaviour in honeybees. Importantly, this body of work does not only increase our understanding of honeybee behaviour, it will also find application in the development of novel, olfactory-based methods to control honeybee aggression.

**Résumé**

Malgré leur domestication il y a plus de 7000 ans, gérer la réponse défensive des abeilles, en particulier contre l'Homme, reste un défi. Cet état de fait est dû en partie à la complexité de cette réponse, qui commence par la détection du danger par quelques individus spécialisés et culmine dans une attaque collective, déclenchée par une phéromone d'alarme. Le comportement agressif des abeilles a fait l'objet de nombreuses études à la fois en laboratoire et sur le terrain, qui ont permis d'identifier les éléments déclencheurs et régulateurs de ce comportement. Cependant les mécanismes neuronaux et moléculaires qui sous-tendent cette réponse agressive sont toujours inconnus. Durant ma thèse, j'ai étudié le rôle des signaux olfactifs et des amines biogènes dans la régulation de l'agressivité des abeilles, en intégrant des approches comportementales, physiologiques et moléculaires.

En utilisant un nouveau test pour mesurer la réponse agressive d'abeilles individuelles en conditions contrôlées, j'ai pu déterminer si certaines odeurs de plantes modulent l'agressivité des abeilles, en particulier en interagissant avec la phéromone d'alarme. J'ai identifié deux composés floraux, le linalol et le 2-phenylethanol, qui bloquent la réponse agressive déclenchée par la phéromone d'alarme. Ces odeurs n'empêchent pas les abeilles de sentir la phéromone d'alarme, mais ont une valeur appétitive importante pour les abeilles. Ces résultats suggèrent qu'une intégration sensorielle complexe a lieu lorsque les abeilles décident de participer ou non à la defense de la colonie. De plus, un test de terrain a montré que le linalol peut aussi être utilisé pour diminuer l'agressivité d'une colonie entière, ouvrant la voie pour des applications pratiques.

Afin de mieux comprendre les mécanismes neuronaux responsables de cette modulation par des odeurs florales, j'ai ensuite regardé comment ces odeurs affectent la représentation de la phéromone d'alarme dans le centre olfactif primaire du cerveau de l'abeille, le lobe antennaire. J'ai ainsi utilisé l'imagerie calcique *in vivo* pour visualiser l'activité des neurones de cette région. Les lobes antennaires sont structurés en unités fonctionnelles appelées glomérules, et l'identité d'une odeur est codée par le patron d'activation des glomérules. Notre hypothèse était que la représentation d'un mélange entre une odeur de plante appétitive et la phéromone d'alarme ne peut pas être obtenu linéairement à partir de la représentation de chaque composé, révélant ainsi les mécanismes neuronaux à l'origine de l'effet de

ces odeurs florales. Cependant l'analyse des données n'a pas mis en évidence ce phénomène, ce qui suggère que l'intégration de la valeur appétitive des odeurs a lieu dans des centres supérieurs.

Finalement, j'ai examiné le rôle des amines biogènes dans le comportement agressif de l'abeille. Les amines biogènes sont d'importants neuromodulateurs qui ont été impliqués dans le contrôle de l'agressivité de nombreuses espèces, mais leur rôle chez l'abeille n'avait pas été démontré. Les abeilles de colonies agressives ont plus de sérotonine dans leur cerveau central que celles provenant de colonies dociles. Dans cette région, les abeilles ayant été exposées à la phéromone d'alarme ont aussi plus de dopamine et de sérotonine. Les niveaux de sérotonine sont aussi plus élevés dans les lobes optiques des abeilles agressives, et dans la zone suboesophageal des abeilles qui répondent à la phéromone d'alarme. Enfin, augmenter artificiellement les niveaux de sérotonine ou de dopamine induit plus de réponses agressives de la part des abeilles, et les diminuer réduit cette réponse. Ceci confirme le rôle clé de ces molécules.

Ces travaux représentent la première étude intégrée des mécanismes moléculaires et neuronaux qui sous-tendent le comportement agressif des abeilles. En plus d'augmenter nos connaissances sur la biologie de l'abeille, ils permettent d'envisager de nouvelles méthodes, basées sur l'utilisation d'odeurs florales, pour contrôler leur agressivité.

**Declaration by author**

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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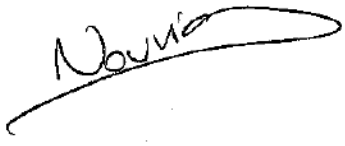

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| Contributor | Statement of contribution |
|---|---|
| Morgane Nouvian (Candidate) | Wrote the paper (70%) |
| Martin Giurfa | Wrote and edited paper (15%) |
| Judith Reinhard | Wrote and edited paper (15%) |

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| Contributor | Statement of contribution |
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| Morgane Nouvian (Candidate) | Designed experiments (60%)<br>Conducted experiments (90%)<br>Analyzed data (100%)<br>Wrote the paper (70%) |
| Lucie Hotier | Conducted experiments (10%) |
| Charles Claudianos | Conceived project (30%) |
| Martin Giurfa | Conceived project (30%)<br>Designed experiments (25%)<br>Wrote and edited paper (15%) |
| Judith Reinhard | Conceived project (40%)<br>Designed experiments (15%)<br>Wrote and edited paper (15%) |

**Contributions by others to the thesis**

Judith Reinhard and Martin Giurfa (principal supervisors) conceived this project and participated in the design of all experiments. Charles Claudianos (associate supervisor) also contributed to the conception of this project. Allen Cheung (associate supervisor) provided advice with statistical analysis. Andrew Barron contributed to the design and realization of the experiments presented in Chapter 5, which were in part conducted in his laboratory at Macquarie University, Sydney, Australia. Charlène Jamme (M1 student) conducted one of the experiments presented in this chapter. Nina Deisig contributed to the realization of the studies presented in Chapter 4, which were conducted in her laboratory at the INRA, Versailles, France. Jean-Christophe Sandoz provided advice on the design of the experiments presented in this chapter, and Maud Combe developed the software used to analyse the data. Mandyam Srinivasan and the Biorobotics laboratory at QBI provided technical assistance to build the experimental set-ups. Lucie Hotier, Peter Anderson and Trevor Weatherhead (beekeepers) managed our honeybee colonies, without which none of this work could have been done.

**Statement of parts of the thesis submitted to qualify for the award of another degree**

None.

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## List of Abbreviations used in the Thesis

| | |
|---|---|
| **5HT** | Serotonin |
| **AL** | Antennal lobe |
| **CB** | Central Brain |
| **Ci** | Citral |
| **CS** | Conditioned stimulus |
| **Cyp** | Cyproheptadine |
| **DA** | Dopamine |
| **DGC** | Deutocerebral giant cell |
| **dMF** | Dimethyl formamide |
| **Flu** | Flupentixol |
| **Ger** | Geraniol |
| **GLM** | Generalized linear model |
| **HPLC** | High performance liquid chromatography |
| **IAA** | Isoamyl acetate |
| **Lav** | Lavender |
| **LH** | Lateral horn |
| **LiA** | Linalyl acetate |
| **Lim** | Limonene |
| **LN** | Local interneuron |
| **Lol** | Linalol |
| **OA** | Octopamine |
| **OL** | Optic lobe |
| **ORN** | Olfactory receptor neuron |
| **PER** | Proboscis extension reflex |
| **PhE** | 2-phenylethanol |
| **PN** | Projection neuron |
| **Pr** | Praescent™ |
| **SAP** | Sting alarm pheromone |
| **SER** | Sting extension reflex |
| **SEZ** | Sub-esophageal zone |
| **TEC** | Triethyl citrate |
| **US** | Unconditioned stimulus |
| **vol** | Volume |
| **β-c** | β-caryophyllene |

# Chapter 1.

# General Introduction

# Introduction

Humans started collecting honey from wild bee colonies over 15,000 years ago, braving the bees' fierce defence to seize this valuable and delectable carbohydrate resource, and often destroying the bee nests in the process. While we have since then domesticated honeybees and developed tools and techniques to handle them without causing too much damage, managing their aggressive response remains a challenge. Selective breeding has been used to create gentle honeybee strains, however the long term benefits of this strategy are questionable, as defensiveness strongly correlates with foraging, productivity and survival of the colony through winter (Wray et al. 2011). Scientific studies of honey bee defensive behaviour may help develop new, better tools to efficiently manage their aggressiveness.

Among the first modern scientific studies characterizing the defensive behaviour of honeybees is the work conducted in 1951 by the French researcher Jacques Lecomte (Lecomte 1951). I would like to hereby pay a tribute to his seminal work on honeybee aggression, summarized in his thesis (Lecomte 1961) and which to this date remains largely unknown (likely because it was never translated into English), because it has been a great inspiration for me. Numerous other studies have investigated the defensive behaviour of honeybees in detail, and many of its mechanisms and regulators have been uncovered. However, how this crucial behaviour is controlled at the physiological level, and especially by the honeybee brain, remains elusive. The main aim of my thesis has therefore been to study neural and molecular mechanisms at play when an individual bee displays aggression.

I focused especially on the olfactory modulation of aggression. There are mostly two reasons for this choice. First, a key element in the defensive behaviour of honeybees is the use of alarm pheromones to signal intruders and coordinate the collective response of defensive bees. Second, the olfactory system of the honeybee has been extensively described and many techniques are available to study it both at the behavioural and neural level. As a result, I was able to use an integrated approach during my thesis research, starting at the behavioural level before exploring neural networks and neuromodulators. More specifically, this thesis aims at providing answers to the three questions detailed below.

## Question 1: Is honeybee aggression modulated by plant odours?

Honeybees live in large colonies of thousands of individuals, which requires effective communication to ensure efficient functioning of the colony as a whole. Pheromones, chemicals used for this purpose (Wyatt 2003), play an important role in many aspects of the honeybee life. Defensive bouts are no exception, and honeybees use potent alarm pheromones to alert their nestmates of the presence of large (usually mammalian) intruders. Nonetheless, pheromones are not the only odorants that are important to bees. These well-known pollinators also rely on their sense of smell to find and identify rewarding flowers when they go out foraging, and the nectar they bring home is often scented (Srinivasan and Reinhard 2009). Thus, floral odours are as central to the biology of honeybees as pheromones. In such cases interactions often happen that can modify or even suppress the insect's response to its pheromone (reviewed in Reddy and Guerrero 2004). Therefore, I first used a series of behavioural assays to investigate if and how common plant odours typically encountered by bees during foraging modulate aggression, in particular when they are combined with the alarm pheromone. This study had both practical and theoretical implications. Indeed, identifying compounds modulating aggression could provide us with new tools to study aggressive behaviour and help its management, but also give us information about how the decision to engage into defence is made by the honeybee brain.

## Question 2: How are the odours modulating aggression processed in the primary olfactory center of the honeybee brain?

Answering Question 1 revealed that some floral odours, but not all of them, indeed modulate aggression in honeybees. More specifically these odours blocked the aggressive response triggered by the sting alarm pheromone. To gain insights into the mechanisms underlying this olfactory modulation, I studied how these mixtures are processed by the primary olfactory center of the honeybee brain, the antennal lobe. This structure is composed of functional subunits called glomeruli, and the identity of an odour is encoded within the spatial and temporal pattern of activation of these glomeruli (Galizia 2014). I used *in vivo* calcium-imaging recordings to visualize

this pattern of activation when the bee was presented with a floral odour (effective against aggression or not), the alarm pheromone or a combination of both. As a control, I repeated this experiment with Geraniol, another pheromonal compound with no function in aggression. I expected two possible outcomes to this experiment: the floral compounds modulating aggression could slightly modify the representation of the alarm pheromone already at this early processing stage, thus revealing part of their mode of action. Alternatively, it could be that no specific effect of these compounds is detectable in the antennal lobe. This would suggest that the modulation of aggression arises from the output of higher brain centers.

## Question 3: Do biogenic amines play a role in honeybee aggression?

Biogenic amines are small molecules produced by the nervous system. Depending on how and where they are released, they can be neurotransmitters (directly generating electrical activity in the post-synaptic neuron), neuromodulators (modifying the response properties of a population of neurons) or neurohormones (circulating in the haemolymph and acting at the periphery). The three major biogenic amines detected in the honeybee brain are octopamine, dopamine and serotonin (Mercer et al. 1983). These molecules regulate aggression in a number of invertebrate species (Kravitz and Huber 2003), however their involvement in honeybee aggression had never been studied. Hence I set out to perform two sets of experiments. First, I searched for correlations between brain biogenic amine levels and components of the aggressive behaviour, both at the colony- and individual-level. Using this information I then used pharmacological approaches to manipulate the levels of the amines of interest to confirm their causal role in honeybee aggression.

## Thesis overview

The structure of this thesis reflects the main questions described above. In Chapter 2, I first review our current knowledge about the defensive behaviour of honeybees. Guard bees, the (temporarily) specialized workers in charge of the important task of defending the nest, exhibit behavioural responses adapted to the different kinds of threats they encounter. They coordinate their efforts and recruit other bees through the use of alarm pheromones, which are described in detail. I also present the sparse information that we already have about the neurobiology of this behaviour, and conclude with an overview of the methods available to study it, including the novel assay that I developed as part of this thesis. This assay allowed me to tackle Question 1, whether plant odours modulate aggression in honeybees, with the outcomes of my investigations presented in Chapter 3. I discovered that interactions between plant odours and the alarm pheromone do exist, with some floral odours blocking the aggressive response triggered by the alarm pheromone. The same odours triggered spontaneous extensions of the proboscis when presented to bees. This holds true even when the bees are raised in cages with unscented food after emergence, thus suggesting that these floral compounds have an appetitive value which may be innate and may interfere with processing of the alarm pheromone in the brain. In Chapter 4, I therefore investigated Question 2 on how these floral odours are represented in the primary olfactory center of the honeybee brain, and if/how they change the representation of pheromonal compounds. In Chapter 5, I then explored the molecular basis of honeybee aggression, presenting results of studies aiming at answering Question 3, namely whether biogenic amines play an important role in honeybee aggression. I validated this long standing hypothesis by showing that serotonin (and dopamine), indeed underlie the aggressive behaviour of honeybees and their response to the alarm pheromone. Finally, Chapter 6 integrates the findings from the behavioural, physiological and molecular-pharmacological studies obtained during my thesis, discusses their theoretical and practical implications, and presents an outlook for future research directions.

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# Chapter 2.

## The defensive response of the honeybee *Apis mellifera*: a review

## Abstract

Honeybees (*Apis mellifera*) are insects living in colonies with a complex social organization. Their nest contains food stores in the form of honey and pollen, as well as the brood, the queen and the bees themselves. These resources have to be defended from a wide range of predators and parasites, a task that is performed by specialized workers, called guard bees. Guards tune their response to both the nature of the threat and the environmental conditions, in order to achieve an efficient trade-off between defence and loss of foraging workforce. By releasing alarm pheromones, they are able to recruit other bees to help them handle large predators. These chemicals trigger both rapid and longer-term changes in the behaviour of nearby bees, thus priming them for defence. Here, we review our current understanding on how this sequence of events is performed and regulated depending on a variety of factors that are both extrinsic and intrinsic to the colony. We present our current knowledge on the neural bases of honeybee aggression and highlight research avenues for future studies in this area. We present a brief overview of the techniques used to study honeybee aggression, and discuss how these could be used to gain further insights into the mechanisms of this behaviour.

## Introduction

The honeybee (*Apis mellifera*) is a eusocial insect. Central to their society is the nest, which contains all the resources of the colony: the queen (the only reproductive female), the brood (attended by nurse bees), the honey produced from nectar collected by foragers, the pollen stores and also the wax combs constructed when the initial swarm moved into the colony housing. Thus, defending this nest [and the main foraging paths emanating from it (Lecomte, 1961)] is of prime importance. Yet, with sociality comes the challenge of coordinating the actions of thousands of bees to achieve an efficient response to potential threats, without depleting the colony of too much of its workforce (Rivera-Marchand et al., 2008). The aim of this Review is to (1) describe how honeybees [of European lineages – see Breed et al. (2004b) for information about Africanized bees] defend their colony at an individual and collective level, (2) highlight the fine tuning of this behaviour, and (3) review our current knowledge about the neurobiology of this response. In doing so, we hope to provide a framework for future investigations of the mechanisms regulating this complex behaviour, which will provide tools to better manage this domestic species. Indeed, with around 3% of the general population (and 14–43% of beekeepers) being allergic to bee venom, the defensive behaviour of honeybees is an important public-health issue (Bilo et al., 2005).

In this Review, after identifying the bee castes involved in colony defence, we describe their behaviour towards different intruders at the hive entrance. Next, we review our current knowledge on alarm pheromones as the key coordinating signals of this social behaviour, before discussing open questions and new research avenues in the study of honeybee aggression, in particular its neural bases. As this analysis requires controlled laboratory assays to study individual aggression, we conclude by presenting the protocols available to study this behaviour.

## Division of labour during colony defence

Honeybee colonies are organized into castes according to a temporal polyethism, with individuals of different ages having different roles in sustaining the community (Winston, 1987). Two populations of bees that perform nest defence have been described: guards and soldiers or stingers. Here, we will use these denominations for simplicity; however, the most striking feature of these populations is that they are not well defined. In contrast to other eusocial species (e.g. some ants and termites), in which defensive individuals can be highly specialized, guard and soldier bees are not morphologically different from other bees. Furthermore, nest defence is a very transient behaviour of honeybees and strongly overlaps with other tasks, particularly foraging; hence, the identity of the defensive bees is constantly changing.

Guarding is typically performed by bees during the transition period from inside duties to foraging. Guards can vary greatly in age but are usually around 2 to 3 weeks old, and they consistently become foragers after or between guarding bouts. Guards are commonly seen sitting at the hive entrance in a characteristic stance, their forelegs off the ground and their antennae pointing forward (Fig. 1A), or, when very excited, with their mandibles open and their wings held away from their body, ready to fly towards any intruder (Fig. 1B) (Breed and Rogers, 1991; Butler and Free, 1952; Free, 1954; Moore et al., 1987; Paxton et al., 1994). The main roles of guards (described in more detail below) are to check whether incoming bees are their nestmates, and to alert the colony to the presence of a predator. The number of bees allocated to guarding is fairly small; only 10 to 15% of workers become guards (Moore et al. 1987) and usually they guard for no more than a day. However, this number increases after a disturbance or when more intruders are trying to enter the hive (Breed et al., 1992; Butler and Free, 1952). Colonies displaying a stronger overall defensive response tend to allocate more workers to guarding, and these guards remain active for a longer period (Arechavaleta-Velasco and Hunt, 2003; Breed et al., 1989; Guzman-Novoa et al., 2004).

The number of guards at the hive entrance correlates with the defensive response of a colony to a disturbance; however, only a small fraction of guards actually participates in the stinging response (Arechavaleta-Velasco and Hunt, 2003). Thus, the main function of guards may be the detection and signalling of

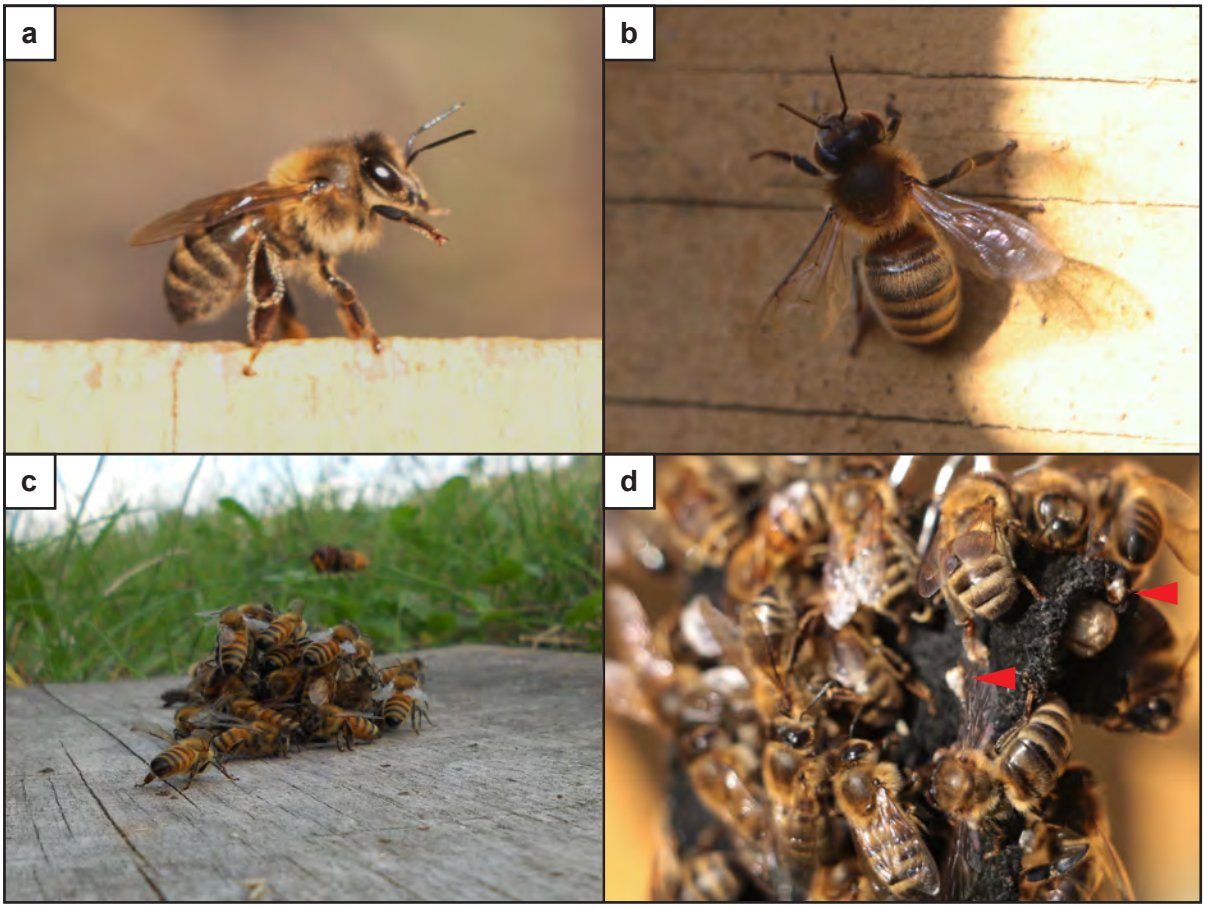


**Figure 1. Guard bees and their behavioural responses to different threats.** Photos are courtesy of David Vogel, CRCA (panels a, b and d) and David Baracchi, CRCA (panel c).
**a.** Guard in the characteristic stance, forelegs off the ground and antennae pointing forward.
**b.** Alerted bee ready to fly off toward the intruder.
**c.** Honeybees engulfing a hornet in the “hot bee ball”. A second hornet (*Vespa Crabro*) is visible in the background.
**d.** Guards recruit nestmates to sting large intruders (here the leather flag used as decoy during a field assay). Sting autotomy is evidenced by the stingers (red arrows) remaining embedded in the leather.

threats. There is some evidence that another population of bees – referred to as ‘soldier bees’ – is responsible for harassing any intruders, but this remains debated. The degree of wear of soldiers’ wings is significantly lower than that of foragers of the same age, so it has been suggested that these bees spend more time inside the hive where they can be quickly mobilized to the entrance (Breed et al., 1990; Breed et al., 1992). In addition, the propensity to sting is regulated by both genetic factors and age, with older bees being more likely to sting (Giray et al., 2000). Indeed, a number of studies have also demonstrated a patrilineal effect, and have mapped quantitative trait loci that are associated with either guarding, stinging or both behaviours (Arechavaleta-Velasco and Hunt, 2004; Breed et al., 2004b; Guzman-Novoa et al., 2002; Hunt, 2007; Hunt et al., 1998; Lenoir et al., 2006; Robinson and Page, 1988; Shorter et al., 2012). More recently, a transcriptional ‘signature’ of aggression has been identified in the bee brain (Alaux et al., 2009; Chandrasekaran et al., 2011). In this Review, we will not include further detail regarding the genetics of honeybee aggression, as this has been extensively reviewed previously (Breed et al., 2004b; Hunt, 2007).

At first glance, the overall defensiveness of a colony correlates with the individual response of its members to noxious stimuli (Avalos et al., 2014), but the link between defensiveness at the individual and at the colony level is far from simple. Complex interactions between individuals are also at play, as evidenced by cross-fostering experiments showing that bees from an aggressive genetic background tend to take over guarding when raised in more gentle colonies, and inversely, gentle bees are less likely to guard when placed in aggressive colonies (Breed and Rogers, 1991). In parallel, cross-fostered bees seem to adopt the propensity to sting of their host colony to some extent (Guzman-Novoa and Page, 1994; Paxton et al., 1994), which suggests that guarding and stinging are differentially regulated but both dependent on colony environment. Finally, when the most aggressive bees of a population are removed, the remaining ones then take over defensive tasks (Lecomte, 1951). This strongly suggests that some kind of defence homeostasis is maintained within the colony. Overall, these studies highlight the sensitivity of guarding and stinging behaviours to both internal (individually based) and environmental factors, and suggest that these behaviours are regulated by interactive, complex and subtle mechanisms. These mechanisms, which have to take place at the individual level, are largely still to be discovered.

## Defence of the hive entrance

### *Intra-specific defence*

When another bee lands at the hive entrance, guards quickly approach and antennate it in order to check whether it is a nestmate. Nestmate recognition is based on the perception of chemical cues carried by the arriving bee (cuticular hydrocarbons, especially alkenes) (Dani et al., 2005; Pradella et al., 2015). These cuticular cues have both a genetic component (Breed, 1983; Getz and Smith, 1983; Page et al., 1991) and an environmental component acquired inside the hive by contact with the comb wax (Breed et al., 1995; Breed et al., 1998; d'Ettorre et al., 2006; Downs and Ratnieks, 1999). Interestingly, emerging bees present a 'blank slate' protecting them from expulsion during the short delay before they are endowed with the proper cues (Breed et al., 2004c).

The task of guards is thus to compare the chemical profile of incoming bees with that of their own colony. Current theories posit that guards have an 'internal template' of the colony odour, although the exact nature of this template remains debated (Breed et al., 2004a; Ozaki and Hefetz, 2014; Page et al., 1991). Because the colony odour can change (e.g. when a new queen takes over, after swarming or when different patrilines are produced), guards continuously update their internal template and accept other bees in accordance with their chemical similarity (Breed et al., 2004a; d'Ettorre et al., 2006). Surprisingly, increased acceptance of non-nestmates after a comb transfer between two hives seems to rely on guards quickly adopting a new template rather than on a change of the bees' odour, suggesting that guards retrieve this information directly from the combs rather than from their kin (Couvillon et al., 2007). Inspections by guard bees are usually very quick (1–5 s), and most of the bees examined do not even stop while they are antennated by guards (Butler and Free, 1952). Sometimes, however, inspections are much longer, up to 30 s or more. On such occasions, the examinee adopts a submissive posture and heats up its thorax, probably to enhance chemical evaporation to facilitate its identification (Butler and Free, 1952; Stabentheiner et al., 2002). If an incoming bee is recognized as an intruder, it is mauled by the guards and dragged away, while remaining in the submissive posture. Even if they gain access to the colony, intruders may still be examined on the combs and dragged back to the hive

entrance, suggesting that guard bees are also present inside the hive (Butler and Free, 1952; Stabentheiner et al., 2002).

The sequence of behaviours described above is mainly directed at returning nestmates or non-nestmate foragers that accidentally land at the wrong hive. Yet bees might also try to steal honey from other colonies' stores. These 'robber bees' are identified even before they land because they exhibit a characteristic swaying flight, moving to-and-fro in front of the hive "as though watching for an opportunity to alight unseen by guard bees" (Butler and Free, 1952). Guard bees dart towards the would-be robber as soon as it lands and start mauling it without any apparent need for an olfactory inspection, although they will release a bee that carries their own colony odour. A robber caught in this way will immediately try to escape and, if successful, will resume its swaying flight. However, if the guards succeed in maintaining their grip, a one-on-one fight ensues in which a guard and the robber try to sting each other (Free, 1954). Such fights end with the death of the opponent successfully stung, with the guard and the robber having a similar probability of winning.

There are a variety of factors that influence the defensive behaviour of honeybees to non-nestmates. The amount of resources available to the colony has a strong influence on the behaviour of guards. They are rarely aggressive to non-nestmates landing at the hive entrance when the colony has sufficient resources; however, under conditions of food shortage, they reject or even kill non-nestmates (Butler and Free, 1952; Ribbands, 1954). This effect could be mediated by the presence of empty combs in the nest, which has been linked to a significant increase in colony defensiveness (Collins and Rinderer, 1985). By contrast, guarding is decreased, along with foraging, under high predation pressure (Rittschof and Robinson, 2013). In addition, guards will more readily reject non-nestmates with activated ovaries – honeybee workers start laying eggs (producing haploid males) if their colony has been deprived of a queen for too long.

The presence or absence of a queen has a strong effect on honeybee defensive behaviour. Without a queen, all bees become generalists and participate in nest defence (Naeger et al., 2013). Furthermore, they reject all non-nestmates to prevent reproductive parasitism (Chapman et al., 2009). However, the prolonged absence of a queen actually causes colonies to become more docile, suggesting that

the queen exerts a direct influence on hive defence in order to ensure her own survival (Delaplane and Harbo, 1987).

***Defence against other insects***

Honey stores also attract other insects, such as ants. When confronted with these pedestrian invaders, the bees at the hive entrance exhibit a stereotyped behaviour: they first turn away from the ants and then blow these small insects off the landing board by fanning their wings at a very high frequency (275Hz on average, exceeding the wing-beat frequency during flight) (Spangler and Taber, 1970; Yang et al., 2009). Ants are faster than bees on foot and do not hesitate to bite them, so this strategy successfully removes the ants while avoiding direct contact. Different subspecies of bees exhibit slight variations of this pattern: *Apis mellifera ligustica* completes this behaviour by kicking its hind legs to strike ants (Spangler and Taber, 1970) but also beetles (Atkinson and Ellis, 2011), whereas *Apis mellifera capensis* performs alternating circles in clockwise and anticlockwise directions to ensure that a large area is covered (Yang et al., 2009). Although defence against ants is rarely observed, the occurrence of this behaviour in two different bee subspecies suggests that it may be widespread.

While ants are mostly opportunistic, other insects have developed strong parasitic associations with honeybee colonies. These pests, well-known to beekeepers, include the mite *Varroa destructor,* the greater wax moth *Galleria mellonella* and the small hive beetle *Aethina tumida*. Defence of the colony against *Varroa destructor* relies on grooming and hygienic behaviours rather than on active guarding, and will therefore not be addressed here (see Rosenkranz et al., 2010 for a recent review on this topic). Wax moths enter the hive through unscreened top entrances and lay eggs in cracks, out of the reach of bees. The emerging larvae feed on wax and hive debris, tunnelling just under the cell caps and feeding on discarded cocoons, thus destroying the combs. Honeybees remove wax moth larvae by biting and dragging them out of the nest (Papachristoforou et al., 2012; Yang et al., 2009). Intruding beetles are usually mauled by several guard bees. However, the small hive beetle has evolved a shielding exoskeleton and reduced appendages that it can retract under its body in a turtle-like manner. This body shape and behaviour make it difficult for guards to grasp or sting this beetle, which often finds a small, out-of-reach place to hide in the hive. Guard bees will surround this area, confining the beetles to

it. Nevertheless, the beetles are still able to survive under such conditions because they can trick their hosts into feeding them through trophallaxis, a mouth-to-mouth food exchange (Atkinson and Ellis, 2011; Ellis and Hepburn, 2006). Within the natural range of this beetle, honeybees (*Apis mellifera capensis*) will further encapsulate the beetles with propolis (Neumann et al., 2001). If the beetle infestation becomes overwhelming, the bees will abscond, a specific form of swarming during which they leave their nest all at once (Ellis et al., 2003). Interestingly, honeybees show a heightened defensive response towards the specialized small hive beetle compared to other beetle species that can accidentally occur within hives, suggesting that they have developed an adaptive strategy towards this specific intruder (Atkinson and Ellis, 2011).

Finally, honeybees also have to face predatory hornets. These large insects prey on adult honeybees, usually hovering near the hive entrance and swooping on returning foragers. A few workers of the Japanese giant hornet *Vespa mandarinia* can exterminate a large honeybee colony within a single day, and later feed on the pupae and larvae (Matsuura and Sagakami, 1973). Because of the hornets' hard cuticles, it is nearly impossible for honeybees to sting them. Thus, the bees' defensive behaviour during such attacks first involves forming large aggregations at the hive entrance. The bees cling to each other to form a 'carpet' and try to catch the hornet with their front legs and mandibles. If successful, they will then quickly trap the hornet within a dense ball of bees (Baracchi et al., 2010). Interestingly, this behaviour is widespread throughout the *Apis* genus but has evolved to fit the particular interactions of each honeybee species/subspecies with the corresponding local species of hornet. *Apis cerana* honeybees, which originate from Asia where there are six species of hornets, are particularly efficient in recruiting over 30 workers to form a 'living ball' inside which the hornet is trapped and killed by the high core temperature of about 45°C. Bees achieve this increase in temperature by contracting their thoracic muscles. The temperature in the centre of the ball is above the thermal limit of the hornet, yet it is harmless for the bees themselves, which have a thermal limit of around 50°C (Ken et al., 2005; Matsuura and Sagakami, 1973). *Apis mellifera ligustica* also use this strategy to confront *Vespa crabro*, a mild predator which occurs in the native range of this subspecies, although only 15 to 20 workers are involved (Fig. 1C), and they raise the ball temperature to 44°C only (Baracchi et al., 2010; Ken et al., 2005). Another subspecies, *Apis mellifera cypria,* is confronted by

*Vespa orientalis*, which has a thermal limit similar to that of honeybees. Consequently, these bees block the hornet's respiration by inhibiting the pumping movements of its abdomen in addition to increasing the temperature, thus asphyxiating it (Papachristoforou et al., 2007). Alternatively, some colonies of this subspecies retreat behind propolis walls with narrow, easy-to-guard openings at the hive entrance and never try to engulf the hornet (Papachristoforou et al., 2011). The reason for the co-existence of these different strategies remains unknown.

Honeybees have been reported to produce piping sounds or 'hisses' when hornets are around, also described as 'shimmering' (Baracchi et al., 2010; Papachristoforou et al., 2008). Hissing seems to be an innate response to noxious stimuli, since this behaviour is also produced in response to electric shocks (Wehmann et al., 2015). Whether these sounds are used as an alarm signal to the colony, as a threat to hornets (which are known to use high-frequency sounds for communication) or are just distress sounds remains to be determined.

***Defence against large predators***

Guards are also the first defensive line against larger predators, such as birds, mice, raccoons, bears and humans. They will fly to check on any disturbance occurring near the hive (Moore et al., 1987), and are mostly triggered by dark colours, rapid movements, mammalian scents and rough textures (Free, 1961). When confronted with a large predator, some guards immediately fly towards it, while others extrude their sting, raise their abdomen and run inside the hive fanning their wings (Collins et al., 1980; Maschwitz, 1964), releasing the alarm pheromones produced by their stinger apparatus (see below), and thus alerting their nestmates to the potential threat. Indeed, guards cannot handle large predators alone. The defence of the colony relies, therefore, on the recruitment of a larger number of bees (Fig. 1D).

Once recruited, a bee will start searching for the possible target. They are primarily attracted by the animal's movement (Wager and Breed, 2000). However, a study of the number and pattern of stings left in two moving targets presented simultaneously revealed that the alarm pheromones left by previous defenders is a powerful attractant, causing the bees to quickly focus on the single most stung target (Millor et al., 1999). Most bees do not actually sting the localized enemy (Cunard and Breed, 1998), but instead harass it by flying rapidly around it and often bumping into it with a characteristic high-pitched buzz (Collins et al., 1980), in what is thought of

as a threatening manner. Because mammalian tissue is elastic, when a bee does sting, the barbed lancets of her stinger (Fig. 2A) – along with its weak connection to the rest of the abdomen – cause this apparatus and the associated muscles to stay in the wound even if the bee itself is quickly removed (Hermann, 1971). This increases the quantity of venom injected into the wound, a single sting thus being equivalent to many injections. This phenomenon, which is followed by the death of the mutilated bee, is called sting autotomy and is found only among eusocial insects where loss of a sterile worker does not have a direct effect on its reproductive fitness (Shorter and Rueppell, 2012). In addition, and contrary to a common belief, the stinging bee does not die right away but lives 18 to 114 hours after losing her sting (Haydak, 1951), thus conserving some value as a defender through pursuing, harassing, biting and hair pulling (Collins et al., 1980; Cunard and Breed, 1998).

## Communication in a defensive context: alarm pheromones

Pheromones are chemicals used for communication between individuals of the same species (Karlson and Luscher, 1959). Two types of pheromones are commonly distinguished: releaser pheromones that cause immediate and short-term responses, and primer pheromones that cause long term physiological changes, eventually leading to behavioural modifications (Wyatt, 2003). The role of these molecules is especially important for colony cohesion in social insects, and the defensive behaviour of honeybees is no exception. Below, we discuss two pheromones that are important for the defensive behaviour of honeybees.

### *The sting alarm pheromone*

*Production and dispersal*

As mentioned above, one of the key elements in the defensive behaviour of honeybees is a pheromonal blend that signals threats to the whole colony. Beekeepers are familiar with this characteristic banana-like scent emanating from the hive whenever the bees are disturbed. Early research demonstrated that the sting apparatus itself carries an alarm pheromone that can alert and attract bees and provokes stinging attacks (Free, 1961; Ghent and Gary, 1962). Anatomical studies showed that the sting alarm substance is produced by both the Koschewnikow

glands and the proximal part of the sting sheaths (Fig. 2A, orange) (Cassier et al., 1994; Grandperrin and Cassier, 1983). The secreted blend flows into the sting chamber, where it accumulates on the setaceous membrane (Fig. 2A, red) (Mauchamp and Grandperrin, 1982). Abundant setae on this structure provide a large surface area, thus enabling a quick discharge of pheromone whenever the sting is extruded (Lensky et al., 1995). Newly emerged bees do not produce iso-amyl acetate (IAA, Fig. 2A), the main component of this pheromonal blend, until they are 3 days old, and levels remain very low for up to a week although they can already perceive it (Allan et al., 1987). When the bee becomes older, however, the volume of alarm pheromone produced rapidly increases to reach about 4–5µg per sting before stabilizing around 2µg per sting. Interestingly, this peak period of production corresponds to the onset of foraging and guarding behaviours, independent of the age of the bee (Allan et al., 1987; Boch and Shearer, 1966). No correlation was found between production of this alarm pheromone and the overall defensive behaviour of a colony, suggesting that aggressive colonies have a lowered response threshold to the pheromone rather than an increased pheromone production (Boch and Rothenbuhler, 1974).

*Composition*

As mentioned above, the first identified and main component of the sting alarm pheromone is IAA (also called isopentyl acetate or IPA). A stationary object marked with IAA at the hive entrance attracts and alerts the guards (Boch et al., 1962), but only a moving object releases their stinging behaviour (Free, 1961; Ghent and Gary, 1962). Although honeybees react strongly to IAA, this odorant does not account on its own for the full response observed with sting extracts (Boch et al., 1962; Free and Simpson, 1968). A second compound present in similar quantities was later identified: (Z)-11-eicosen-1-ol. This compound attracts bees to a moving target but not to a stationary one, unlike IAA. A mixture of these two molecules is sufficient to trigger a full response, and prolongs IAA activity on stationary items (Pickett et al., 1982). However, over 40 other compounds have been identified as part of this pheromonal blend (Blum et al., 1978; Collins and Blum, 1983; Pickett et al., 1982). The reason for such complexity is unknown, although it could serve to create a unique signature of this pheromone (Sandoz et al., 2007; Wang et al., 2008).

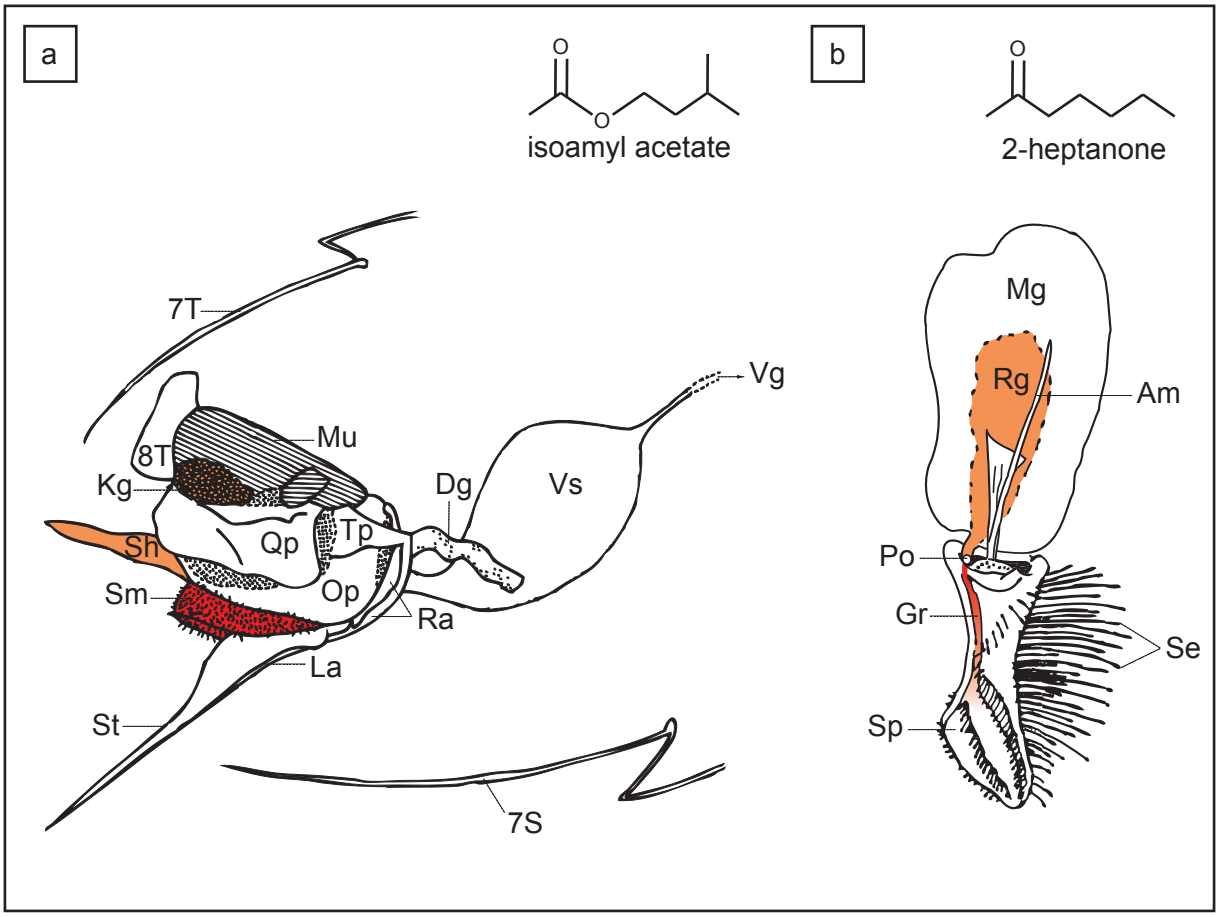


**Figure 2. Organs producing and dispersing the alarm pheromones.** Organs producing the alarm pheromones are shown in orange, and those that disperse the pheromones are shown in red. Adapted from Lensky and Cassier, 1995; Snodgrass, 1956, with permission.

**a.** The sting apparatus and the chemical structure of the main component of the sting alarm pheromone, isoamyl acetate. 7S, 7th sternum; 7T, 7th tergum; 8T, 8th tergum; Dg, Dufour gland; Kg, Koschewnikow gland; La, lancet; Mu, muscle; Op, oblong plate; Qp, quadrate plate; Ra, rami; Sh, sheath lobe (in this image it is abnormally folded upwards instead of laying along the stylet); Sm, setaceous membrane; St, stylet; Tp, triangular plate; Vg, venom gland; Vs, venom sac.

**b.** The mandible and its gland. The chemical structure of the alarm compound 2-heptanone is shown in the upper right corner. Am, apodeme of the adductor muscle; Gr, groove; Mg, mandibular gland; Po, pore; Rg, reservoir of the mandibular gland, Se, setae; Sp, spatula.

*Functions: alert and orientation*

All alarm compounds are releaser pheromones and, as such, trigger fast responses. Table 1 presents some of the molecules identified and their efficiencies in causing agitation of young cage-reared bees (data pooled from Collins and Blum, 1983; Collins and Blum, 1982). Wager and Breed (2000) further tested the functions of some of these molecules by placing them on moving or stationary targets in front of the hive entrance. They found that some were strictly involved in recruiting more defenders (e.g. 1-butanol, 1-octanol), whereas others were orienting the bees towards the target (octyl acetate), and some had both properties (IAA, 1-hexanol, butyl acetate). IAA was the only compound tested that increased flight activity (Wager and Breed, 2000). Interestingly, the alerting function of the sting alarm pheromone seems to be restricted to encounters with other species: contrary to what could be expected, guard bees do not reject non-nestmates more readily when IAA is blown at the hive entrance (Couvillon et al., 2010). The quick decrease in the guards' acceptance threshold observed after a high number of non-nestmate bees have been trying to enter the hive must therefore rely on another mechanism which remains to be determined.

*Long-term exposure and primer effects*

A few studies have investigated the consequences of long-term exposure to IAA on behaviour and physiology. First, it was demonstrated that bees adapt to their own alarm pheromones. When a dispenser containing synthetic alarm substances is placed into a hive, within one hour the bees become less inclined to sting and do not differentiate between scented and control targets (Al-Sa'ad et al., 1985; Free, 1988). Under natural conditions, however, the high volatility of IAA makes adaptation very unlikely. Second, it is known that disturbed colonies remain aroused for a long period. Indeed, repeatedly stimulating a colony with IAA caused the number of bees recruited to the entrance to increase over trials before reaching a plateau (Alaux and Robinson, 2007). IAA also induces the expression of the immediate early gene and transcription factor *c-Jun* in the antennal lobes, which suggests that it has a role as a primer pheromone, prompting long term changes in brain gene expression (Alaux and Robinson, 2007).

Finally, IAA has been reported to induce analgesia through activation of an opioid system (Núñez et al., 1998), an effect that would ensure that the recruits are

**Table 1. Effectiveness of individual compounds in eliciting an alarm response in young caged bees.** M, compounds from the mandibles; St, compounds from the sting; C, control chemicals not produced by the bees. For comparison purposes, we created an Alarm Score. Alarm Score=($N_{weak}$+2*$N_{medium}$+3*$N_{strong}$+4*$N_{very\ strong}$-$N_{no}$)/$N_{total}$. The compounds marked by the same group letter in the 'Statistical groups' column elicited similar reactions in the original studies. The Alarm Score closely matches the original statistics run by Collins and Blum. Data are taken from Collins & Blum, 1982, 1983.

| Chemical | Alarm response | | | | | Total | Alarm Score | Statistical groups | |
|---|---|---|---|---|---|---|---|---|---|
| | No | Weak | Medium | Strong | Very Strong | | | 1982 | 1983 |
| 2-heptanone (M) | 2 | 2 | 22 | 42 | 4 | 72 | 2.583 | | a |
| 2-nonanol (St) | 2 | 1 | 29 | 32 | 8 | 72 | 2.569 | | a |
| 1-hexanol (St) | 1 | 7 | 31 | 32 | 10 | 81 | 2.519 | a | |
| n-hexyl acetate (St) | 1 | 3 | 33 | 31 | 4 | 72 | 2.458 | | a |
| IAA (St) | 2 | 13 | 102 | 89 | 10 | 216 | 2.417 | b | a |
| n-butyl acetate (St) | 5 | 5 | 31 | 27 | 4 | 72 | 2.208 | | a |
| benzyl acetate (St) | 5 | 7 | 34 | 25 | 1 | 72 | 2.069 | | a |
| 2-heptanol (St) | 0 | 16 | 43 | 13 | 0 | 72 | 1.958 | b | |
| iso-pentyl alcohol (St) | 17 | 29 | 70 | 33 | 4 | 153 | 1.745 | b | b |
| 1-acetoxy-2-nonene (St) | 0 | 32 | 29 | 11 | 0 | 72 | 1.708 | c | |
| 1-butanol (St) | 6 | 24 | 35 | 7 | 0 | 72 | 1.514 | d | |
| 2-nonyl acetate (St) | 53 | 96 | 65 | 9 | 2 | 144 | 1.444 | e | |
| 1-octanol (St) | 6 | 24 | 30 | 3 | 0 | 63 | 1.381 | d | |
| n-octyl acetate (St) | 17 | 12 | 28 | 15 | 0 | 72 | 1.333 | | b |
| 2-heptyl acetate (St) | 10 | 26 | 30 | 6 | 0 | 72 | 1.306 | de | |
| 1-acetoxy-2-octene (St) | 14 | 43 | 15 | 0 | 0 | 72 | 0.819 | f | |
| n-decyl acetate (St) | 35 | 5 | 28 | 4 | 0 | 72 | 0.528 | | c |
| benzyl alcohol (St) | 37 | 12 | 19 | 4 | 0 | 72 | 0.347 | | c |
| 1-decanol (St) | 57 | 11 | 14 | 7 | 2 | 81 | 0.136 | h | |
| phenol (St) | 41 | 24 | 7 | 0 | 0 | 72 | -0.042 | g | |
| trans-cinnamaldehyde (C) | 40 | 28 | 4 | 0 | 0 | 72 | -0.056 | g | |
| methyl benzoate (C) | 46 | 23 | 3 | 0 | 0 | 72 | -0.236 | g | |
| beta-ionone (C) | 61 | 11 | 0 | 0 | 0 | 72 | -0.694 | g | |

unlikely to withdraw from the fight. This could play an important role in social coordination, facilitating recruitment during intense and/or long-lasting defensive events. In addition, prolonged exposure to IAA impairs appetitive learning for up to 24h (Urlacher et al., 2010). This could be part of a general mechanism priming the bees for defence by causing them to focus on stimuli that would be relevant for colony defence rather than on appetitive stimuli. Interestingly, honeybees exposed to IAA while foraging in a food patch stop other bees from recruiting foragers to this particular location when they return to the hive (Nieh, 2010; Srinivasan, 2010). We recently discovered that appetitive floral odours can, in turn, prevent the bees from stinging in response to IAA (Nouvian et al., 2015). This blocking effect of floral odours could thus be part of an adaptive, long-term strategy to avoid predator-infested areas: by preventing bees from engaging in defence – and potentially dying – this mechanism makes sure that they come back to the colony and communicate the danger at the foraging site.

### *The mandibular alarm pheromone*

*Composition and production*

Another compound with alarm function is stored in the worker mandibular glands (Shearer and Boch, 1965). Identified as 2-heptanone, this substance is produced in relatively high amounts (15–23µg per bee). A pore on the internal face of the mandibles allows this secretion to flow out of the mandibular glands, and a groove directs it towards the sharp edges at the tip of the spatula (Fig. 2B, red) (Papachristoforou et al., 2012; Vallet et al., 1991). Young bees produce very small amounts of 2-heptanone, but as they get older this quantity slowly increases. Bees performing indoor tasks have the lowest level of production of mandibular alarm pheromone, guards show intermediate levels and it peaks in foragers (Vallet et al., 1991), which has raised some doubts about the postulated defensive role of this substance (see below). Although a correlation between high levels of 2-heptanone and stinging behaviour has been reported (Kerr et al., 1974), later studies showed no such link (Lensky and Cassier, 1995; Vallet et al., 1991). This may be because the first study used related colonies (Kerr et al., 1974), so the results might simply indicate a genetic linkage between these two elements.

*Functions*

The efficacy of 2-heptanone as an alarm pheromone has been much debated. When applied on corks at the hive entrance, it elicits defensive behaviour in guard bees (Shearer and Boch, 1965). Similarly, other studies found that it causes agitation in young cage-reared bees (Table 1) (Collins and Blum, 1982), that it increases sensitivity (measured by the sting extension) to electric shocks (Balderrama et al., 2002) and that bees preferentially attack a ball treated with 2-heptanone over a control one (Free and Simpson, 1968). However, the dose of 2-heptanone required is 20 to 70 times larger than the dose of IAA necessary to trigger similar behaviours (Balderrama et al., 2002; Boch et al., 1970). Only one study found that 2-heptanone and IAA had similar efficiency: when presented simultaneously on two moving balls, the bees did not attack either one preferentially (Free and Simpson, 1968). In fact, in some studies 2-heptanone acts as a repellent or does not elicit any reaction from the guard bees (Butler, 1966; Papachristoforou et al., 2012; Vallet et al., 1991).

Recently, a different function for 2-heptanone in the context of colony defence was revealed: when this substance is injected into parasites through biting, it causes local anaesthesia and paralysis, facilitating their removal from the hive. The injection of 2-heptanone can even kill small parasites such as *Varroa* mites (Papachristoforou et al., 2012). This finding, together with the weak efficacy of 2-heptanone as a recruiting pheromone in simulated mammalian attacks, suggests that this molecule may be more important in the context of defence against other insects. For example, it could help to recruit nestmates not to sting but in order to remove parasites, or be released as a threat to non-nestmates trying to enter the hive.

Finally, 2-heptanone is used differently in a foraging context. There, it serves as a forage-marking pheromone, repelling foragers from flowers that were just visited and depleted of nectar, thereby saving them time and energy. This allows a bee to forage efficiently in a patch of flowers and to coordinate its activity with the other workers (Giurfa, 1993; Giurfa and Núñez, 1992). This function of 2-heptanone is consistent with its peak production in foragers, and may indicate that its recruiting role in colony defence is of secondary importance, thus explaining the discrepancies between the results of previous studies.

## Neurobiology of honeybee aggression

### *Olfactory processing of alarm pheromones*

Neurophysiological studies have analysed how odorants and their individual components are processed in the olfactory circuits of the bee brain (Sandoz, 2011). Odorants are first detected by olfactory receptor neurons (ORNs) located within specialized structures on the antennae. ORNs send their projections to the brain where they contact local interneurons and projection neurons within specific subunits (termed glomeruli) of the primary olfactory centre, the antennal lobe. The number of glomeruli corresponds to the number of molecular receptors existing in the bee genome (around 160), because all ORNs carrying the same molecular receptor converge within a single glomerulus. As olfactory receptors tend to be broadly tuned (i.e. responsive to a wide range of odorants), odours are encoded in the antennal lobe as specific spatio-temporal patterns of glomerular activation (Galizia, 2014; Sandoz, 2011). The olfactory message is then conveyed to higher-order structures, the mushroom bodies and the lateral horn (LH), via parallel tracts (Carcaud et al., 2015).

In contrast to ants, in which a cluster of five ‘alarm-sensitive’ glomeruli has been identified (Mizunami et al., 2010), no specific brain structure dedicated to alarm pheromones has been found in the honeybee so far. Rather, components of these pheromones seem to be processed like general odours (Carcaud et al., 2015; Sandoz et al., 2007). Nonetheless, there are some distinctions between the processing of alarm pheromones and that of other odorants. In the antennal lobe, the representation of a mixture of general odours can be predicted based on the linear combination of responses to its individual components (elemental processing) (Deisig et al., 2006; Deisig et al., 2010), yet this is not the case for components of the sting alarm pheromone (Wang et al., 2008). This supports the hypothesis that the large number of compounds found in this pheromone could serve to create a unique signature. Little information is available about alarm pheromone processing beyond the antennal lobe. 'However, one study found that pheromone components elicited patterns of activity in the LH that were similar for compounds carrying the same message (alarm, aggregation, presence of the queen or of brood) (Roussel et al., 2014). This is in agreement with current views positing that this structure is a pre-

motor centre mediating fast, innate responses in insects (Galizia, 2014; Parnas et al., 2013).

### *Central and peripheral control*

Our recent results show that honeybees integrate all stimuli – relevant ones, such as the alarm pheromone, but also contextual odours – before taking the decision to engage in stinging, thus suggesting that this process is more complex than previously thought (Nouvian et al., 2015). However, the central neural network controlling aggression is still unknown. More is known about peripheral control, particularly about the regulation of the movements of the stinger by the terminal abdominal ganglion. This structure contains a central pattern generator consisting of two loosely connected oscillators, each controlling the thrusting movement of one of the stinger's lancets. The activity of each oscillator is further regulated by afferent inputs from proprioceptors located throughout the sting apparatus: campaniform sensilla, which detect the stress and strain in the cuticle of the stylet and lancets (Fig. 2A), and hairplates between the cuticular plates, which provide information about the relative position of the different elements of the stinger (Ogawa et al., 2011; Shing and Erickson, 1982). The rhythmic movements produced simultaneously bury the stinger deep into the tissue and push the venom towards the tip of the sting, thus maximizing venom delivery (Ogawa et al., 1995). Severing the ventral nerve cord either behind the head or behind the thorax produces activity in the sting muscles (Burrell and Smith, 1994) and triggers the release of alarm pheromones (Balderrama et al., 1996), thus revealing a general inhibitory effect from the brain.

### *Biogenic amines*

Biogenic amines are small molecules synthesised by the nervous system which play a variety of roles, from local neurotransmitters and neuromodulators to peripheral neurohormones (Farooqui, 2012; Libersat and Pflueger, 2004; Scheiner et al., 2006). Using isolated abdominal preparations, it was shown that octopamine reduces the rhythmic activity of the stinger (Burrell and Smith, 1995), but the nature of the effectors (muscles or neurons) remains unknown. Studies of other invertebrate species also suggest that central biogenic amines may play a crucial role in shaping aggression (Alekseyenko et al., 2013; Hunt, 2007; Kravitz and Huber, 2003; Zhou et

al., 2008). Indeed, the serotoninergic system has been linked to the fight-or-flight response in crustaceans (Edwards and Kravitz, 1997; Livingstone et al., 1980). More recently, the molecular tools available in the fruit fly *Drosophila melanogaster* enabled the localization of subsets of serotoninergic (Alekseyenko et al., 2010; Dierick and Greenspan, 2007), dopaminergic (Alekseyenko et al., 2013) and octopaminergic neurons (Dierick, 2008; Hoyer et al., 2008; Zhou et al., 2008), functional alteration of which caused significant changes in the aggressive behaviour displayed by male flies. Activation of the octopaminergic system has also been linked to a transient increase in aggressiveness in crickets (Rillich et al., 2011; Rillich and Stevenson, 2011; Stevenson et al., 2005; Stevenson et al., 2000). In the honeybee, the sting extension reflex (SER), an innate response elicited by noxious stimuli, has been coupled with injections of biogenic amine antagonists in the bee brain (Fig. 3A) in an attempt to determine whether and how these amines modulate stinging responsiveness. Dopamine and serotonin antagonists up-regulate responsiveness (Fig. 3B,C). It has been proposed that both amines act on attention processes, avoiding excessive responsiveness to irrelevant stimuli (Tedjakumala et al., 2014). Overall, these studies strongly suggest that biogenic amines are main regulators of invertebrate aggression, and that studying their involvement in honeybee aggression in more detail would be an important first step towards the identification of the underlying neural mechanisms.

***Brain metabolism***

The first hint that the brain metabolism of honeybees was altered during aggressive bouts came from a transcriptomic study identifying functional clusters of genes which were consistently up or down-regulated in the brains of aggressive bees (Alaux et al., 2009). These results were confirmed recently by studies revealing that mitochondrial oxidative phosphorylation is inhibited in the brain of aggressive bees in favour of aerobic glycolysis (Barros et al., 2015; Chandrasekaran et al., 2015; Li-Byarlay et al., 2014; Rittschof et al., 2015b). This holds true when comparing genetically aggressive bees to gentle ones, but also when comparing bees from the same background before and after exposure to IAA (Chandrasekaran et al., 2015). Direct manipulation of the brain metabolism of bees confirmed this relation to be causal, since inhibiting oxidative phosphorylation increased the aggressiveness of treated bees (Li-Byarlay et al., 2014). How this shift in energy metabolism is acting is

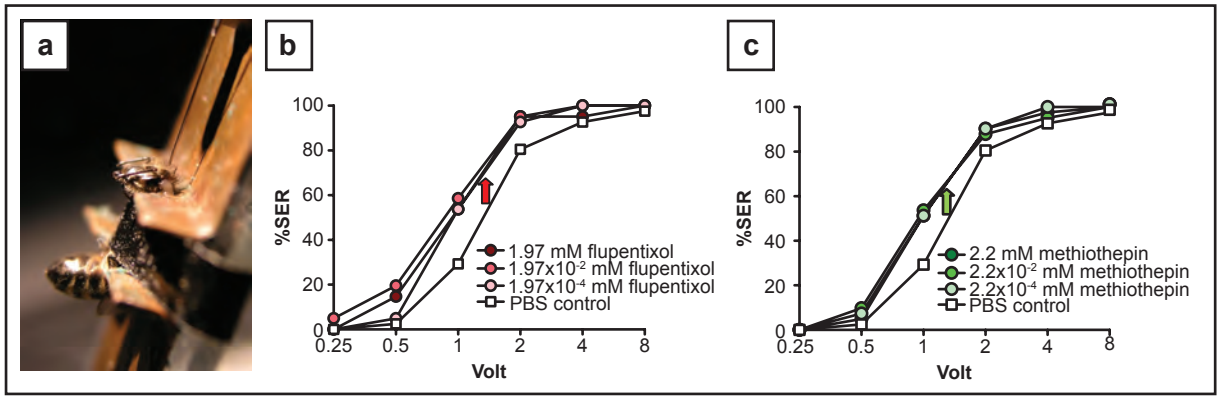


**Figure 3. Dopamine and serotonin modulate the honeybees' responsiveness to noxious stimuli.** Adapted from Tedjakumala et al., 2014.

**a.** Brain injection via the ocellar tract in a honey bee harnessed on a shock delivery setup. A tiny hole was pricked into the cornea of the median ocellus to allow the insertion of a Hamilton syringe located above the bee. The syringe allows delivery of the drug to be tested in the median ocellar tract, which runs medially and caudally from the dorsal margin of the head capsule into the protocerebrum.

**b.** Effects of DA blocking on stinging responsiveness. Three different groups of bees were injected with three different concentrations of the DA antagonist flupentixol (1.97 mM: n = 41; 1.97x10-2 mM: n = 41; 1.97x10-4 mM: n = 41). A fourth group was injected with PBS as a control (n = 41). Sting responsiveness was measured in response to increasing voltages during shock trials. All three flupentixol concentrations induced an increase of responsiveness to electric shocks compared to PBS controls.

**c.** Effects of 5-HT blocking on aversive responsiveness. Three different groups of bees were injected with three different concentrations of the 5-HT antagonist methiothepin (2.2 mM: n = 41; 2.2x10-2 mM: n = 41; 2.2x10-4 mM: n = 41). Each methiothepin concentration induced a significant increase of stinging responsiveness with respect to the PBS control.

unknown. It may involve the accumulation of metabolic by-products, including neurotransmitter precursors, thus increasing neuronal excitability. Another hypothesis is that aerobic glycolysis, although less energy efficient than oxidative phosphorylation, may be faster, thus providing the aroused bee with a more immediate supply of energy to cope with this short, energy-demanding state.

## Assessing aggression experimentally

A major constraint to the study of the elements modulating and underlying the honeybee's defensive behaviour has been the lack of reliable methods to quantify this behaviour (Guzman-Novoa et al., 1999; Kolmes and Fergusson-Kolmes, 1989; Shorter and Rueppell, 2012; Uribe-Rubio et al., 2008). Field assays are influenced by numerous uncontrolled environmental conditions, causing huge variability across trials (Guzman-Novoa et al., 1999; Guzman-Novoa et al., 2003; Southwick and Moritz, 1987). They are, however, necessary to study aggression in its natural context. Laboratory-based assays are better controlled and provide more detailed information about the behaviour of individual bees, but they sometimes use stimuli that are difficult to relate to those occurring in the field, such as electric shocks (Kolmes and Fergusson-Kolmes, 1989; Núñez et al., 1983). Here, we summarize these techniques and suggest ways to improve the assessment of aggression in order to facilitate its study.

### *Colony assays*

The majority of assays developed over the years to assess the defensive response of a honeybee colony use either a moving target (flag or ball covered in leather) jerked above or in front of the hive, the alarm pheromone IAA or a combination of both: Table 2 presents a number of these methods, and the variables used to measure the bees' response. This overview reveals the lack of consensus on a single assay to measure aggression in the field, a fact that renders comparisons between studies difficult. This diversity of approaches to measuring aggression may allow precise dissection of the different traits underlying this behaviour (such as responsiveness to the alarm pheromone, responsiveness to visual stimuli, propensity to sting, etc.). However, we believe that the study of honeybee aggression would

## Table 2. Colony-level assays of aggressive traits.

| Bee containment | Prior disturbance | Target | Measures | First described in[1] |
|---|---|---|---|---|
| Whole hive (field) | None | Ball moving at the hive entrance | Number of stings in gloves | Free 1961, Stort 1974 |
| | | | Number of stings in ball | |
| | | | Time before 1st sting | |
| | | | Time before fierce | |
| | | | Pursuit distance | |
| | | Suede flag waved at the hive entrance | Number of stings | Villa 1988 |
| | | | Time before 1st sting | |
| | Brick dropped on the hive | | Number of stings | Giray et al. 2000 |
| | | | Time before 1st sting | |
| | | Honey bee temper tester[2] | Number of hits | Guzman-Novoa et al. 1999 |
| | | | Time before hits | |
| | Puff of breath | | Number of hits | Spangler et al. 1990 |
| | Opening of the hive | Suede flag passed above top frames | Number of stings | Delaplane & Harbo 1987 |
| | Opening of the hive + alarm pheromone | | Number of stings | Moritz et al. 1987 |
| | Alarm pheromone(s) | None | Number of bees recruited | Breed & Rogers 1991 |
| | | Ball in front of the hive entrance | Number of stings | Moritz et al. 1985 |
| | Alarm pheromone(s) + marble shot | Suede flag waved at the hive entrance | Number of bees recruited | Collins & Kubasek 1982, Collins & Rinderer 1985 |
| | | | Time before recruitment | |
| | | | Number of stings | |
| | | | Time before 1st sting | |
| | Opening of the hive + manipulation of brood frames + smoke | None | Ratings of the tendency to run, fly, hit and sting | Guzman-Novoa et al. 2003 |
| Transparent box at the hive entrance | None | Moving suede flag | Number of stings | Guzman-Novoa et al. 2003 |
| | | | Time before 1st sting | |
| Cages (laboratory based) | Odours | None | Agitation of young bees | Collins & Blum 1982 |
| | None | Live bee or moving dummy | Frequency of attack | Lecomte 1951 |
| | Alarm pheromone(s) | None | Metabolic rate | Moritz et al. 1985 |

[1]To the best of our knowledge. [2]The Honey bee temper tester is a black bottle containing a small microphone recording the noise made by the bees impacting it.

also benefit from a more careful design of field assays. In many cases, the bees are disturbed before the presentation of the moving target (Table 2). As pointed out by Collins and Kubasek (1982), such prior disturbances affect different components of the defensive sequence, so they can create confounding effects. In particular, the use of alarm pheromones circumvents all the initial regulatory steps during which guards detect and signal a threat.

***Individual assays***

Honeybees are rarely aggressive when they are alone and away from the hive. Thus, measuring aggressiveness at the individual level has proved challenging. To our knowledge, only five assays are available. In the sting extension reflex (SER) assay (Fig. 4A) (Núñez et al., 1983), as well as in its free-walking equivalent, the Petri dish assay (Fig. 4B) (Kolmes and Fergusson-Kolmes, 1989), stinging is provoked by electric shocks. These assays can be used to measure a threshold voltage at which the bees start responding (Balderrama et al., 2002; Kolmes and Njehu, 1990; Núñez et al., 1998; Paxton et al., 1994). Alternatively, at a constant voltage, the frequency of response (Núñez et al., 1983), degree of response (Lenoir et al., 2006) or time needed for the bee to respond can be recorded (Uribe-Rubio et al., 2008). Since current, not electrical tension, has physiological impacts, improved versions of these assays should benefit from technological progress and control this parameter rather than voltage. A third assay was developed in order to measure the stinging response of a honeybee exposed to alarm pheromones (Tel-Zur and Lensky, 1995). In this assay, a bee is placed in an apparatus made of a delivery compartment and a recording chamber, in which the abdominal contractions of the bee are recorded upon alarm-pheromone stimulation (Fig. 4C). A fourth assay, recently introduced by our laboratory, measures the aggressiveness of honeybees confronted with a dummy rotated by a step motor (Fig. 4D) (Nouvian et al., 2015), based on a previous version in which the dummy was moved manually (van der Burg et al., 2014). We also added a feather that touched the bees during dummy rotation, which reliably induces a stinging response. This assay uses the same stimuli as in field assays, hence providing a new opportunity to investigate the mechanisms regulating honeybee aggression at the individual level. In addition, in this assay the bees exhibit their full defensive repertoire, including lower-level behaviours such as threatening, chasing and ‘hair-pulling’ (of the feather), so it could also be used to

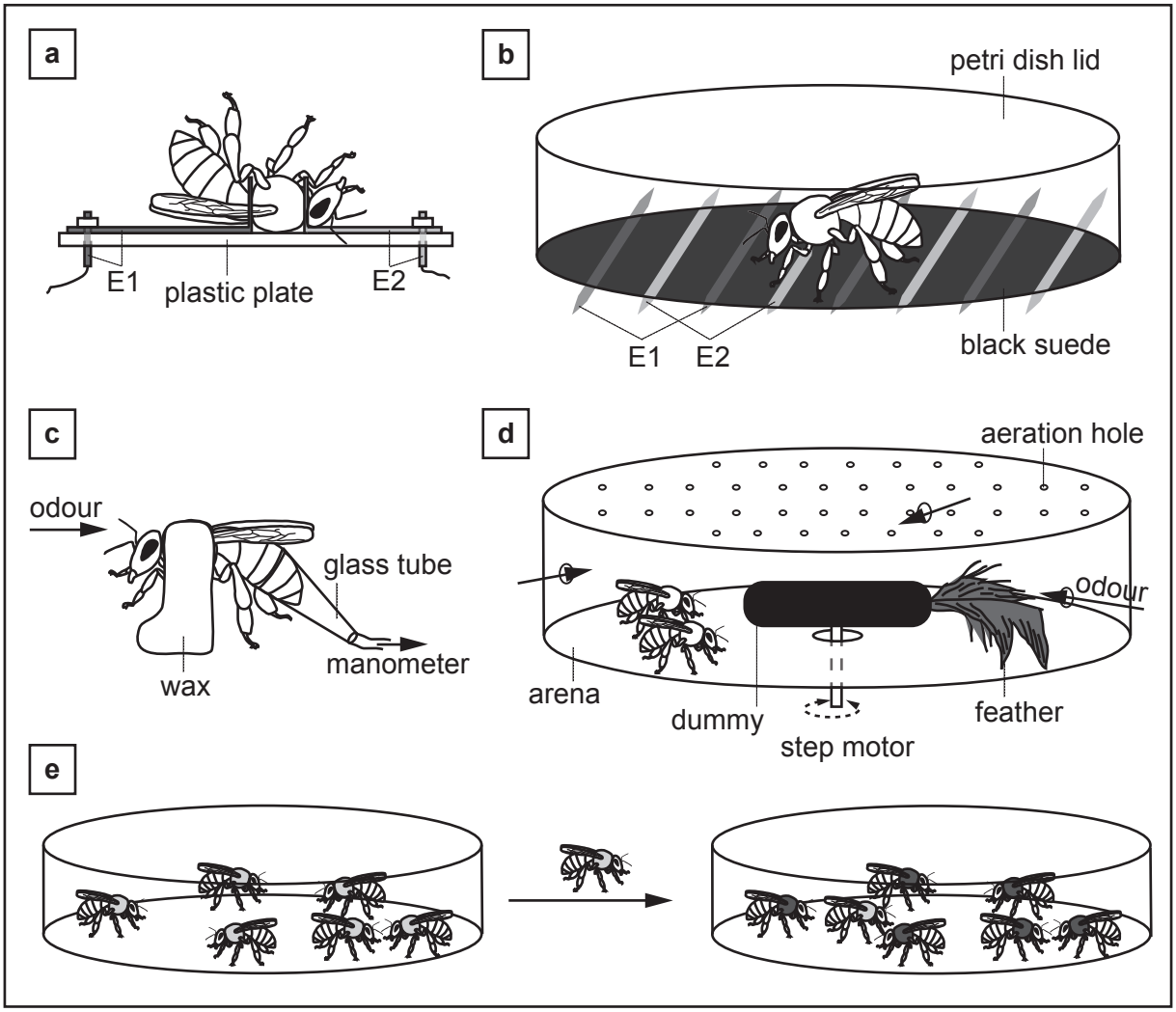


**Figure 4. Individual assays of defensive behaviour**

**a.** In the sting extension reflex assay, the bee is completely restrained in a holder made of two stainless steel plates connected to a power unit which delivers the electric shocks. The extension of the bee's sting in response to the shocks is analysed. E1, E2: electrodes. (Núñez et al., 1983).

**b.** The Petri dish assay involves parallel wires set upon a black suede patch. Adjacent wires are connected to opposite poles of the DC power unit (E1, E2) such that a connection between them will cause a short circuit. The honeybee is placed on this surface under a Petri dish cover. The bee receives a shock when she contacts adjacent wires simultaneously (while walking), and can react by stinging the suede patch. (Kolmes and Fergusson-Kolmes, 1989).

**c.** Set-up to assess the stinging response to alarm pheromones from Tel-Zur and Lensky (1995). The tip of the bee abdomen is sealed into a glass tube and abdominal contractions are recorded by a manometer as the differences in air pressure inside the tube.

**d.** In the rotating dummy assay, the bees are placed in a small arena and confronted with a rotating dummy to which a light feather is attached. Stinging of the dummy is the response assessed. A continuous air flow can deliver odours into the arena (Nouvian et al., 2015).

**e.** In the intruder assay, groups of bees are placed in small containers. After some time, individuals from one group are then introduced into recipient groups, and the aggressive behaviour (mauling, biting, stinging) that resident bees display against this intruder is scored (Breed, 1983).

study these responses. Finally, the intruder assay (Fig. 4E) rates the aggressiveness of small groups of bees towards an intruding conspecific (Breed, 1983). This assay was originally designed for the study of nestmate recognition, but in recent years it has been adapted to investigate how honeybee aggression is affected by different treatments such as early-life experience (Rittschof et al., 2015a), metabolic manipulation (Li-Byarlay et al., 2014) and compromised immunity (Richard et al., 2012). The various assays described here provide interesting possibilities for characterizing honeybee aggression, as they cover different aspects of this behaviour. Thus, combining them may be a good way forward in the study of honeybee aggression.

## Conclusion

Despite thousands of years of honeybee domestication, managing the defensive responses of this insect is still a current issue. A wealth of knowledge on this behaviour has been accumulated over the decades: not only has the behaviour been described in detail, but many details on the sensory triggers, environmental factors and pheromonal regulation of the behaviour have been reported. However, there is still a need to uncover the biochemical and neural mechanisms regulating aggression in honeybees. Knowledge of these mechanisms may allow us to understand at what level environmental factors act on individual responsiveness to potential threats, and may allow the development of new tools to manage aggressive colonies or the selection of lines with desirable physiological or neural traits in order to improve colony handling.

The defensive behaviour of honeybees requires sophisticated multisensory integration, involving olfactory, visual and mechanosensory cues. It constitutes, therefore, an interesting case study in terms of multimodal analysis and decision-making. The dissection of its neural bases offers a rich opportunity to understand how neural circuits mediate coordinated behaviour, and the resulting coordination between individuals producing a collective defensive response provides an appropriate framework for studies on collective intelligence and adaptive evolution. The availability of cutting-edge technology and techniques to study cellular and

molecular mechanisms in the honeybee brain, combined with the appropriate behavioural assays, will allow us to take on these challenges.

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# Chapter 3.

# Appetitive floral odours prevent aggression in honeybees

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## Abstract

Honeybees defend their colonies aggressively against intruders, and release a potent alarm pheromone to recruit nestmates into defensive tasks. The effect of floral odours on this behaviour has never been studied, despite the relevance of these olfactory cues for the biology of bees. Here we use a novel assay to investigate social and olfactory cues that drive defensive behaviour in bees. We show that social interactions are necessary to reveal the recruiting function of the alarm pheromone and that specific floral odours – linalool and 2-phenylethanol – have the surprising capacity to block recruitment by the alarm pheromone. This effect is not due to an olfactory masking of the pheromone by the floral odours, but correlates with their appetitive value. In addition to their potential applications, these findings provide new insights about how honeybees make the decision to engage into defence and how conflicting information affects this process.

## Introduction

Aggression is a crucial element in the competition for food, mates and territory, as well as a defense mechanism against predators. The defensive behaviour of the honeybee *Apis mellifera* aims at the protection of its nest, which contains the food, the brood and the only reproductive individual of the colony, the queen. A specific subset of worker bees, the guards, are responsible for responding to any disturbance occurring close to the colony (Moore et al. 1987). Guards are highly responsive to visual cues such as movement and dark colours, which allow them to identify and locate potential intruders (Free 1961). Guard bees signal this threat to soldier bees (Breed et al. 1990) inside the nest by releasing the Sting Alarm Pheromone (SAP) (Maschwitz 1964), which triggers collective aggressive responses. Over 40 compounds have been identified in this pheromonal blend, but its main component, isoamyl acetate (IAA), is sufficient to elicit most of the behavioural response to SAP (Boch et al. 1962; Collins and Blum 1983). When bees are stimulated by SAP, excitement soon unfolds and they fly out, harass and eventually sting the intruder (Collins et al. 1980); if the stinger apparatus pierces elastic tissue such as human skin, it is detached from the abdomen and stays in the wound after stinging, causing the death of the mutilated bee a few hours later (Haydak 1951; Hermann 1971; Shorter and Rueppell 2012). This extreme cost of aggression may explain why engaging into defensive behaviour is tightly regulated both at the individual and colony level by a variety of factors such as the foraging conditions (Ribbands 1954), the state of the reserves of the colony (Collins and Rinderer 1985) and the defensiveness of a guards' nestmates (Moritz and Burgin 1987; Breed and Rogers 1991; Paxton et al. 1994; Hunt et al. 2003), to name a few. However, the mechanisms underlying this regulation remain to be elucidated.

Olfaction plays a major role for worker honeybees in a variety of behavioural contexts including nest defense (Sandoz 2011). Many odorants (in particular pheromones) are only released in a specific context and thus trigger stereotyped behavioural responses (Sandoz et al. 2007). Yet, odour-specific behaviours may be subject to the modulatory action of odorants that are ostensibly irrelevant for the task considered. For instance, exposure to the sting alarm pheromone impairs appetitive olfactory learning in which bees learn to associate a neutral odorant with sucrose

solution (Urlacher et al. 2010). In this case, the learning impairment may be a response to the alarm signal, which would detract bees from responding to appetitive stimuli in a situation in which such responses would be of secondary importance compared to hive defense. In this study, we investigated the reverse scenario, i.e. whether plant odours, referring to an appetitive foraging context, affect the response to the alarm pheromone in a defensive context. Semiochemical interactions between two sets of odours are known to occur in insects: in the silkmoth for example, host plant odours change the response to sex pheromones and vice-versa (Reddy and Guerrero 2004; Namiki et al. 2008; Party et al. 2009; Chaffiol et al. 2012; Chaffiol et al. 2014).

The aggressive behaviour of the honeybee is an excellent model to study this question because it is reliably triggered by a pheromonal odour (IAA) and results in a stereotypic and easily measured behaviour (stinging). Here, we demonstrate for the first time that the floral compounds linalool (Lol) and 2-phenylethanol (PhE), as well as the odour mixture lavender (Lav), block the aggressive response triggered by IAA. This decreased response is not due to IAA being masked by these specific compounds, but rather correlates with the fact that these floral odours act as appetitive signals for bees. The fact that honeybees weigh and integrate different olfactory stimuli before taking action provides new insights regarding the possible neural circuitry that regulates aggressive behaviour. Furthermore, determining if and how honeybee aggression can be modulated by exposure to natural odorants that are not related to a defensive context may have important practical and economic implications.

## Materials and Methods

### *Honeybees*

For all experiments except the field test, bees were collected from several unrelated honeybee colonies (*Apis mellifera ligustica*) housed on the University of Queensland St Lucia campus (Brisbane, Australia), from April 2013 to October 2014, excluding the winter months – June to August. All colonies were freely foraging and underwent routine beekeeping inspections and honey collection during the course of the experiments. An equal number of bees from 4 different colonies participated. The

bees were caught on sunny days in two rounds (around 9.30am and 11am, alternatively for each colony), in a pattern ensuring that no colony was disturbed more than once every 48 h. This delay allowed the hives to fully settle down in between disturbances, and indeed no increase in aggressiveness was observed over time. In order to select for the population of bees involved in colony defense (guards and soldiers), the bees were collected by waving a large black feather in front of the hive entrance for a few seconds. Once the feather was covered in about 30 to 40 attacking bees, it was quickly placed into a sealable plastic bag and in a freezer at -20°C. The state of the bees was checked after 5 min and then every 1 to 2 min until they were all motionless (on average 8.25 min in the freezer). The bees showing the quickest recovery were selected and placed alone or in pairs into 50 mL syringes (Terumo) containing a wet tissue and 3 droplets of sugar water (50% sugar water, vol/vol). The tip of the syringe was cut and replaced with a plastic sliding door held with a paper clip. In case of pairs, one honeybee was marked with a red dot on the thorax (enamel paint) while the other was left unmarked. Similarly, half of the single honeybees were marked in the same fashion while the other half remained unmarked in order to control for a possible effect of the enamel paint. The data revealed no difference in aggressive behaviour between marked and unmarked bees ($\chi^2$=1.575 , df=2, p=0.455). Once this step was complete, all honeybees were allowed to recover for another 10 min and up to 80 min before being tested in the set-up investigating aggressive behaviour. If one or both bees showed signs of poor recovery when put in the set-up (difficulty to hold upside down, clumsy and/or slow walk), the whole trial was excluded from further analysis. All the materials used to contain the bees were washed with detergent, rinsed and dried after each use.

For the main aggression experiment, a large number of different odorants were tested. Because it was not technically possible to test them all simultaneously, the odorants were distributed into 4 sets, each including IAA and the solvent TEC as reference points. Since there was no statistical difference between these references across the 4 sets (see results), the data was pooled. As a consequence, the IAA and TEC groups include 128 pairs of bees while every other group includes 32 pairs. The experiment testing the role of social interactions included 32 pairs or individuals per group. This sample size was chosen based on pilot experiments. The experiment using the full SAP included 48 pairs of bees per odour condition. The sample size for this experiment was increased to gain the statistical power necessary to detect this

smaller effect. No bee was tested more than once nor released (they were euthanized).

The field test was performed at the apiary of the University Paul Sabatier (Toulouse, France) at the end of summer 2015 (August-September). Three colonies of the same subspecies (*Apis mellifera ligustica*) participated in this experiment. They were all freely foraging, treated against Varroa and underwent routine beekeeping inspections during the course of the experiment. The colonies were tested no more than once every 24 h, in the morning of sunny days.

***Odorants***

All odorants were pure chemicals (98-99.9% purity) from Sigma-Aldrich and kept in the freezer (-20° C). Prior to each set of experiments a fresh batch of odorant dilutions was prepared using triethyl citrate (TEC) for solvent and kept for the whole length of this experiment. These odorants were delivered at room temperature (25° C) and kept in the fridge (4° C) when not in use. Table 1 presents all the odorants used and their concentrations. The concentration of 0.075% (vol/vol) for all plant odours was chosen taking as reference the concentration of Praescent$^{TM}$, the combination of plant-derived odours used in our work (0.03% Z-3-hexen-1-ol, 0.03% E-2-hexenal, and 0.015% α-pinene in triethyl citrate). This concentration was shown to reduce corticosterone, glucose, and redox responses elicited by psychological stress in rats(Einstein and Lavidis 2007; Spiers et al. 2014) and was thus used as a starting point for our study. For the field test, the concentration of the odorants was increased to 1% to cope with the large volume of air in which the odour had to be delivered.

***Aggression assay***

Assessment of the bees' aggressiveness was done in circular arenas (Figure 1a; 14 cm diameter, 4 cm high) made of transparent plastic. A sliding door on the side allowed introduction of the honeybees from the syringes. The various odorants used were blown into the arena through three entry points (4 mm ID) regularly spaced and at middle height along the wall. The arena lid was regularly drilled with about 40 holes (1 mm ID) to avoid building up of the odour inside the arena. A 1 cm hole was also opened in the middle of the arena floor to allow passage of the step motor axle (Aviosys DYO AK27PCB). The step motor was connected to a DC power unit set to

**Table 1: Chemical and biological information on the odorants used to investigate the effect of olfactory cues on honeybee aggression.**

| Odorant | Abbreviation | Composition | Background information | |
|---|---|---|---|---|
| None | None | N/A | Control: no odour. | Controls |
| Triethyl citrate | TEC | pure | Control: solvent. Odourless. | |
| 2-phenylethanol | PhE | 0.075% 2-phenylethanol in TEC | Common floral compound | Floral odours |
| Lavender | Lav | 0.04% linalyl acetate + 0.035% linalool in TEC | Lavender odour simplified to its 2 main components | |
| Linalool | Lol | 0.075% linalool in TEC | Common floral compound (inc. lavender) | |
| Linalyl Acetate | LiA | 0.075% linalyl acetate in TEC | Common floral compound (inc. lavender) | |
| R-(+)-Limonene | Lim | 0.075% limonene in TEC | Common floral compound | |
| Praescent™ | Pr | 0.03% cis-3-hexanol + 0.03% trans-2-hexenal + 0.015% α-pinene in TEC | Green odour. Decreases the noxious effects of chronic stress on vertebrates[1] | Plant odours |
| β-caryophyllene | β-c | 0.075% β-caryophyllene in TEC | Found in many essentials oils (e.g. clove, rosemary) | |
| Citral | Ci | 0.075% citral in TEC | Main component of the Nasanov pheromone, attractant | Pheromones |
| Iso-amyl acetate | IAA | 10% IAA in TEC | Main component of the sting alarm pheromone | |
| Sting Alarm Pheromone | SAP | 30 stings crushed in 500µl TEC | Complete alarm pheromone extracted from the sting | |

1. (Einstein and Lavidis 2007; Spiers et al. 2014; Spiers et al. 2015)

9 V and 0,25 A. Before each trial, the arena was wiped clean using a 70% ethanol solution; and a wet filter paper was put on the floor to maintain the humidity. A dummy was placed horizontally on top of the step motor axle with blue tack. Four dummies were made, each consisting of the barrel of a 3 mL syringe (cylinder of 5-6 cm long, 1 cm in diameter) covered with a rectangular patch of black suede leather (4,5x7 cm) and prolonged on one end with a soft black feather. The use of the 4 dummies was always balanced across the different conditions. The leather patch was held with four pieces of yellow electrical tape and was changed whenever it had been stung. Stung leather patches were rinsed with clear water and left to dry outside for at least 24 h before being used again; the feather was also cleaned with 70% ethanol. In order to increase the jerkiness of the movement, the step motor was used at its lowest speed: as a result, the dummy rotated horizontally across the middle of the arena floor while the black feather gently brushed the sides. The size and shape of the dummy allowed the honeybees to freely move along the sides and lid of the box without touching it. The purpose of the black feather was to disturb the bees without causing them pain. Indeed, this feather was merely touching the bees and was not strong enough to change the path of a walking honeybee.

***Odour delivery in the arena***

Medical grade air (BOC) was delivered from a 680 L tank and fed into a custom-designed olfactory stimulus controller. This olfactometer delivered a constant clean air flow of 1 L per min. PTFE Teflon tubing (3 mm ID) led this air flow to the base of a 15 mL Falcon tube, inside which filter papers carrying the odorants were placed. Further up the sides of the Falcon, three more Teflon pipes were connected, which terminated on the other end into truncated pipette tips. The resulting device could be easily plugged in and out of the three odorant entry points of the arena (Figure 1). In order to avoid contamination, eight of these devices were made and each one was used for the delivery of a single odorant (or combination of odorants) during the course of an experiment. In between experiments, they were thoroughly washed with 70% ethanol and let to dry for at least 24 h before being used for another set of odours.

During each trial two pieces of filter papers were put in the Falcon tube dedicated to the odour delivery. Depending on the odour combination tested, they were either both blank (none, no odour control), one soaked with 10 µl of an odorant

and the other with 10 µl of solvent (odorant alone) or one soaked with 10 µl of an odorant and the other with 10 µl of the alarm pheromone (IAA + odorant or SAP + odorant). For example, for the TEC control both papers were soaked with TEC, for testing limonene alone the combination was Lim+TEC and for testing the interaction between limonene and the alarm pheromone one filter paper carried IAA and the other limonene. To ensure homogeneity of the data, presentation of the different odorants was balanced over colonies and time of the day.

***Trials and scoring of the aggression assay***

All trials were recorded with an HD camera positioned above the arena. Each trial went as follows: first the camera and the step motor moving the dummy were switched on. The tip of the syringe containing the honeybees was then inserted inside the arena. The olfactometer was always switched on just before introduction of the honeybees in the arena, while the arena door was already open but not yet the syringe door. As a result the bees received a quick puff of odour just before facing the dummy, thus mimicking the successive steps of colony defense usually occurring in nature. The syringe door was then opened and if necessary the bees were gently pushed inside the arena with the plunger. The odorant air flow was left running during the whole length of a trial (3 min). During the trial, the rotating direction of the dummy was manually and randomly changed multiple times.

The stinging response of a bee was scored visually and defined as the bee holding onto the dummy for at least 3 s, with the tip of the abdomen pressed against it in the characteristic stinging position, the vast majority (90.2%) of the attacks recorded were further confirmed by the presence of the stinger apparatus still embedded in the dummy leather. Another 5.7% of the aggressive bees stayed exclusively on the feather, which was considered the reason why their stinger was not pulled away. Finally, the remaining 4.1% of attacks scored correspond to bees either choosing to bite the dummy, or (in very few cases) to bees clearly attempting to sting the dummy although the reason why the stinger could not be recovered was unknown. Fewer than 1% of the trials were considered borderline (for example, when an agitated bee contacted the dummy multiple times but did not exhibit any of the other criteria) and were excluded. For each trial, the aggressive response was scored as 1 if at least one of the bees attacked the dummy and 0 if all bees remained calm.

***Field test***

Before the beginning of the experiment, the size of the landing board was standardized (5.5x53 cm) for all the colonies and an open box was created around the hive entrance by placing two vertical wooden walls (10 cm high) on each end of the landing board and a transparent plastic roof on top (Figure 2c). This box was closed at the beginning of each test by the addition of a front door, thus creating a stable atmosphere of about 2.5 L at the hive entrance in which an odour could be delivered. To this end, two 15 mL Falcon tubes containing a filter paper carrying 10 µL of the odour were inserted into holes in the lateral walls (Figure 2c). Four small holes were drilled at the bottom and the lid was modified so that the tubes could be easily connected to the output of an aquarium air pump (Rena 300, delivering a total air flow of about 3.3 L per min). In order to avoid contaminations, a pair of tubes was made for each odour tested. To measure aggressiveness at the colony level, a black leather patch (4.5x7 cm) on a wooden pole was placed in front of the hive entrance, 1 to 2 cm away from the landing board, and jiggled via a small motor (Lego® Power Functions XL-Motor) (Collins and Kubasek 1982; Guzman-Novoa et al. 2002). A square marker on the ground ensured that the flag positioning was the same across days. The tests started by closing the front door of the box and switching on the air pump to deliver the odour, while the flag remained motionless just outside the box. The order of presentation of the odorants was randomized. After 2 min of odorant exposure, the flag motor was switched on and the door was removed, thus allowing the bees to confront the moving flag (with the odour delivery still on, Figure 2c). This step lasted for another minute (hence a total of 3 min for the whole test), after which the motor was stopped and the flag quickly sealed in a plastic box so that no additional bees could access it. The number of stingers embedded in the leather was then counted and used as a measure for aggressiveness at the colony level. The flags were discarded after they were stung and all the material was washed with 70% ethanol between trials. All trials were recorded with a camera placed above the landing board. Each of the 3 colonies was tested 6 times with each odour (n=18 per group), and data were normalized per colony (see below) to account for different inter-colony aggressiveness before being pooled.

***Masking experiment***

In the morning, equal numbers of bees from the 4 colonies were caught at the hive entrance, using Falcon tubes. They were then cold-anaesthetized in the freezer during 5 min and tethered in the restraining tubes used for PER conditioning (Matsumoto et al. 2012). They were fed with a droplet of sugar water (50% vol/vol) before being placed in a dark incubator (26° C, 85% humidity) for 3 h. This is a standard procedure to homogenize the satiation level of the bees and habituate them to the restraining tube (Matsumoto et al. 2012).

The conditioning of the Proboscis Extension Reflex (PER) is a classical conditioning assay in which harnessed bees learn to associate odorants with the appetitive reward of sucrose solution (Giurfa and Sandoz 2012). When the antennae of a hungry, harnessed bee are touched with sucrose solution, the animal reflexively extends its proboscis to reach out to and suck the sucrose. If an odorant is presented immediately before sucrose solution (forward pairing), an association is formed which enables the odorant to release the PER in a following test. In our experiments, bees were exposed to an odour (CS) for 6s followed by the presentation of sucrose solution (US, 50% vol/vol) for 3 s. The CS and US overlapped during 3s. Bees were conditioned with 4 trials spaced by 13 min. Forty-five minutes after the last conditioning trial, the bees' responses to 3 or 4 odours was tested, in a randomized order and without any sugar reward. There was a 13 min inter-trial interval between the tests. Three sets of experiments were conducted. In the first set, the bees were conditioned with IAA and tested either with IAA, PhE and IAA+PhE or with IAA, Pr and IAA+Pr. In a second set, the bees were trained with a mixture (IAA+PhE or IAA+Pr) and tested with the same mixture, IAA, the plant odour alone (PhE or Pr) and β-c (novel odour). Finally, in a third set of experiments the bees were trained with the plant odour (PhE or Pr) and tested with the same plant odour, the corresponding mixture (IAA+PhE or IAA+Pr), IAA and β-c. These 6 test groups include respectively 53, 54, 56, 56, 53 and 56 honeybees. These sample sizes are within the standard range used to ensure statistical power during analysis of PER experiments (Matsumoto et al. 2012).

***Experiment testing the appetitive value of odours***

Two populations of honeybees were tested during this experiment. Defensive bees were caught directly from colonies as described above. To test whether some odours

were innately appetitive, we produced odour naïve bees by placing a capped brood frame in a dark incubator (34° C) and collected the newly emerged bees every day. Groups of 20 age-matched bees were then raised in meshed cages in the same incubator for 10 days. They had *ad libitum* access to water and an unscented sugar solution (50% vol/vol), except during the night before testing when the sugar solution was removed to increase their motivation. Fresh food and water were provided every day. All the bees were cold-anaesthetized on the morning of the test day, placed in the restraining tubes used for PER testing, fed a droplet of sugar water and then left in a 26° C dark incubator for 4 h before testing. A total of 101 naïve bees and 110 aggressive bees participated in this experiment.

Each bee was presented once with the 6 odours tested, in randomized order and spaced by 13 min. Importantly, no training was performed before testing, and no reward was given during testing. At the end of the testing session, the PER was triggered by touching the honeybees' antennae with sugar water, and the few bees that did not respond to this stimulation were excluded from the analysis.

***Statistics and calculation of theoretical data***

We used $\chi^2$ tests to analyse the data produced by the experiment investigating the role of social interactions as the observations were independent and all expected cell counts were greater than 10. To calculate the theoretical data, we considered that the frequency of aggressive trials for single bees under given conditions represent the probability p of one bee from this population to sting under these conditions. The probability of scoring an aggressive trial from two such bees was then calculated using the classical probability laws for two independent events. As a result,

P(aggressive trial) = P(1 of the 2 bees stinging) + P(both bees stinging)

$= 2p(1-p) + p^2$

Or more generally,

P(aggressive trial) = $1-(1-p)^n$ ; where n is the number of bees in the arena.

The expected results were then obtained by multiplying this probability by the sample size.

All the other aggression data was analysed using a generalized linear model (GLM) set-up with a logit link function appropriate for binomial data.

In the field test data set, two outliers had to be removed in each group. They all corresponded to extremely aggressive trials during which 2 to 9 times more stingers than usual were collected. Removing them did not change the overall pattern of responses observed but allowed the data set to meet the normality assumption (Shapiro-Wilk tests) necessary to run an ANOVA with repeated measures. The data was also normalized per colony by subtracting the colony average from each data point and dividing by the colony standard deviation (standard score). This was done to homogenize the data since each colony had a different baseline aggression level (from 1.76 to 17.6 responding bees on average for the most aggressive colony). Post hoc pairwise comparisons were corrected with a Bonferroni procedure.

A potential difference between the percentages of bees exhibiting a PER response when presented with the different odorants was tested with Cochran Q test as it is adapted to repeated measures with dichotomous responses. If this test was significant, a post hoc analysis was performed using multiple McNemar tests and a significance threshold adjusted with a Bonferroni correction. The correlation between two data sets was tested using Pearson's r test.

## Results

### *Effect of IAA and social interactions on honeybee aggression*

To investigate honeybee aggression in a controlled environment, we developed a novel assay in which individual or small groups of bees are confronted with a moving, dark target (a rotating dummy) inside a cylindrical arena (14x4 cm) into which various odours can be released via an automated olfactometer (Figure 1a). Honeybees involved in colony defense (guards and soldiers) were selected from natural hives by briefly waiving a black feather in front of the colony entrance and collecting the bees attacking the feather. After a short anaesthesia and at least 15 min of recuperation, the bees were tested for their aggressive behaviour towards the target in the arena for 3 min in the presence of different odours. Aggressiveness was measured as the percentage of trials during which at least one bee attempted to sting the dummy.

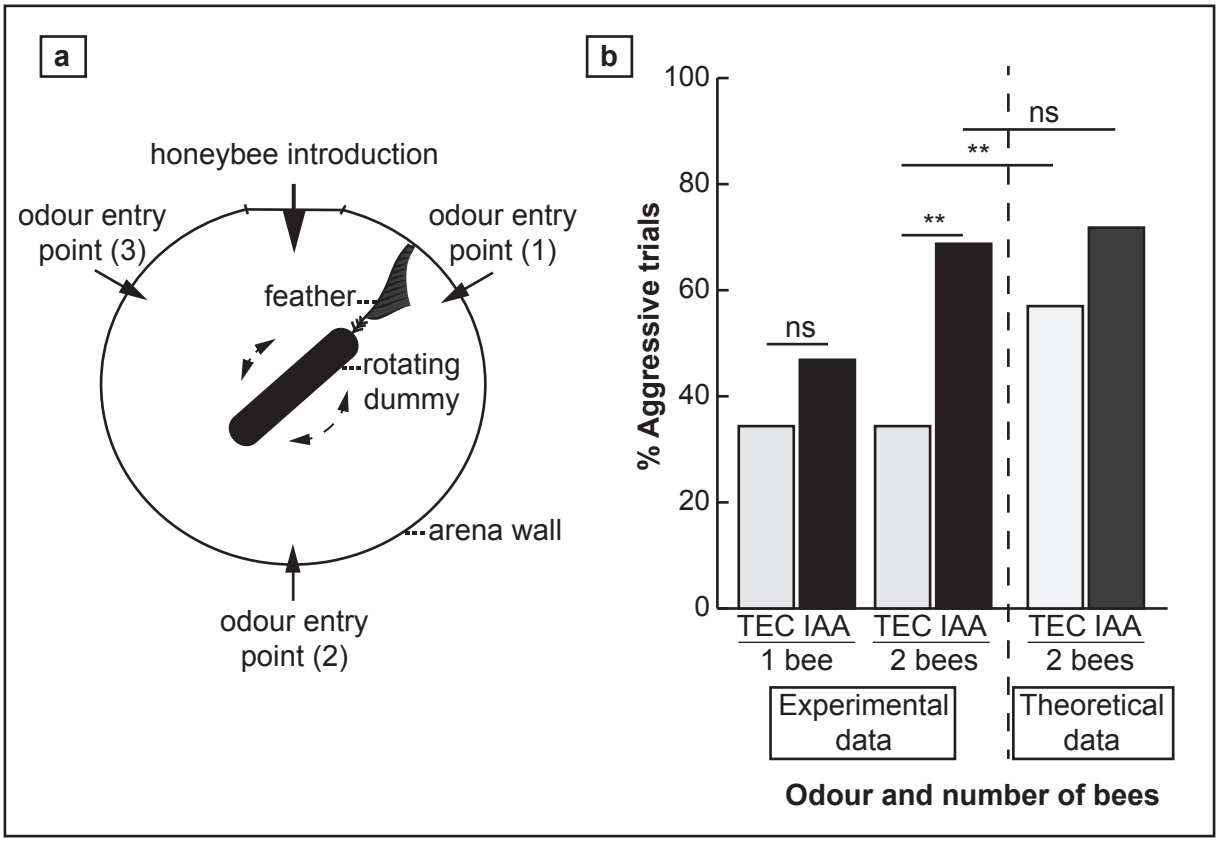


Figure 1: Aggression assay

a. Top view of the arena showing the location of the odorant and honeybee entry points, as well as the rotating dummy.

b. “Experimental data”: percentage of trials in which at least one of the bee stung the dummy, recorded as a function of the odor present (TEC: solvent; IAA: alarm pheromone) and the number of bees introduced inside the arena. “Theoretical data”: results expected if the 2 bees were acting independently from each other, calculated from the probability of attack of a single bee. $\chi 2$ tests, “ns” $p>0.05$, ** $p<0.01$, n=32 single bees and 32 pairs of bees.

Because it was previously reported that single workers rarely respond to SAP and that the defensive behaviour of honeybees is subject to a positive group effect (Moritz and Burgin 1987), we first evaluated the aggressive response of single (n = 32 bees) or paired honeybees (n=32 pairs). The bees in the arena were exposed either to a solvent control (triethyl citrate, TEC) or to IAA (10% in TEC, Table 1). When a single bee was present in the arena, no significant difference between the proportion of attacks to the dummy was observed between IAA- and TEC-exposed bees ($X^2$=1.036, df=1, p=0.309), although a slight increase in aggressiveness could be observed in the presence of IAA (Figure 1b). When pairs of bees were tested in the arena, they reacted strongly to IAA and increased significantly their attacks compared to the controls (69% vs. 34%, respectively; $X^2$=7.570, df=1, p=0.006).

While these experimental results are in agreement with the previous observations of a positive group effect during honeybee aggression (Moritz and Burgin 1987), they do not exclude a purely additive rather than a synergistic effect. To decide between these alternatives, we calculated the hypothetical aggression levels of paired bees under the assumption that social interactions would have no effect on aggression, i.e. that the bees in the arena would behave as single bees did. In this scenario, the aggressive behaviour recorded for the bee pairs would be purely additive. The results of this calculation are presented on Figure 1b as “Theoretical data”. Surprisingly, comparison of these theoretical values (paired bees acting independently) with the experimental data (paired bees acting socially) revealed that social interactions did not cause an increased response to IAA ($X^2$=0.148, df=1, p=0.700). Rather, social interactions between paired bees led to a decreased baseline of aggressiveness in absence of IAA, as the proportion of aggressive trials in the TEC-exposed group was lower in the experimental group than in the theoretical group ($X^2$=6.683, df=1, p=0.010). While the reasons for this lowered baseline of aggression in paired bees remains to be determined, this experiment highlights that a minimum level of social interactions (i.e. the presence of another bee in the arena) is necessary to reveal the natural recruiting function of IAA in our experimental set-up. Therefore, we used pairs of bees to investigate the role of olfactory cues on aggressive behaviour.

***IAA-induced aggression is blocked by specific floral odours***

Next, we studied the effect of several plant-derived odours as well as one other pheromonal compound (citral) on the aggressiveness of honeybees (see Table 1). We exposed the bees to these odorants, either on their own or in combination with IAA, while confronting them with the dummy in the arena. The test odorants were chosen among the most common floral compounds The test odorants were chosen among the most common floral compounds, which are likely to be encountered by bees. Praescent$^{TM}$, a mixture of plan-derived odours, was also chosen because of its known relieving effect on vertebrate stress (Spiers et al. 2014; Spiers et al. 2015). Indeed, honeybee colonies are known to become more aggressive when the resources are scarce (Ribbands 1954) (i.e. in response to a stressor), hence we wanted to explore whether Praescent could also modulate honeybee aggression. IAA was presented as a 10% (vol/vol) solution while all other odorants were at 0.075% (Table 1). This ratio was chosen to ensure the salience of IAA. Because it was not technically possible to test all the odorants simultaneously, they were divided into 4 sets of experiments. No statistical difference could be observed between the two reference stimulations across the 4 sets (TEC and IAA, GLM, $p>0.05$ in both cases), hence the data were pooled. As a result, the data points for TEC and IAA include 128 pairs of bees while all the others include 32 pairs.

As expected (see “Controls” section of Figure 2a), the bees attacked the dummy much more frequently when IAA was blown inside the arena than when there was only TEC, the solvent control (GLM, $p<0.001$), thus confirming the data from the first experiment (Figure 1b). We also included a control with no odour (None) that resulted in a level of aggression similar to the one occurring with TEC, confirming that the solvent itself did not have any effect (GLM, $p=0.874$ vs TEC, $p<0.001$ vs IAA).

When the bees were exposed to plant odours alone (Figure 2a “Odorants alone”, green and brown bars), their aggressiveness did not differ from the baseline level measured during the TEC trials (GLM, all p values >0.1). In addition, in all cases, aggression levels remained significantly lower than that displayed during IAA trials (GLM, $p<0.05$ to $p<0.001$ for all comparisons). The pheromonal compound citral (Ci), which is part of the attractant ‘Nasonov’ pheromone(Butler and Calam 1969) did not have any effect either (GLM, $p=0.156$ vs TEC and $p=0.035$ vs IAA). However, when the same odours were presented simultaneously with IAA (Figure

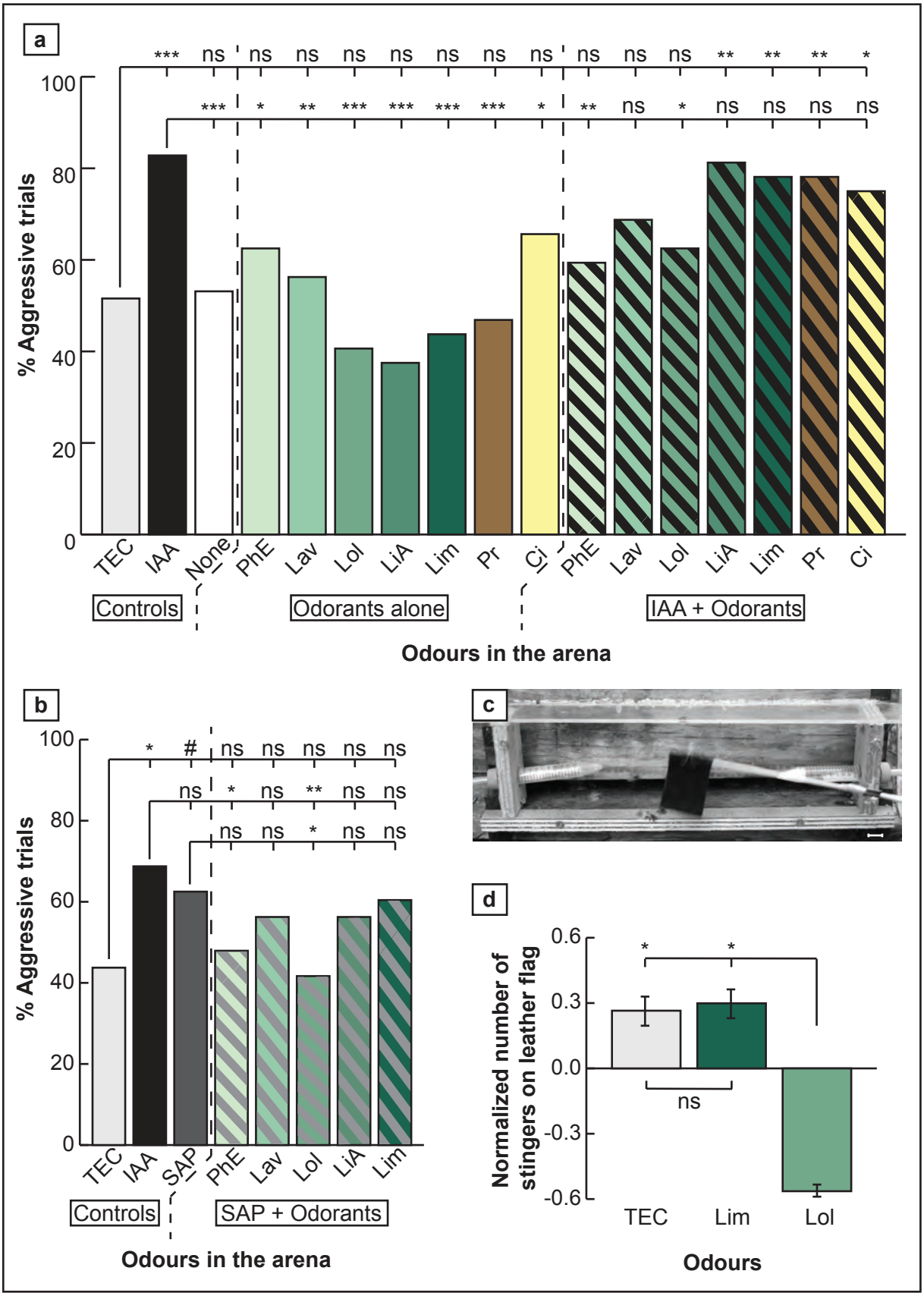
a
% Aggressive trials
100
80
60
40
20
0
TEC
IAA
None
PhE
Lav
Lol
LiA
Lim
Pr
Ci
Controls
Odorants alone
IAA + Odorants
Odours in the arena
b
% Aggressive trials
TEC
IAA
SAP
PhE
Lav
Lol
LiA
Lim
Controls
SAP + Odorants
Odours in the arena
c
d
Normalized number of stingers on leather flag
0.6
0.3
0.0
-0.3
-0.6
ns
TEC
Lim
Lol
Odours

**Figure 2: Some floral odours block the aggressive response to the alarm pheromone a.** Percentage of aggressive trials recorded as a function of the odours blown inside the arena. “Controls” include TEC (solvent), IAA (alarm pheromone) and None (no odour). “Odorants alone” shows that when the compounds were not associated with IAA, none of them had an impact on aggression. In the “IAA+Odorants” section of the graph, the same compounds are presented alongside the alarm pheromone. PhE and Lol significantly decrease the response to IAA, and Lavender to a lesser extent. GLM, “ns” $p>0.1$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n=128 pairs of bees in the TEC and IAA groups and n=32 pairs in all the other groups.
**b.** Percentage of aggressive trials recorded as a function of the odours blown inside the arena. “Controls” include TEC (solvent), IAA (main component of the alarm pheromone) and SAP (sting alarm pheromone). The floral compounds have similar effects when presented alongside SAP (“SAP+Odorants”) than when they were presented alongside IAA. GLM, “ns” $p>0.1$, # $p=0.067$, * $p<0.05$, ** $p<0.01$, n=48 pairs of bees in each group.
**c.** Field test set-up around the entrance slit of a hive. The landing board, small wooden walls and plastic roof form the sides of the box (open here) used to create a stable atmosphere for odour delivery, which is done through the Falcon tubes. The black leather flag is jiggled via a small motor (not visible on the picture). Two aroused bees can be seen under the flag. Scale bar: 1.5 cm.
**d.** Number of stingers embedded on the leather flag depending on the odours blown in front of the hive, normalized per colony (mean ± s.e.m.). Lol significantly decreases the number of bees engaging into defense of the colony. ANOVA with repeated measures, Bonferroni corrected threshold $\alpha=0.017$, “ns” $p>\alpha$, * $p<\alpha$, n=16 trials per odour treatment.

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2a, “IAA+Odorants”), different types of response could be observed. On the one hand, addition of linalyl acetate (LiA), limonene (Lim), citral (Ci) or the green-odour mixture Praescent™ (Pr) did not affect the response to IAA. When bees were exposed to these odorants combined with IAA, the percentage of aggressive responses measured was significantly higher than the baseline control (GLM, $p<0.05$ to $p<0.01$ vs TEC for all comparisons) and similar to the aggression level elicited by IAA alone (GLM, $p>0.1$ for all comparisons). By contrast, bees exposed simultaneously to IAA and 2-phenylethanol (PhE), or IAA and linalool (Lol) did not attack the dummy as frequently as those exposed to IAA alone (GLM, $p<0.05$ vs IAA in both cases). In these cases, the bees’ aggressiveness was reduced to levels similar to the baseline (GLM, $p>0.1$ vs TEC in both cases). Finally, the mixture lavender (Lav), which is composed of Lol and LiA (Table 1), presented together with IAA provoked an intermediate state where the percentage of aggressive trials was not significantly different from that induced by IAA alone (GLM, $p=0.084$) but also similar to the one of the solvent control (GLM, $p=0.080$). Since this mixture is

composed of Lol and LiA (Table 1), the small reduction of aggression may be driven by the presence of Lol.

While IAA is sufficient to trigger a full defensive response, over 40 compounds have been identified in the honeybee SAP(Collins and Blum 1982; Collins and Blum 1983). We therefore also investigated the effect of natural SAP on aggressiveness. We excised 30 stings from defensive bees and crushed them into 500 µl of TEC to extract the SAP. This preparation of SAP proved less effective than synthetic IAA in triggering aggression (Figure 2b; GLM, p=0.519 vs IAA but p=0.067 vs TEC), possibly because the final concentration of IAA was lower in the extract than in the solution prepared with synthetic IAA, or because not all the SAP components were soluble in TEC. Nevertheless, a significant reduction of aggression could also be observed upon stimulation with Lol + SAP compared to stimulation with SAP alone (GLM, p=0.042). Although PhE also seemed to reduce aggression in the presence of SAP, the effect was not significant (GLM, p=0.152). However, SAP+PhE was the only mixture other than SAP+Lol that induced an aggression level significantly different from the one observed during IAA trials (GLM, p=0.040), thus confirming the clear blocking of aggression by Lol and Phe (Figure 2a).

To determine whether these laboratory results can be transferred to the colony level, we conducted a field experiment in which we investigated whether Lol could also decrease aggressiveness in the more relevant context of nest defense. Bees at the hive entrance were exposed to an odour for 2 min and then confronted to a standard stimulation used to measure aggressiveness (Collins and Kubasek 1982; Guzman-Novoa et al. 2002) (a jiggling black leather flag for 1 min), with the odour still present (Figure 2c). Aggressiveness was measured as the number of stingers collected on the flag and the data were normalized per colony to correct for the different levels of overall aggressiveness displayed by the three colonies that participated in this experiment (see Materials and Methods for details). The average number of stingers collected did not differ between the trials in which bees were exposed to the solvent control TEC and the trials in which they were exposed to the control odour Lim (Figure 2d, ANOVA with repeated measures, Bonferroni corrected threshold α=0.017, p=1.000). However, when Lol was blown at the hive entrance significantly fewer bees stung the leather flag (Figure 2d, ANOVA with repeated measures, Bonferroni corrected threshold α=0.017, p=0.014 vs TEC and p=0.014 vs

Lim). Thus, the blocking of aggression by Lol observed in the laboratory assays could be reproduced in the test field at the colony level.

***IAA is not masked by floral compounds blocking aggression***

When two odours are presented simultaneously to honeybees, one of these odours can potentially overshadow or block the other so that the bees only respond to the more salient odour (Smith 1998; Guerrieri et al. 2005; Reinhard et al. 2010; Schubert et al. 2015). This effect could explain the decrease in aggression induced by floral compounds such as PhE or Lol when presented with IAA. To examine this possibility, we conducted a series of experiments using the well-established olfactory conditioning of the proboscis extension reflex (PER) (Bitterman et al. 1983; Giurfa and Sandoz 2012) in which immobilized bees are trained with paired presentations of an odour (the conditioned stimulus or CS) and sucrose reward (the unconditioned stimulus or US). We conditioned bees with a single odour or odour mixture (absolute conditioning) to investigate if IAA was masked by Lol and PhE, the effective plant odours blocking aggression.

In a first experiment, honeybees were trained to associate IAA (CS) with a sugar reward during 4 conditioning trials. Forty-five minutes after the end of the conditioning phase, bees were tested with the CS alone, the mixture of IAA + the plant odour, and the plant odour alone. If IAA was masked by the plant odour in the mixture, the bees should respond significantly less to the mixture than to IAA. The plant odours used were PhE (n=53) since this molecule was effective in reducing the honeybee response to IAA, and Pr (n=54) as a control odour with no effect on aggression (see Figure 2a). During the tests, honeybees trained to IAA responded similarly to IAA and to the mixtures containing IAA and a plant odour (Figure 3, “CS=IAA”; McNemar tests, Bonferroni corrected threshold $\alpha=0.025$, $p>\alpha$ in both cases), but significantly less to the plant odour alone which was novel to them (McNemar tests, Bonferroni corrected threshold $\alpha=0.025$, $p<0.01$ for both odorants). These results suggest that the mixtures were perceived by bees as being similar to IAA. However, these results do not allow us to conclude without doubt that the bees perceived IAA as a separate element of the mixture.

Therefore, in a second experiment, we conditioned groups of 56 honeybees with a mixture of IAA and one of the plant odours, Phe or Pr, as CS (i.e. IAA+PhE or IAA+Pr). We then tested if they would also respond to IAA alone, the plant odour

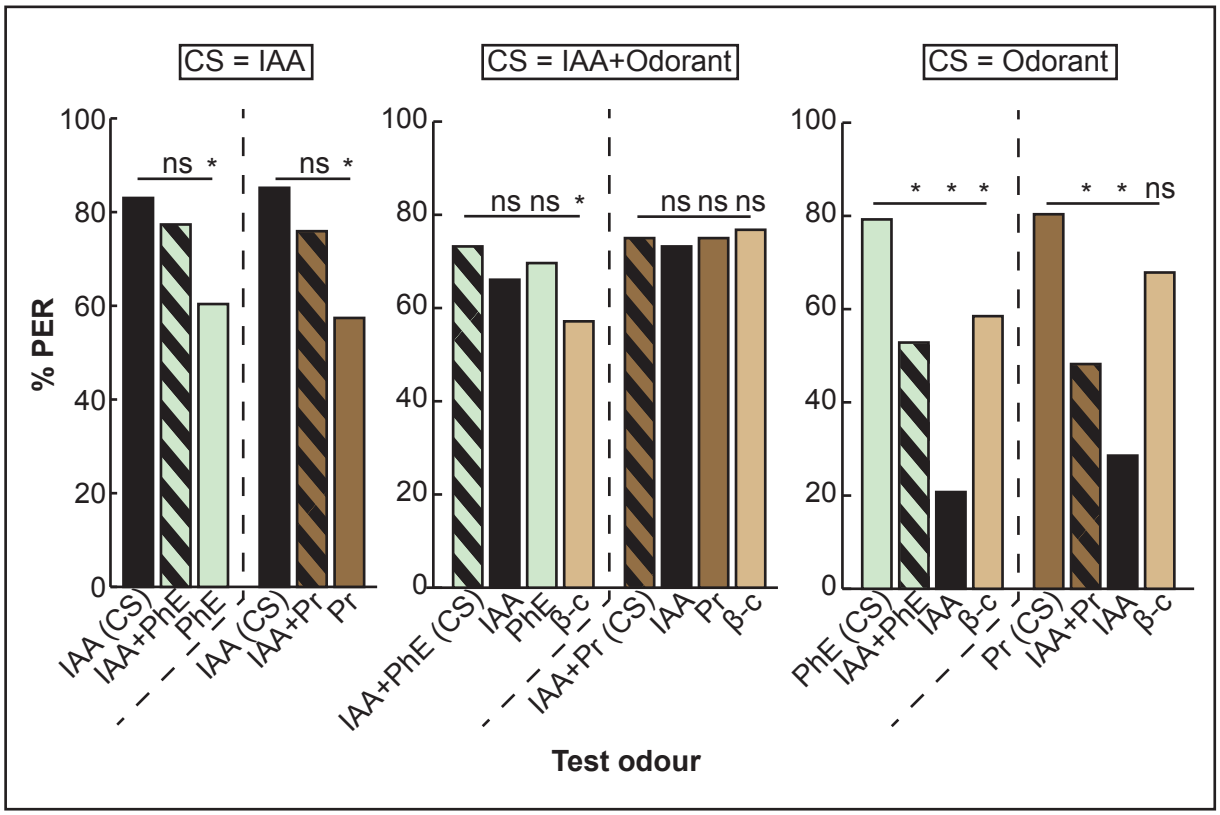


**Figure 3: The impaired response to IAA was not caused by masking of this pheromone by the floral compounds**

"CS=IAA": Bees trained to associate a reward with IAA also responded well when IAA was mixed with the plant odor but less to the untrained plant odor alone. "CS=IAA+Odorant": Bees trained with the mixture also responded to IAA and the plant odor when they were presented alone. However in the case of Pr they also generalized to the novel plant odor β-c. "CS=Odorant": Bees trained to the plant odor did not respond well when this odorant was mixed with IAA or when IAA alone was presented. Again in the case of Pr they generalized to the novel plant odor β-c. McNemar tests, Bonferroni corrected threshold α, "ns" $p>\alpha$, * $p<\alpha$, n=53 to 56 bees per conditioned group.

alone or a novel odour β-caryophyllene (β-c). A high response to IAA would indicate that bees recognize IAA as one of the mixture components, thus precluding overshadowing by the plant odour. Indeed, honeybees responded similarly to IAA, the plant odour and the mixture (Figure 3, “CS=IAA+Odorant”; McNemar tests, Bonferroni corrected threshold $\alpha=0.016$, $p>\alpha$ for all four odour conditions), thus suggesting that IAA and the plant odour are learnt and processed separately even if presented as a mixture during conditioning. After training to IAA+PhE, the response to the novel odour β-c was significantly lower than to the conditioned odour (McNemar test, Bonferroni corrected threshold $\alpha=0.016$, $p=0.012$). However, after training to IAA+Pr the bees’ response to β-c was high, and similar to that of IAA+Pr (the conditioned stimulus CS) (Cochran Q test, $p=0.914$ on this data set as a whole). Thus, we cannot exclude that the bees’ response to IAA was not due to a non-specific response to all odorants (generalization) at least in the case of IAA+Pr training.

Hence, in a final experiment we trained the bees to one of the plant odours, Phe (n=53) or Pr (n=56), and quantified in subsequent tests their responses to the novel odorants IAA and β-c, and to the mixture of the plant odour conditioned and IAA, thus reversing the conditions of the first experiment (Figure 3, “CS=Odorant”). The response to IAA, which was a novel odour in this case, was very low. Surprisingly only few bees responded to the mixture that contained the conditioned plant odour. When compared to the percentage of bees responding to the plant odour on its own, these differences were highly significant (McNemar tests, Bonferroni corrected threshold $\alpha=0.016$, $p<<\alpha$ for these four test odours). Response to β-c as novel odour was also significantly lower for bees trained with PhE (McNemar test, Bonferroni corrected threshold $\alpha=0.016$, $p=0.001$) but, again, not for bees trained with Pr (McNemar test, Bonferroni corrected threshold $\alpha=0.016$, $p=0.039$). This suggests that β-c may be perceptually similar to Pr for honeybees, a fact that could explain why bees trained with IAA+Pr respond to this odour on the basis of similarity rather than non-specifically.

It is intriguing that few bees responded to the mixture of IAA+PhE or IAA+Pr after training with the plant odour alone (third experiment), while all learners responded to this same mixture after training with IAA (first experiment). This strongly suggests that IAA is the dominant component of the mixture, negatively affecting the perception of the plant odour. Indeed, IAA seems to mask the plant

odour, which is not surprising considering that IAA is present at much higher concentrations in the mixture than the plant odour (10% vs 0.075%, Table 1). Bees conditioned to odour mixtures respond more to a dominant component in the mixture (Reinhard et al. 2010; Schubert et al. 2015). Based on this finding, bees trained to IAA+PhE or IAA+Pr should respond more to IAA and less to the plant odour given the concentration differences of these odorants. However, this was not the case (Figure 3, "CS=IAA+Odorant"). This result can be due to the fact that appetitive conditioning to IAA induces high generalization levels to plant odours in honeybee (Sandoz et al. 2001). Taken together, these experiments demonstrate that the decreased response to IAA observed during the aggression assay when some floral compounds were also present cannot be explained by a masking of IAA by these floral odours.

***Aggression-reducing odorants have an appetitive value***

A possible explanation why certain floral compounds prevent bees from stinging in response to IAA could be that these compounds are associated with floral rewards and elicit feeding or foraging, thus preventing the bees from engaging into defense even in the presence of IAA. To test this hypothesis, we measured the spontaneous PER of honeybees participating in the colony defense (guards and soldiers collected as described above) when they were presented with the five floral odours (PhE, Lav, Lol, LiA and Lim) as well as with TEC as solvent control (n=110). Each bee was presented with all 6 odours, and the order of presentation was randomized between bees.

Honeybees extended their proboscis significantly more often when exposed to PhE and Lol (McNemar test, Bonferroni corrected threshold $\alpha=0.01$, $p<\alpha$ vs TEC for both odorants, Figure 4a) but not when presented with LiA or Lim (McNemar test, Bonferroni corrected threshold $\alpha=0.01$, both $p>\alpha$ vs TEC), which had no effect in reducing aggression. As also observed during the aggression assay, Lav elicited an intermediate PER response, not as strong as Lol or PhE (McNemar test, Bonferroni corrected threshold $\alpha=0.01$, $p=0.013$ vs TEC, Figure 4a). Further analysis of the spontaneous response data with regards to the aggression data (Figure 2a) revealed a strong correlation between the appetitive value of the tested floral compounds and the extent to which they decreased aggressive responses by IAA (Pearson's r test, $r=-0.99$, $p<0.001$, Figure 4b).

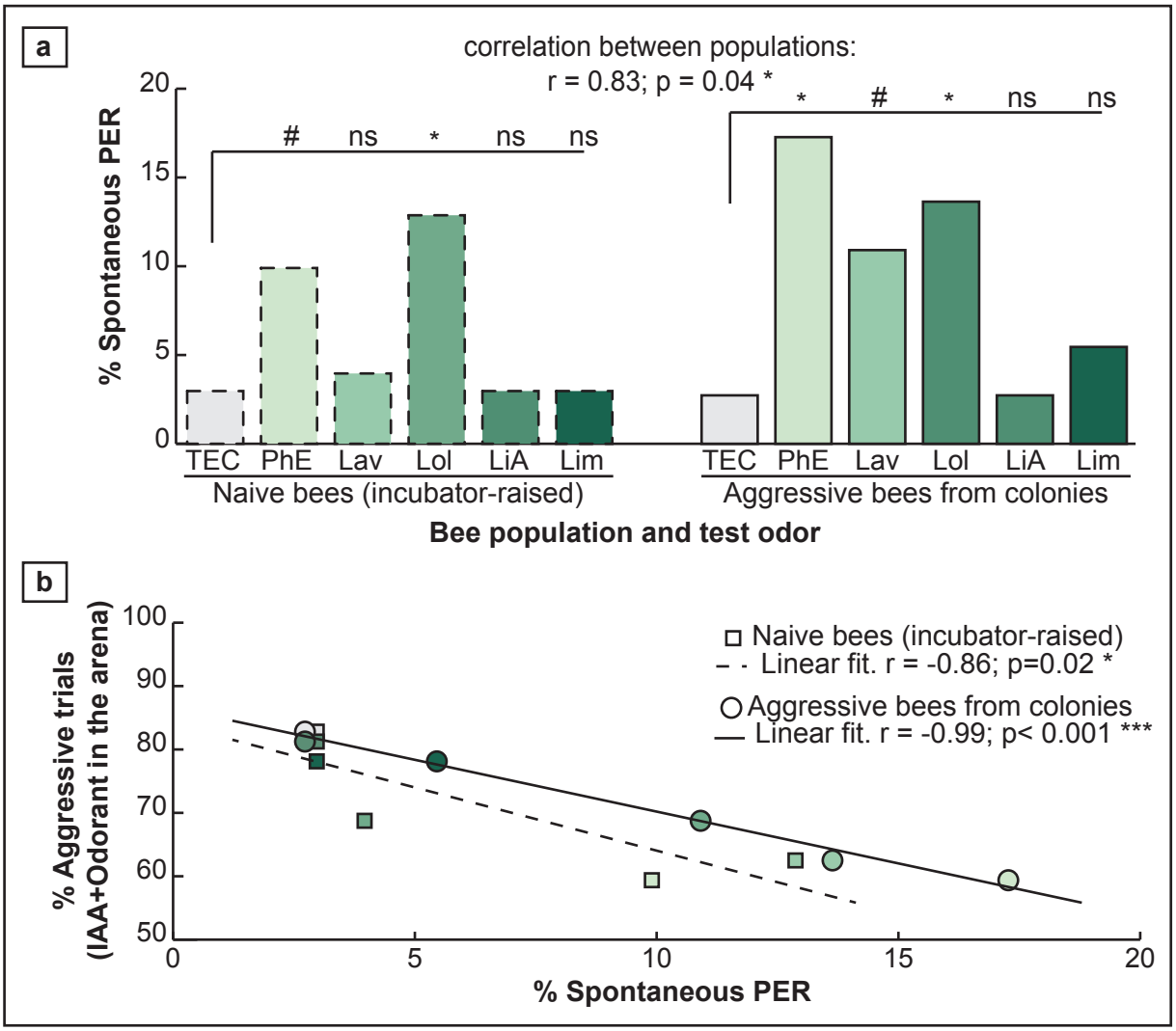


**Figure 4: Floral compounds compete with IAA to an extent directly proportional to their appetitive value**

**a.** Bees participating in the colony defense or naive bees raised in an incubator exhibit spontaneous PER responses to some floral compounds, in particular to PhE and Lol. McNemar tests, Bonferroni corrected threshold α=0.01, "ns" p>α, #PhE p=0.020, #Lav p=0.013, * p<α, n=101 naïve bees and n=110 aggressive bees. The responses of naïve and aggressive bees are correlated. Pearson's r test, r=0.83; p=0.043.

**b.** The appetitive value of each floral compound correlates with the extent to which it affected the response to IAA during the aggression assays. Pearson's r tests, aggressive bees: r=-0.99, p<0.001, naïve bees: r=-0.86, p=0.027.

Spontaneous responses to floral odours are well-known in honeybees and can be explained by prior foraging experience (Gerber et al. 1996). However, our experiments on aggression were conducted over a year using bee colonies that were free to forage all year round in a seasonally changing environment. Their experience with floral odours varied and as a consequence, their olfactory processing and responses to plant odours should have varied accordingly(Claudianos et al. 2014). How then can we explain the consistent effect over a whole year of LoI and PhE on aggression in our study? We postulated that the preference for certain floral odours found in the guards and soldiers that participated in our experiments, and whose main task is not foraging, may be determined at the time of emergence rather than shaped by foraging experience. To test this hypothesis, we collected newly emerged bees from a brood frame and kept them in groups of 20 bees in cages in an incubator for 10 days with unscented sugar solution as food. After emergence these bees were thus raised without any olfactory experience emanating from the colony, food stores or floral sources found in nature. After ten days, we tested the spontaneous PER response of these 'naïve bees' to the same floral compounds (n=101). The naïve bees exhibited a pattern of PER responses similar to that of the aggressive guard and soldier bees (Figure 4a, Pearson's r test, r=0.83, p=0.043). In particular, we observed a higher level of responses to LoI than to the solvent TEC (McNemar test, Bonferroni corrected threshold $\alpha$=0.01, p=0.008), while PhE induced only marginally more proboscis extensions than TEC (McNemar test, Bonferroni corrected threshold $\alpha$=0.01, p=0.020). None of the other tested floral odours induced significant PER response in naïve bees.

Crucially, the data set from the naïve bees also correlates well with the results from the aggression assay (Pearson's r test, r=-0.86, p=0.027, Figure 4b). This strongly suggests that the olfactory preferences of guard and soldier bees for the specific floral compounds LoI and PhE are already determined at the time of emergence, and that the appetitive value of LoI and PhE may be the factor that reduces honeybee aggression in the presence of IAA.

## Discussion

The aggressive behaviour of the honeybee is a considerable public-health issue, with 0.3 to 7.5% of the population allergic to bee venom and a prevalence reaching 14 to 49% for beekeepers (Bilo et al. 2005). Understanding the biological mechanisms at play is a crucial step in developing tools for its management. Here, we used a novel bioassay to investigate if plant odours could decrease the aggressive behaviour of honeybees. We found that the floral compounds linalool (Lol) and 2-phenyethanol (PhE) reduce the aggressive response triggered by the alarm pheromone, thus exerting a calming effect on disturbed bees. We further show that this effect directly correlates with the appetitive value of the floral odours used as detractors from aggression: the higher the appetitive value, the lesser the aggression elicited by a concomitant exposure to alarm pheromone.

Our novel arena-based bioassay combines aggression-triggering elements detected by honeybees in nature and reliably induces bees to sting a target, while allowing the experimenter to easily record reproducible behavioural elements of aggression. This robust and technically simple assay has the potential to become a standard assay used to study the molecular and neural mechanisms underlying aggression in honeybees.

Using this assay, our first experiment challenged the view that single, individual honeybees rarely react to the alarm pheromone (Moritz and Burgin 1987). Our analysis shows that single bees do in fact react to IAA, but that in the absence of this pheromone the baseline aggressiveness of single bees is higher than for paired bees. Two alternative interpretations are possible for these results. Single bees may be more reactive to the dummy, possibly because being alone is a stressor in itself. Alternatively, paired bees could be less reactive, because being in a group would diminish the threat of the dummy (statistically). Importantly, previous studies reporting that single honeybees do not react to SAP determined the response to SAP as an increase in metabolic rate rather than as stinging behaviour (Moritz et al. 1985; Moritz and Burgin 1987). Taking this fact into consideration makes our first explanation more likely: if being alone is a stressor, then the metabolic rate of single bees would be high even before the presentation of SAP, rendering the detection of a metabolic change difficult.

Two types of pheromones are commonly distinguished: releaser pheromones, which provoke immediate and short-term responses, and primer pheromones, which cause long term physiological changes, eventually leading to behavioural modifications. The honeybee SAP and its main component IAA belong to both categories. In addition to eliciting a defensive behaviour, IAA has long-lasting physiological effects on honeybees. First it induces opioid-like analgesia which is thought to prevent the bees from withdrawing from the fight (Núñez et al. 1998). Second, it impairs appetitive learning for up to 24 h after exposure (Urlacher et al. 2010). In the latter case, it was concluded that IAA detracts the bees from responding to appetitive signals that are irrelevant in the context of colony defense. In our study however, we found that bees exposed to a floral odour with an innate appetitive value exhibit reduced aggression levels. While these results seem contradictory at first sight, a major difference between both studies is that our defensive encounter only lasted 3 min, while in the other study the bees experienced 30 min of IAA exposure prior to conditioning (Urlacher et al. 2010). Thus, the learning impairment might reflect a slow behavioural shift that could play an important role during intense and long-lasting defensive events.

The crucial question remains why some floral odours (and not others) have an inhibiting effect on the response to the alarm pheromone. We demonstrated that these floral compounds were already appetitive to newly emerged honeybees, which were not in contact with combs or comb odorants since their emergence. This result supports the idea that some odorants may be innately appetitive to bees with no foraging experience. An alternative explanation could be, however, that the naïve bees tested in our experiments were eventually imprinted by these odorants during larval development if they were present in the wax comb. It is, nevertheless, unknown if larval honeybees can learn olfactory cues and retain this information throughout the development and metamorphosis until the adult stage. On the contrary, innate preferences for colours are well known in flower-naïve honey bees which correlate with the colour of flowers producing high-quality nectar rewards (Giurfa et al. 1995). In the same manner, innate preparedness for floral odour cues could help inexperienced bees to find food sources in their first foraging flights (Butler 1951). Interestingly, flower-naïve honey bees do not land on artificial coloured flowers unless they are scented (Giurfa et al. 1995).

Both linalool and 2-phenylethanol, but none of the other compounds we tested, have previously been shown to elicit spontaneous appetitive responses in honeybees (Dötterl and Vereecken 2010). Similarly, these two compounds often feature among the key components that the bees use to learn complex mixtures (Pham-Delegue et al. 1993; Reinhard et al. 2010). Nonetheless it is, to our knowledge, the first time that the existence of preferences for some floral odours has been formally shown using naïve honeybees whose exposure to these odours during adult life has been controlled for. Further work would be needed to determine if the slight differences observed between the preferences of naïve bees and guard/soldier bees are caused by a refinement through olfactory experience or by further maturation of the olfactory system after 10 days.

The most striking result, however, is not the existence of olfactory preferences but the fact that exactly the odours that are associated with reward are the ones that affect IAA-triggered aggression. After having excluded perceptual interference during olfactory processing of plant odours and IAA via the PER assay, this is the first lead towards the underlying regulatory mechanisms how Lol and PhE may block aggressive behaviour. The fact that exposure to IAA reduces learning of floral odours in an appetitive context (Urlacher et al. 2010) and that floral odours reduce in turn the response to IAA in an aggression context implies that an integrative mechanism in the bee brain has to weigh different odour values, in different contexts, against each other.

Numerous studies support the idea that the division of labour in honeybee colonies is caused by differences in response thresholds to environmental stimuli (Collins et al. 1980; Robinson 1987; Pankiw 2005; Page and Amdam 2007). Based on this model and on our new findings, we present a possible mechanism for the decision making process underlying honeybee aggression (Figure 5). In this model, we postulate the existence of an integrative mechanism in the bee brain which weighs the different stimuli (olfactory but also visual and mechanical) and computes an overall “defensive score”. This score would then be compared to an individual threshold in order to choose between possible behavioural outputs, which in our model are limited to engaging into defense or continuing to perform other non-lethal colony duties (e.g. foraging). The individual threshold itself would be determined by a range of factors including the internal state of the bee (Paxton et al. 1994; Breed et al. 2004) and the state of the colony (Ribbands 1954; Collins and Rinderer 1985;

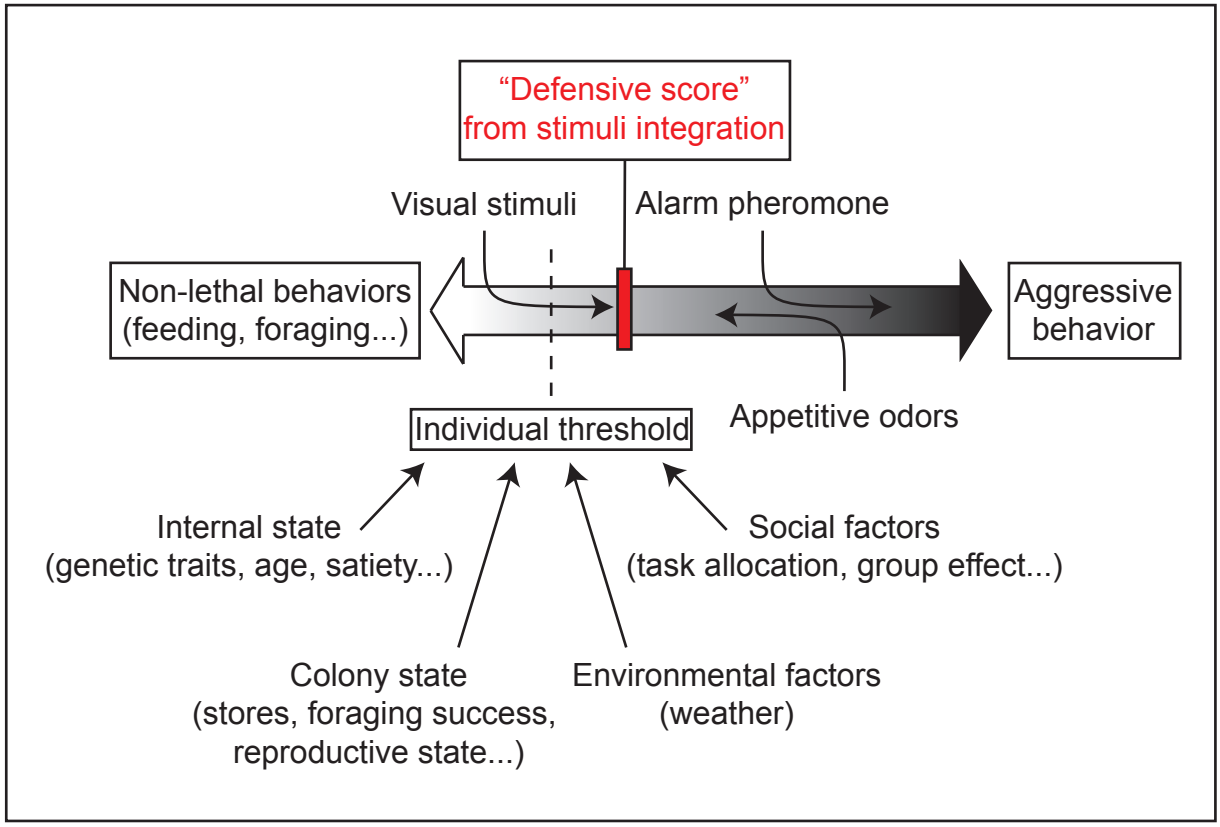


**Figure 5: A possible model for the decision making process underlying honey-bee aggression**

We postulate the existence of an integrative mechanism, which computes a "defensive score" from all the stimuli (visual, mechanical, olfactory…). This score, represented here as the red cursor, is compared against an individual threshold determined by internal and colony state as well as by environmental and social factors. Appetitive floral odours and the alarm pheromone exert opposite actions on the defensive score moving it towards and away the individual threshold, respectively. The resulting state determines if the bee engages into active defence.

Delaplane and Harbo 1987; Paxton et al. 1994). We also suggest that this individual threshold might already take into account social (Moritz et al. 1987; Giray et al. 2000; Hunt et al. 2003) and environmental (Southwick E.E. 1987) factors as a way of enhancing the computational speed of the integrative mechanisms, but these parameters may also be considered as changing stimuli and feed directly into the integrative mechanism. The changing individual threshold along with variations in the weight attributed to each stimulus would thereby create the diversity of reactions observed during a defensive event. Our findings constitute a first step towards the elucidation of the mechanisms regulating honeybee aggression. Further research may also shed light on the adaptive evolutionary value of plant odours modulating this complex and little understood behaviour. For example, floral odours are usually encountered during foraging trips away from the colony, a context in which stinging is not a primary adaptive response. Therefore, floral odours may detract bees from aggressive interactions by acting as markers of distant foraging locations. A decrease in aggressiveness correlated with the perceived distance from the nest has already been demonstrated in another social insect, the desert ant (Knaden and Wehner 2004). In the honeybee, a similar effect could be triggered by the perception of appetitive floral odours.

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# Chapter 4.

# Processing of odours modulating aggression in the antennal lobes of honeybees

## Abstract

Honeybees exhibit heightened aggressiveness and stinging behaviour when they smell the alarm pheromone produced by their stinger. However, the appetitive floral odours linalool and 2-phenylethanol block this response to the alarm pheromone, while the non-appetitive floral compounds limonene and linalyl acetate have no such effect. Here, we use *in vivo* calcium imaging to investigate how these odours are represented and interact in the primary olfactory center of the bee brain, the antennal lobe. We quantified neural responses both at the input and the output layer of the antennal lobe to determine if and how in this structure the processing of the alarm pheromone might be affected by the simultaneous presentation of appetitive floral odours. At both neuronal levels and at the odour concentrations used in the behavioural assay, we find no evidence that the spatial activity pattern elicited by the alarm pheromone is affected by the presence of floral compounds. The activity pattern elicited by mixtures of the alarm pheromone and a floral odour can be computed linearly from the patterns of both components. Moreover, appetitive floral odours seem to be processed like non-appetitive ones. While it is possible that our analysis may not have detected very subtle interactions, these results suggest an absence of neural interactions between the appetitive odours and the alarm pheromone in the antennal lobe, thus indicating that the behavioural modulation exerted by the former on the latter may take place in higher brain centers such as the lateral horn, a structure recently identified as an odour “evaluator”.

## Introduction

In honeybees, olfactory perception starts in specialized structures called *sensilla placodea* located on the antenna (Figure 1). These sensilla comprise numerous pores and host olfactory receptor neurons (ORNs). Odorants penetrate the sensillum via the pore and reach the membrane of ORNs either passively or transported by odorant-binding proteins. Each ORN carries on its membrane only one of the 163 olfactory receptors identified in honeybees. ORN axons are bundled into the antennal nerve which reaches the primary olfactory center in the insect brain, the antennal lobe (AL). This structure is constituted by functional, globular subunits, called glomeruli, which are sites of synaptic interaction between different populations of neurons. While ORNs expressing the same receptor are scattered all over the antenna, their axons converge into the same glomerulus. Within each glomerulus the ORNs contact projections neurons (PNs) which innervate higher brain centers such as the lateral horn (LH) and the mushroom bodies. Furthermore, a dense population of inhibitory local interneurons (LNs) branch across glomeruli. They are responsible for the first processing of the olfactory information in the antennal lobe, refining odour representation (see Sandoz 2011; Galizia 2014 and references therein for this whole paragraph). Consequently to this organization of the AL, the identity of an odour can be visualized as a specific spatio-temporal pattern of glomerular activation. Except for slight variations likely linked to individual experience, this pattern is the same species-wide (Joerges et al. 1997; Galizia et al. 1999).

Honeybees use olfaction to detect and discriminate objects such as flowers (Butler 1951; Srinivasan and Reinhard 2009), predators (Free 1961) or other bees (Breed et al. 1998). The honeybee olfactory system is also essential for communication within the colony, through the detection of chemicals released by the bee's nestmates. Adult and larval honeybees produce a wide range of compounds that intervene in intraspecific communication and act as chemical messengers in a variety of behavioural contexts. These substances, called pheromones (Karlson and Luscher 1959), inform their nestmates about the state of the colony and trigger appropriate responses (reviewed in Pankiw 2004; Le Conte and Hefetz 2008; Trhlin and Rajchard 2011). In this study, we used two pheromonal compounds with very distinct functions. The first one is isoamyl acetate (IAA), the main component of the

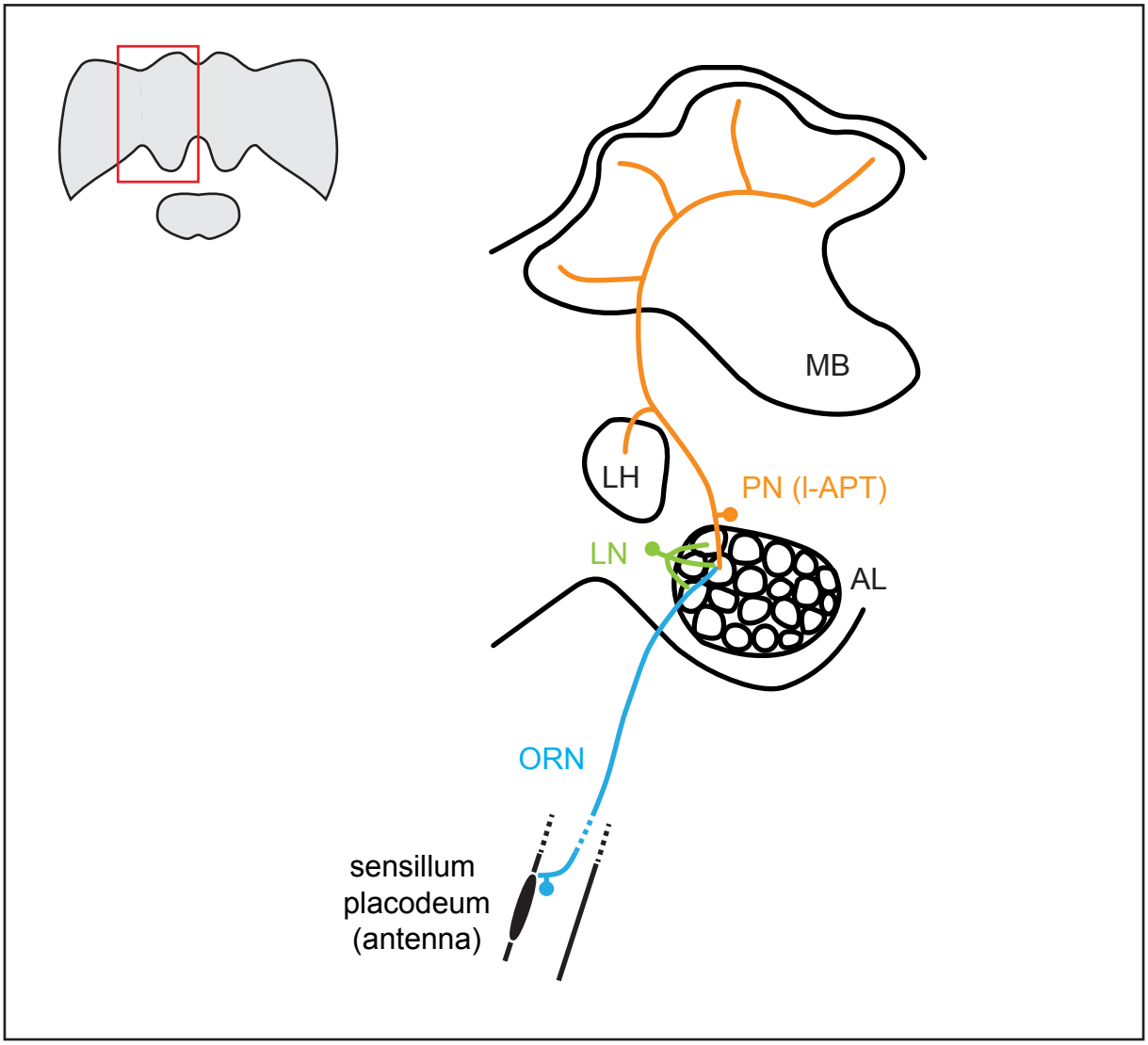



**Figure 1: Schematic organization of the olfactory pathway in the honeybee.** Only the part of the brain within the red inset is represented. Odorants pass through pores in the sensillum placodeum and bind to receptors on the membrane of olfactory receptor neurons (ORNs). These neurons contact projection neurons (PNs) in the antennal lobe (AL), which then arborize into the lateral horn (LH) and the mushroom bodies (MBs) via two tracts. Here only the lateral antenno-cerebralis tract (l-APT), which was targeted for retrograde staining of PNs, is represented. The second, medial tract (m-APT) project first to the MB and then to the LH. In the AL, lateral interneurons (LNs) connect multiple glomeruli and refine the olfactory signal.

sting alarm pheromone (Boch et al. 1962). As its name suggests, this pheromone is carried by the sting and released to signal a threat to the colony. When honeybees smell IAA, they are aroused and react by attacking the object that they perceive as threatening (Free 1961). The second pheromonal compound, which we used as a control, is geraniol (Ger). This molecule is produced by the Nasanov gland and is part of an attractive blend (Butler and Calam 1969) released either at the hive entrance to help returning foragers find their way or at profitable food sources to attract foragers; it also triggers aggregation during swarming (Trhlin and Rajchard 2011).

The AL processes both general odorants and pheromonal compounds. However in many species, specialized glomeruli within this region (often enlarged) are dedicated to the perception of pheromones, mostly those involved in sexual attraction. This is the case, for example, in male moths (Hansson and Anton 2000) and bees (Kropf et al. 2014) in which a macroglomerular complex specializes in the processing of odorants of the female sex pheromone. Enlarged glomeruli of this complex receive inputs exclusively from sex-pheromone sensitive ORNs. In ants, five “alarm-sensitive” glomeruli have also been identified (Mizunami et al. 2010) and a macroglomerulus specialized for a trail odorant has been suggested (Kleineidam et al. 2005). However in the worker bee no evidence for a spatial separation between the detection of pheromones and general odorants has been found in the AL so far (reviewed in Sandoz et al. 2007). This does not mean that bees treat pheromones like general odours: for example when they are conditioned to associate pheromonal compounds with a sugar reward, they generalize this association to other odorants more than when they are trained with general odours (Sandoz et al. 2001).

The fact that pheromones and general odours are processed by the same structures in worker bees suggests that interactions may occur between these two kinds of compounds. In a recent behavioural study, we indeed found that some floral odours can block the aggressive response triggered by IAA. Interestingly these floral compounds also triggered appetitive responses in bees (Nouvian et al. 2015). We therefore investigated how these odours are processed in the AL, and especially how mixtures of a pheromonal and a floral odour are represented. Linear or elemental processing is characterized by the fact that the representation of mixtures can be predicted from the representation of its components (albeit with some gain control to avoid saturation). Alternatively, during synthetic or configural processing

the information on the components is lost in favour of a novel percept specific to the mixture (Lei and Vickers 2008). Strikingly the neural representation of a mixture of general odours can be predicted linearly from the pattern elicited by each of its components (Deisig et al. 2006; Deisig et al. 2010) but this does not hold true for components of the sting alarm pheromone (Wang et al. 2008). Thus our study aimed at identifying which of these rules prevails when it comes to mixtures of pheromones and general odours. In particular since the appetitive value of the floral odours blocking the response to IAA seems to be innate, we wondered if the processing of mixtures containing these specific compounds would follow pheromonal rules (non-linearity) rather than general ones (linearity), thus hinting at the mechanism underlying their effect on aggression.

To answer this question, we recorded the glomerular pattern of activity elicited by the presentation of the floral odours blocking aggression, linalool (Lol) and 2-phenylethanol (PhE), the alarm pheromone component IAA and their mixtures (IAA+Lol and IAA+PhE). To allow for comparisons we also did the same recordings using floral odours with no effect on aggression, limonene (Lim) and linalyl acetate (LiA). Finally, we used Ger as a pheromonal control to check if the possible interactions would be specific to the alarm pheromone or not. We applied two approaches. Using two different staining methods, we recorded activity patterns from both the AL input layer (ORNs) and output layer (projection neurons, PNs) to explore whether and where the appetitive value of Lol and PhE influences processing of pheromonal compounds. The activity patterns obtained with the odour mixtures in these experiments suggested an absence of neural interactions between the appetitive odours and the alarm pheromone in the antennal lobe, thus indicating that the behavioural modulation exerted by the former on the latter may take place in higher brain centers.

## Materials and Methods

### *Honeybees*

Experiments were performed in winter; 15 bees were collected every Monday by opening the top of a hive placed in a warmed room. They were then kept in a small cage, with *ad libitum* honey and water, until being used in the experiment. To

prepare the bees for calcium-imaging experiments, they were first shortly cold-anaesthetized and fixed into a small plastic holder with low-melting dental wax (Siladent®). A small plastic piece sealed with a bi-compound epoxy glue (SADER®) separated the antennas from the top of the head. The head capsule was then opened, and the salivary glands and trachea sacks were removed to reveal the two antennal lobes. The brain was immersed in bee Ringer during the dissection and subsequent recordings (in mM as follows: 130 NaCl, 6 KCl, 4 MgCl2, 5 CaCl2, 160 sucrose, 25 glucose, 10 HEPES, pH 6.7, 500mOsmol).

***Staining with Calcium-sensitive dyes***

For the first experiment, 50 µg of Ca-Green 2-AM were dissolved in 50 µl of Pluronic-127 (20% in DMSO, Molecular Probes, Eugene) and then diluted in 800µl of bee Ringer. Each brain was incubated with 15 µl of this solution for at least 1 h. Ca-Green 2-AM potentially stains all neuronal populations of the AL. For the second experiment, Fura-2 dextran (potassium salt, 10,000 kDa, in 2% BSA; Invitrogen) was injected into the lateral tract (l-ALT) using a glass electrode coated with crystals of the dye. This staining method allows recording of PNs activity exclusively. The bee was then left in a dark, moist container for at least 3h, so that the dye could migrate back to the antennal lobes along the PNs axons.

***Olfactory stimulations***

We used the plant odours linalyl acetate (LiA), limonene (Lim), linalool (Lol) and 2-phenylethanol (PhE). Lol and PhE are the effective compounds preventing aggression in honeybees, while LiA and Lim have no effect on aggression and were thus used as controls. These compounds were diluted to either 0.075% or 10% (vol/vol) in triethyl citrate (TEC). The higher concentration was added to better visualize the activity patterns elicited by the plant odours, which were very weak when presented at 0.075% (the concentration that was previously used when testing the effect of these odours on aggression). We also used two pheromonal compounds: isoamyl acetate (IAA), the main component of the sting alarm pheromone, and geraniol (Ger) as a control, an attractant molecule produced by the Nasanov gland. Both were diluted to 10% in TEC. All pure chemicals were ordered from Sigma-Aldrich (France).

When placed under the imaging set-up, the bees received a constant clean air flow. The olfactory stimulation consisted in switching the path of this air flow for 1 s, so that it went through a pipette containing two filter papers. These papers carried 5 µl of each odorant's solution in case of a mixture presentation, or of the odorant and of the solvent TEC in case of the presentation of a single odorant. The full set of stimuli was presented to the bee 1, 2 or 3 times depending on the survival time of the preparation, and the order of presentation of each stimulus within a set was randomized.

***In vivo calcium-imaging***

For recordings, a T.I.L.L. Photonics imaging system was coupled to an epifluorescent microscope (Olympus BX-51WI, Olympus) equipped with a 10x(NA 0.3) water immersion objective. Signals were recorded using a 1004x1002 pixel 14-bit monochrome CCD camera (Andor iXON, cooled to -70°C). The microscope was equipped with a GFP-BP filter set composed of a 490 nm dichroic beamsplitter and a 525/550 nm emission filter. Ca-Green was excited with 470 nm light, while Fura-2 was alternatively excited with 340 and 380 nm lights using a monochromators (T.I.L.L. Polychrom V). Each measurement consisted of 100 frames (or double frames), at a rate of 5 Hz (interval between frames: 200 ms), with 4x4 binning on chip. The olfactory stimulation was on from frame 15 to 20 (for 1 s).

***Data processing and activity maps***

Fura-2 is a ratiometric dye, hence the fluorescence ratio between the two excitation wavelengths (340/380 nm) was calculated before any other processing. To reduce photon noise, the raw data was filtered in the three dimensions (2 spatial and 1 temporal) using a median filter with a size of 3 pixels. Second, a bleach correction was applied by subtracting a logarithmic curve fitted to the median brightness decay of the entire image frames, excluding the frames during the stimulus until 5 sec after stimulus onset. Changes in fluorescence ($\Delta F/F$) were calculated with respect to a reference frame just before stimulus onset (frame 10). Activity maps were obtained by subtracting the mean signal of three frames during the inhibitory phase (59-61) to the mean signal of three frames around the response's peak (21-23). To facilitate

visualization of the pattern of glomerular activation, these maps were further filtered with a Gaussian filter (9x9) and presented in a false colour code.

***Analysis***

Clustering analysis was performed separately for each individual bee, using the pixels of the final activity maps as variables. After testing a range of values for k (3 to 5), we determined that the k-means algorithm worked best on our datasets with k=4. Furthermore, five replicates of the clustering analysis were performed on each bee to avoid potential local minima due to the randomly selected starting points of the algorithm. By pooling the results from all bees, we could then measure how often two odours were associated. To compare these frequencies we used Chi-square tests ($X^2$).

Pixel-wise Euclidean distances between two odour maps (*i* and *j*) were calculated as follow, with *p* being the total number of pixels and *X* the intensity (ΔF/F) of each pixel:

$$d_{i,j} = \sqrt{\sum_{k=1}^{p} \left(X_{ik} - X_{jk}\right)^2}$$

The relative distance between one component i and the mixture i+j was then calculated as:

$$D_{i,i+j} = \frac{d_{i,i+j}}{d_{i,i+j} + d_{j,i+j}}$$

Finally, the weight of an odorant was defined as the Euclidean distance between its activity map and the one obtained for the solvent control TEC. Since TEC has no odour, its activity map represents background noise. Thus, the weight of an odour as we just defined it is a measure of the overall intensity of activation triggered by this odour in the AL: the larger the distance between an odour and the TEC representation, the more significant the weight of the odour. As a consequence the relative weight of a component i in the mixture i+j was obtained from the following equation:

$$W_{i,i+j} = \frac{d_{i,TEC}}{d_{i,TEC} + d_{j,TEC}}$$

All linear correlations were tested for significance with Pearson's r test. Furthermore, analyses of co-variance (ANOCOVA) were performed to compare the different regressions obtained.

## Results

Two different experiments were conducted to explore whether the appetitive value of Lol and PhE influences how pheromonal compounds presented simultaneously are processed, and at which level of the computation performed by the AL they would do so. The first experiment used a staining method that acts on all neuronal populations of the AL; however as ORN afferences constitute the largest population, this method reveals mostly ORN activity, the input of the AL (Galizia and Vetter 2005). In the second experiment we retrogradely stained projection neurons (PNs) so that we selectively quantified AL output.

### *Odour representations in ORNs*

The results from Ca-Green 2-AM stainings are shown in Figure 2, presenting the AL activity maps obtained for an individual bee during the presentation of all odours, alone or combined. TEC being the odourless solvent, maps of an odour "+TEC" represent this odour alone. The four floral compounds elicited clear patterns of activity when they were presented at 10% concentration. However, when presented at 0.075% (the concentration used in the previous aggression study), the signals were very weak. The patterns elicited by the pheromonal compounds Ger and IAA were highly consistent across bees, and clearly predominant in mixtures. This is in line with the behavioural results of the previous study (Nouvian et al. 2015), in which the bees responded more to the mixture after training with IAA than after training with the plant odour in a learning paradigm. Thus, at the concentrations used in both studies, the pheromonal compound seems to overshadow the floral one rather than the other way around. For all odours, the activity maps we observed were consistent with the recordings reported for the same odorants in previous studies using this staining method (Deisig et al. 2006; Wang et al. 2008; Carcaud et al. 2015).

Nonetheless, slight variations in the neural representation of the pheromone might still hinder its identification by the bees. To investigate if such a phenomenon occurs, we performed a clustering analysis by the k-means algorithm (with k=4). Because we did not use defined glomeruli but used all the pixels of the activity maps as variables, we performed this analysis separately for each individual bee (Figure 3a). We then evaluated the strength of the association between each pair of

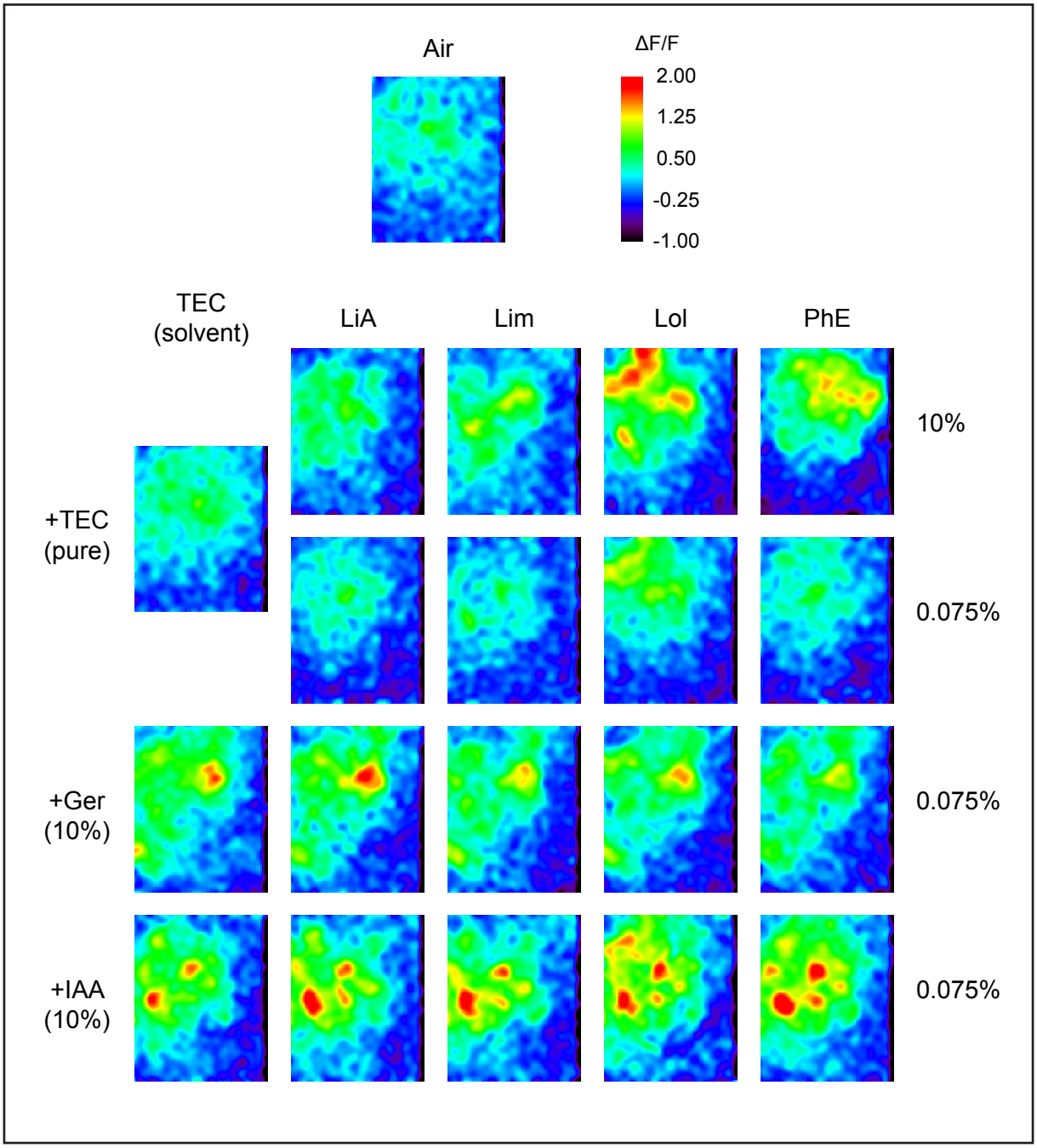



**Figure 2: Spatial patterns of odour-induced activity in ORNs in the left antennal lobe of a single bee.** Each map was obtained by averaging the results of two stimulus presentations. The odours carried by each of the 2 filter papers during stimulation are indicated at the top and on the left of the panels, and the concentration of the floral compounds is indicated on the right. The floral compounds elicit specific patterns which are clearly visible at 10% concentration, but weak at 0.075%. The representation of the two pheromonal compounds (Ger and IAA) is clearly predominant in the mixtures. In all figures TEC: triethyl citrate (solvent control), LiA: linalyl acetate, Lim: limonene, Lol: linalol, PhE: 2-phenylethanol, Ger: geraniol (aggregation pheromone), IAA: isoamyl acetate (alarm pheromone).

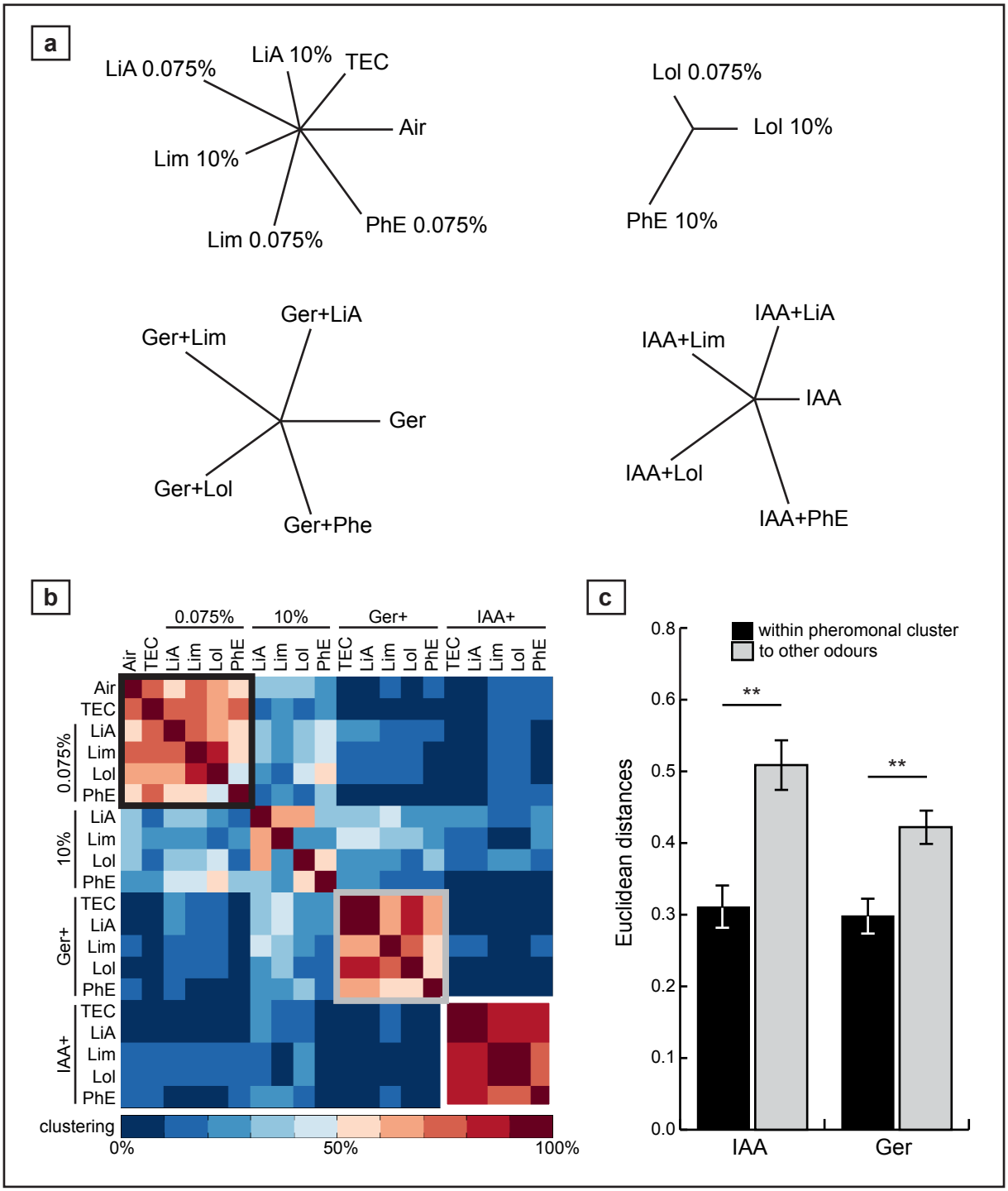
odorants by determining how often they were classified in the same cluster across all bees (Figure 3b). This analysis revealed three over-arching clusters in our dataset. A first cluster (outlined in black in Figure 3b) regrouped all maps with no or weak signals: the controls (Air and TEC) and the floral odours presented at low concentration (0.075%). The second cluster (outlined in grey) consisted of all the stimuli containing Ger. Finally, the third and strongest cluster (outlined in white) grouped all the stimuli containing IAA. The presence of these two pheromonal clusters is also evidenced by the fact that the Euclidean distances between the stimulus maps within these clusters are significantly smaller than between stimuli belonging to different clusters (related samples Wilcoxon signed rank test, p=0.08 for both IAA and Ger). This result confirms that the floral compounds do not strongly affect the neural representation of the pheromones at these concentrations.

Furthermore, the mixtures containing the floral odours blocking the aggressive response to IAA (IAA+Lol and IAA+PhE) fell within the same cluster as IAA+TEC as often as those containing the other floral compounds (IAA+LiA and IAA+Lim; $X^2$, p=0.670). Also, the Euclidean distance between IAA+TEC and IAA mixed with a floral compound did not vary significantly depending on the floral odour (data not shown, Friedman test, p=0.706). Thus, the representation of IAA at the periphery (input to the AL) is not more affected by the presence of Lol or PhE than it is by LiA or Lim.

***Linear processing of mixtures in ORNs***

Previous studies have shown that the representation of mixtures of general odours recorded at the input level of the AL can be computed linearly from the representation of its components (Deisig et al. 2006), while mixtures of pheromonal compounds create unique patterns that cannot be linearly predicted (Wang et al. 2008). Which one of these rules holds true when we present the bee with mixtures containing both a pheromone and a general odour?

To answer this question, we followed the same methodology as in those two studies. Namely, we first calculated the relative distance between each component and the mixture (Figure 4a). The weight of each component was originally defined as the number of glomeruli activated by this component (Deisig et al. 2006). We defined it as the Euclidean distance between the activity map of a compound and the map obtained for the solvent control TEC. The map for TEC being background noise, this


a
LiA 0.075%
LiA 10%
TEC
Air
Lim 10%
PhE 0.075%
Lim 0.075%
Lol 0.075%
Lol 10%
PhE 10%
Ger+LiA
Ger+Lim
Ger
Ger+Lol
Ger+Phe
IAA+LiA
IAA+Lim
IAA
IAA+Lol
IAA+PhE
b
0.075%
10%
Ger+
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Air
TEC
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Lim
Lol
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clustering
0%
50%
100%
c
within pheromonal cluster
to other odours
Euclidean distances
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.8
**
**
IAA
Ger

measure is a good proxy of the overall intensity of activation elicited by each compound. As a result we could also calculate the relative weight of each component in a mixture (Figure 4b). A correlation analysis revealed a clear linear relationship between the relative distance of a component to the mixture and its relative weight (Figure 4c, Pearson's test, $r = -0.989$, $p<0.001$). Thus, the processing of mixtures containing a pheromone and a general odour follows linear rules like the one observed for mixtures of general odours at the level of the ORNs. This was true for mixtures containing the floral odours with no effect on IAA (Lia and Lim), but also for those that blocked the aggressive response to IAA (Lol and PhE). Indeed, all mixtures followed virtually the same linear relationship (Figure 4d, ANOCOVA, df=1, $F=0.34$, $p=0.563$). Therefore the appetitive value of these floral compounds did not seem to modify how they are processed at the level of ORNs.

***Odour representations in PNs***

In the second experiment, PNs were stained retrogradely by injection of Fura-2 Dextran in the l-ALT tract so that we recorded exclusively output signals from the AL. Figure 5 presents the signals typically recorded for a single bee. In PNs, some weak activity was sometimes observed in 1 or 2 glomeruli during control stimulations (Air and TEC). Like in recordings emphasizing ORN signalling, the floral odours elicited

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**Figure 3: The pheromonal representation is not strongly affected by the presence of floral odours.** For simplicity, "+TEC" is not specified throughout the figure (thus "Odour X+TEC" is noted "Odour X").
a. Cluster analysis using k-means with k=4 in the same individual as in Figure 2. The algorithm identifies two clusters containing all plant odours and controls and two "pheromonal" clusters. The euclidean distance between each stimulus and the cluster centroid is the only distance represented accurately.
b. Frequency at which each pair of stimuli clusters together across all bees (n=9): 0% indicates odours that never fall within the same cluster, while 100% indicates odours that are associated in all bees. All stimuli containing IAA are strongly associated between them (clustering >75%, white square) and separated from the other stimuli (<25%). The same is true with Ger, although less strongly (grey square). The representation of floral odours at low concentration tends to cluster with the Air and TEC controls (black square), likely because of the weakness of the calcium signals. At high concentration they do not cluster consistently with any other stimuli, thus confirming the specificity of each activity pattern.
c. The similarity between all stimuli containing one of the pheromone is also evidenced by smaller pixelwise euclidean distances between the activity maps of two stimuli within the cluster than between a stimulus in the cluster and a stimulus outside of it. Related samples Wilcoxon signed rank test, **: $p<0.01$.

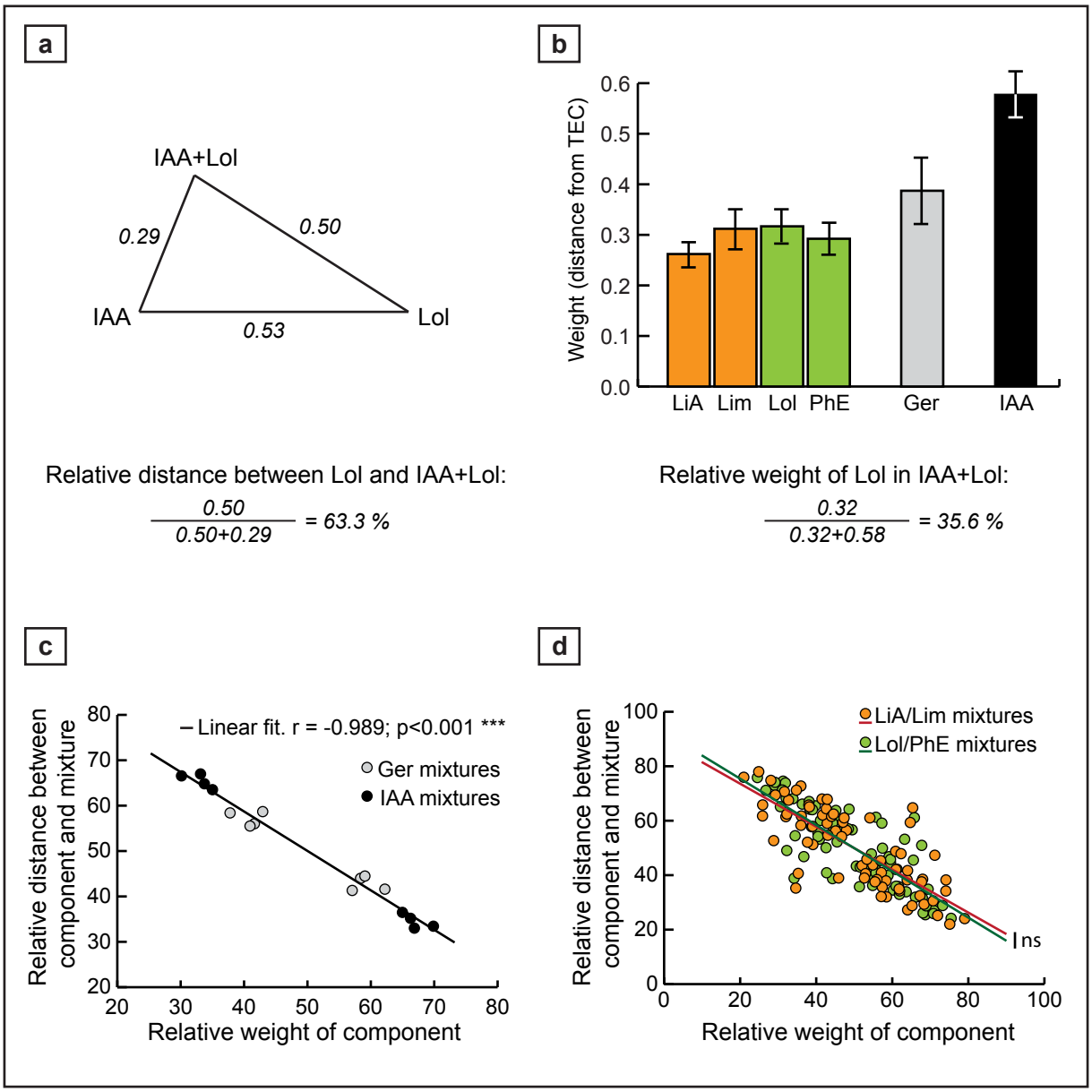


**Figure 4: All pheromone+floral odour mixtures are processed following the rule for general odours in ORNs.**

**a.** Example of the calculation of the relative distance between a component and a mixture (following the methodology defined in Deisig et al, 2006).

**b.** The weight of each component is a measure of the intensity of the activity it elicits. We used the pixelwise euclidean distance between the activity map of the solvent TEC (which corresponds to background noise) and the activity map elicited by each odour as a measure of this intensity. The relative weight of each component within the mixture is then defined as in Deisig et al, 2006.

**c.** There is a clear linear relationship between the relative weight of each component and the relative distance of its representation to the mixture, irrespective of the identity of the pheromone (data averaged across all bees, n=9). Pearson's test, ***: p<0.001.

**d.** Mixtures containing the appetitive floral odours blocking the aggressive response to IAA (Lol or PhE) follow virtually the same rule as mixtures containing the other floral odours (LiA or Lim). ANOCOVA, ns: p=0.563 on the interaction term. All individual data points are shown to better visualize possible differences in the dispersion of the data.

specific patterns of activity, which were clearly visible at high concentration (10%) but weak at low concentration (0.075%). Strikingly, the presentation of IAA triggered strong responses while that of Ger only elicited weak activity in a few glomeruli. These patterns of activation are consistent with those reported by another study (Carcaud et al. 2015). The pheromonal patterns were still visually predominant in mixtures, as they were in Ca-green recordings staining ORNs.

We performed the same clustering analysis as before (k-means with k=4 on each individual bee, then measure of the frequency at which two stimuli clustered together across all six bees). The results (Figure 6a) were markedly different from those obtained with the ORNs dataset (Figure 3c). Indeed, at the level of PNs only the stimuli containing IAA formed a well-defined cluster (outlined in white). All the other stimuli seemed to be randomly associated (black square). In particular the stimuli containing Ger did not group together anymore, likely because of the weak activation elicited by this pheromone in the population of PNs recorded. Nonetheless, the similarity of mixtures containing the same pheromone could still be detected when looking at Euclidean distances (Figure 6b, related samples Wilcoxon signed rank test, p=0.028 for IAA and p=0.046 for Ger). Thus, at least the representation of IAA, the pheromonal compound of interest in this study, was not strongly affected by the simultaneous presentation of floral odours.

In addition, the properties of the floral odour did not affect how often a mixture clustered with its pheromonal control. Indeed the mixtures IAA+LoI and IAA+PhE belonged to the same cluster as IAA+TEC as often as the mixtures IAA+LiA and IAA+Lim, despite the blocking effect of LoI and PhE during aggression assays ($X^2$, p=0.564). Thus, this clustering did not reflect the particular effect of these two odours on aggressive behaviour. The same was true when looking at Euclidean distances between IAA+TEC and IAA mixed with a floral compound: no significant variation of this measure depending on the identity of the floral odour could be detected (data not shown, Friedman test, p=0.122).

### *Linear processing of mixtures in PNs*

We found that the representation of mixtures containing both a pheromonal and a general compound follow linear rules in recordings emphasizing ORN signals (see above). Processing within the AL could change the way in which mixtures are represented in PNs (AL output level), due to inhibitory lateral connections between

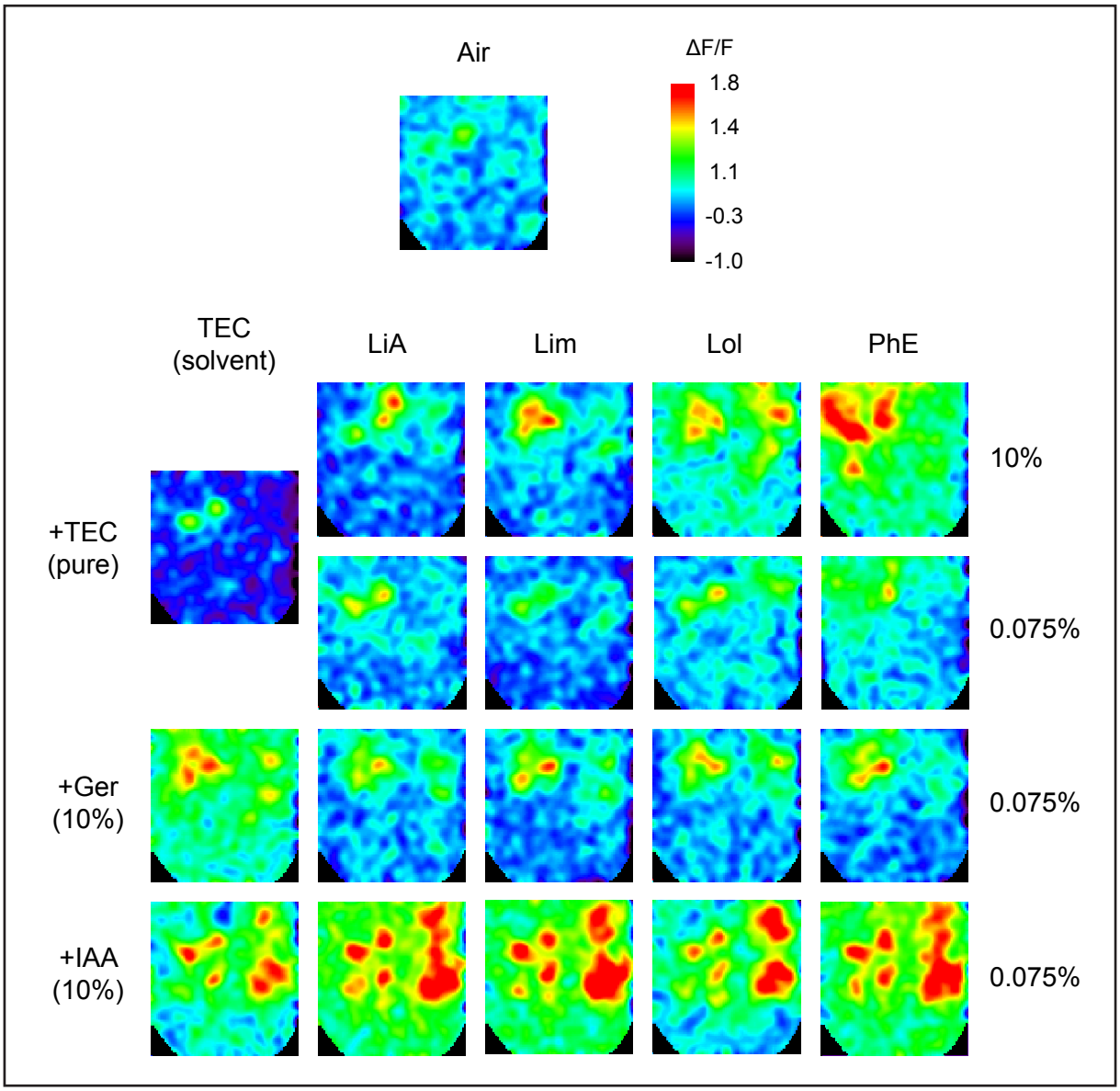


**Figure 5: Spatial patterns of odour-induced activity in PNs in the right antennal lobe of a single bee.** Each map was obtained by averaging the results of three stimulus presentations. The odours carried by each of the 2 filter papers during stimulation are indicated at the top and on the left of the panels, and the concentration of the floral compounds is indicated on the right. In PNs, some weak activity was sometimes observed in one or two glomeruli during control stimulations (Air and TEC). Like in ORNs, the patterns elicited by floral compounds are visible at 10% concentration but weak at 0.075%. The pheromonal compound Ger elicit weak PNs activation in this part of the antennal lobe, while IAA induces a very strong response.

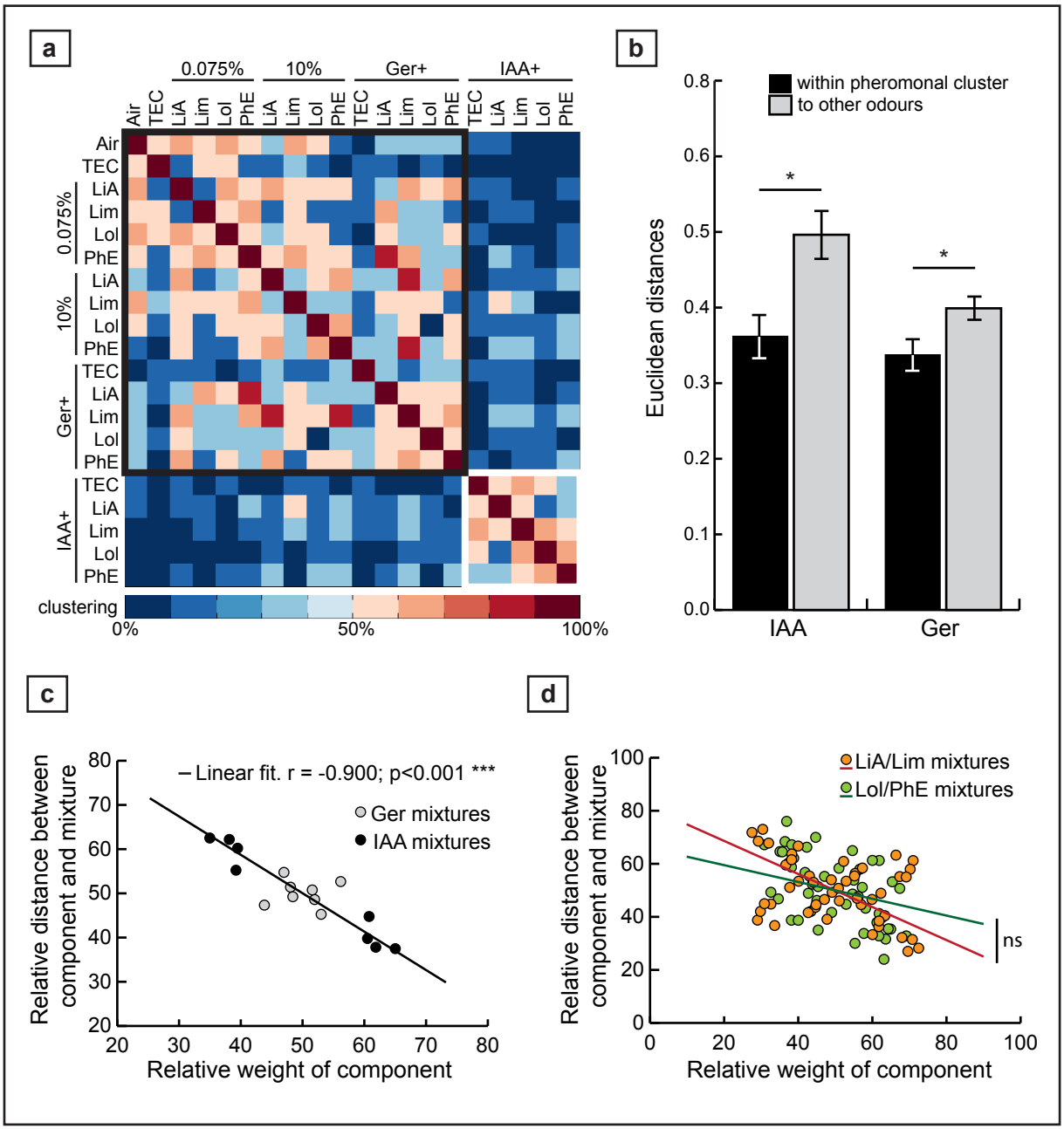


**Figure 6: Processing in the PNs.**

**a.** Clustering analysis in projection neurons (n=6 bees): 0% indicates odours that never fall within the same cluster, while 100% indicates odours that are associated in all bees. Clustering in PNs is not as strong as in ORNs, and mainly segregates stimuli containing IAA (white square) from all the others (black square).

**b.** Euclidean distances are still smaller between two stimuli containing the same pheromone than from one containing a pheromone to one not containing the same pheromone. Related samples Wilcoxon signed rank test, *: $p<0.05$.

**c.** There is a clear linear relationship between the relative weight of each component and the relative distance of its representation to the mixture (data averaged across all bees, n=6). Pearson's test, ***: $p<0.001$.

**d.** The relationship between relative distance and relative weight is similar in mixtures containing the appetitive floral odours blocking the aggressive response to IAA (Lol or PhE) and in mixtures containing the other floral compounds (LiA or Lim). ANOCOVA, ns: $p=0.094$ on the interaction term. All individual data points are shown to better visualize possible differences in the dispersion of the data.

glomeruli. Thus, we performed the same correlation analysis done before (see above) also on the PNs dataset. The relationship between the relative weight of a component and the relative distance of its representation to the mixture still followed linear rules (Figure 6c, Pearson's test, $r = -0.900$, $p<0.001$): namely the more activity an odour evoked in the AL, the more its pattern was conserved in the mixture representation. Furthermore, the coefficients of this regression were not different from those of the ORNs dataset (ANOCOVA, df=1, F=0.27, p=0.608). Finally the mixtures containing the floral compounds blocking aggression (Lol and PhE) followed a similar rule to those with no effect on aggression (LiA and Lim, ANOCOVA, df=1, F=2.86, p=0.094). Thus, we conclude that at the output of the AL, there is no specific information reflecting the blocking of the aggressive response triggered by IAA by the appetitive odours Lol and PhE.

## Discussion

In a previous study (Nouvian et al. 2015), we identified two floral compounds, Lol and PhE, that blocked the aggressive response triggered by the alarm pheromone in honeybees. Using a behavioural paradigm, we showed that these compounds do not mask the alarm pheromone. Rather, their effect on aggression strongly correlated with the frequency of spontaneous appetitive responses they triggered. Here, our aim was to search for neural correlates of this odour interaction in the primary olfactory center of the honeybee brain, the AL. We recorded the activity of neurons within this region using *in vivo* calcium imaging, first at the input level using a staining method that emphasizes ORN responses, and then at the output level using a different method that yields responses of PNs exclusively. By comparing input and output of the AL we aimed at determining whether and how potential reshaping of odour information occurring within this structure reflects the modulatory effect of appetitive floral odours on alarm pheromone components. We hypothesized, that if such effects were visible at the AL level, mixtures of IAA, the main alarm pheromone component, and the two appetitive floral odours could induce particular neural signatures in which IAA signals would be somehow modified reflecting our behavioural observations (Nouvian et al. 2015).

However, using the experimental approaches described above, we did not detect any specificity in the processing of the appetitive floral odours that could explain their effect on aggression. The neural representation of the alarm pheromone was not more affected by the presence of these odours than by control odours with no calming effect. The appetitive value of LoI and PhE may be innate as these odours elicit spontaneous extension of the proboscis even in naïve bees with no olfactory experience during adulthood (Nouvian et al. 2015). Thus, we postulated that these odours might be more related to pheromones than to general odours for honeybees. Consequently we expected that the processing of mixtures containing these compounds might produce configural rather than elemental patterns. This hypothesis was not supported by our results: we found that mixtures containing these odours and a pheromonal compound are processed linearly in the antennal lobes, just like general odours (Deisig et al. 2006). Configural processing in the AL was observed for components of the sting alarm pheromone, and interpreted as a way of creating a unique “signature” for this pheromone (Wang et al. 2008). An interesting experiment testing the phenomenon of configural pheromonal processing, which to our knowledge has never been performed, would be to stimulate the bees with a mixture of the two pheromonal compounds IAA and Ger. This would verify if configural processing also happens when the components belong to ecologically distinct pheromonal blends.

When interpreting the results from the current experiments we need to take into account certain technical limitations. The most striking difference between our analysis and those performed in previous studies is that we did not try to identify individual glomeruli activated by each odour. Instead, we chose to use all the pixels of the activity maps as dimensions in a putative olfactory space. Using this analysis we were able to reproduce the results obtained by the previous studies on the processing of mixtures in the honeybee AL (Deisig et al. 2006; Deisig et al. 2010), hence we are confident in the validity of this approach. Nonetheless, defining individual glomeruli could facilitate performing a more in-depth analysis of our data, as it would reduce dimensionality and allow more direct comparisons of all the bees. At the glomerular level, three types of interactions have been recorded: suppression happens when the response to the mixture is lower than that elicited by the most effective component, hypoadditivity is defined by a response to the mixture similar to that of the most effective component, and synergy when the mixture elicit higher

responses than any of the component (Duchamp-Viret et al. 2003). Quantifying the response of each glomerulus rather than considering the pattern as a whole could potentially allow detecting more subtle changes induced by the presence of the appetitive floral compounds. For example, they may cause suppression in a single, key glomerulus for the signalling of the alarm pheromone while leaving the rest of the pattern intact. An analysis at the glomerular level would also allow a closer look at the temporal activity pattern. Clearly, there are a range of additional analyses one could conduct, but given the complexity of these, they will be the focus of future investigations.

An important theoretical result from our experiments is that we observed linear processing of mixtures both at the input and output of the AL. The conservation of this form of processing from ORNs to PNs is somewhat surprising considering the complex architecture of the antennal lobe (Lei and Vickers 2008), and the fact that intracellular recordings of local interneurons and PNs revealed rather unpredictable variations between responses to mixtures and to their components at this level (Sun et al. 1993). While this property of the AL network was already demonstrated in another study (Deisig et al. 2010), the authors also found that processing of a mixture's components is more homogeneous in the PNs forming the l-ALT tract than in ORNs. This supports the hypothesis that processing in these PNs might be concentration invariant to a certain extent, accentuating the information about the identity of the components rather than about their relative contribution to the mixture (Deisig et al. 2010). In our datasets we did not detect any difference between the regression coefficients of ORNs and PNs. This may be because our experiments were designed to follow-up on the aggression study, hence we only used one set of concentrations and the emphasis was always on the pheromonal compound, which was presented at higher concentration (10%) than the floral compound (0.075%). However in many situations, this ratio may be reversed. Would the relationship between odorants still follow the same parameters in such a mixture? One could imagine that the mechanism identified by the previous study could be critical to enhance the social signal, potentially important for the animal, over the contextual background.

The objective of this study was to identify potential neural correlates of the effect of floral odours on the response to IAA in the honeybee AL. While we did not find any interaction within the AL accounting for this effect, we cannot exclude that

these correlates may exist in forms that we have not been able to detect with current approaches and analyses. Another intriguing explanation would be that this olfactory modulation of aggression takes place in higher brain centers. PNs carry the olfactory information to two main structures in the bee brain: the lateral horn (LH) and the mushroom bodies. In the LH of fruitflies and bees, odours are still represented by a spatial pattern of activation, albeit one more distributed than in the AL (Parnas et al. 2013; Roussel et al. 2014). Furthermore, in *Drosophila* the connectivity between the AL and the LH is highly stereotyped, i.e. PNs from specific combinations of glomeruli project to the same area of the LH in all animals (Fisek and Wilson 2014). This is in sharp contrast with the apparently random organization of the mushroom body input (Caron et al. 2013) and supports the idea that the LH, described as a pre-motor center, is responsible for fast, innate responses to odours, while the mushroom bodies achieve odour identification (classification) and learning (Parnas et al. 2013; Galizia 2014). In *Drosophila*, this hypothesis is further supported by a clear segregation between the inputs corresponding to general odours and those of pheromones in the LH (Jefferis et al. 2007; Liang et al. 2013). In honeybees, such strong segregation was not observed. Nonetheless the representations of pheromonal compounds of different meanings were more clearly separated in the LH than in the AL, thus the honeybee LH also performs some classification of odorants according to their biological relevance (Roussel et al. 2014). This wealth of studies strongly suggests that the LH would be an excellent candidate region for future investigations into the neural mechanisms and correlates that underlie the modulatory effect of appetitive floral odours on aggression.

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# Chapter 5.

# The role of biogenic amines in honeybee aggression

## Abstract

Biogenic amines are important neuromodulators and have been implicated in the regulation of the aggressive behaviour of a number of species, both vertebrate and invertebrate. However, their potential role in regulating the honeybee's stinging behaviour had not been investigated so far. Honeybees band together in a collective stinging response to protect their nest against large predators, through the use of an alarm pheromone. To investigate the role of biogenic amines in this defensive response, we measured the total amount of octopamine, dopamine and serotonin in the brain of bees: 1) from colonies of known aggressiveness (colony-level) and 2) individually tested for their aggressive response (individual-level). We found that bees from aggressive colonies have higher serotonin levels in their central brain. The central brain of bees exposed to the alarm pheromone during the aggression test also contained more dopamine and serotonin than control bees, irrespective of the bee's behaviour. Serotonin levels were also higher in the optic lobes of aggressive bees, and in the sub-esophageal zone of bees responding to the alarm pheromone. To confirm the role of serotonin and dopamine in honeybee aggression, we pharmacologically manipulated their levels. We found that agonistic treatments induced higher aggressiveness in bees, while antagonists decreased aggressiveness. Our results evidence the key role of serotonin and dopamine in regulating honeybee aggression, and we suggest that they do so by setting the threshold of responsiveness to relevant stimuli.

## Introduction

Biogenic amines are small, ubiquitous molecules synthetized by the nervous system of vertebrates and invertebrates. Among the most important biogenic amines found in invertebrate brains are octopamine (OA, analogous to the vertebrate norepinephrine) dopamine (DA), and serotonin (5HT). Their functions are extremely diverse, ranging from classical neurotransmitters to neuromodulators and neurohormones circulating both at the periphery and at the central level (Libersat and Pflueger 2004). They participate in the regulation of a large number of sensory perceptions (Linn and Roelofs 1986; Grosmaitre et al. 2001; Farooqui 2007; Tedjakumala et al. 2014), underlie different behavioural and motivational states (e.g. arousal (Andretic et al. 2005)) and coordinate complex behavioural responses (Barron et al. 2002; Beggs et al. 2007). In particular, these molecules modulate aggression in a number of species. In the fruit fly, populations of octopaminergic (Hoyer et al. 2008; Zhou et al. 2008), dopaminergic (Alekseyenko et al. 2013) and serotoninergic (Dierick and Greenspan 2007; Johnson et al. 2009; Alekseyenko et al. 2010) neurons are implicated in the control of male fighting behaviour. Similar trends, yet without the cell resolution enabled by *Drosophila* neurogenetics, have been obtained from the study of crustaceans (Livingstone et al. 1980; Edwards and Kravitz 1997; Kravitz 2000; Pedetta et al. 2010), spiders (Jones et al. 2011), crickets (Adamo et al. 1995; Dyakonova et al. 1999; Rillich and Stevenson 2011; Rillich and Stevenson 2014), bumble bees (Bloch et al. 2000), ants (Szczuka et al. 2013) and termites (Ishikawa et al. 2016). The precise role of each amine seems to vary from species to species, probably because the particular form of aggression studied differs across species: for example in some species, it serves to establish dominance or social status, while in others it is a defensive response against members of another species. Nonetheless, 5HT is a common denominator in the majority of these studies where it has been positively linked to aggression.

In honeybees, OA tends to increase arousal and sensory perceptions, acting as a facilitator of different behaviours (Orchard 1982; Barron et al. 2002; Farooqui 2007; Farooqui 2012), in particular appetitive ones. DA and 5HT have overall opposite effects (Mercer and Menzel 1982; Erber et al. 1993; Rein et al. 2013), with DA acting as a general depressor of different behaviours and 5HT having a less

clear role (Mercer and Menzel 1982; Erber et al. 1993; Perk and Mercer 2006). As a consequence, through the modification of the response threshold to a number of stimuli, OA has been found to play an important role in the division of labour, which is a hallmark of honeybee societies (Schulz and Robinson 1999; Wagener-Hulme et al. 1999; Barron et al. 2002). The role of biogenic amines in honeybee aggression has not been investigated so far, but some data are available on stress responses, defined either as a physical stressor (leg clamping for 10 min or more), anaesthesia or spinning of the bees. Stress seems to modify the level of some biogenic amines in the bee brain, but the results are not very consistent across studies: Harris and Woodring found that stress increases OA and 5HT levels (Harris and Woodring 1992), while Chen and collaborators found a decrease in OA and DA brain levels (Chen et al. 2008). These discrepancies may be due to the very different types of stressors that these studies used. Moreover, the responsiveness to electric shocks in the form of a series of increasing voltages depends on 5HT and DA signalling (Tedjakumala et al. 2014).

Here, we studied the role of biogenic amines in honeybee aggression using a novel laboratory assay to quantify the bees' response to the perceived threat of a rotating dummy (Nouvian et al. 2015). We focused on OA, DA and 5HT and first measured the levels of these amines in the brain of bees using two approaches: in a first experiment, biogenic-amine contents were measured in bees caught at the entrance of colonies with known different overall defensive responses (colony-level comparisons); in a second experiment, the bees were first tested for their stinging response and then flash-frozen in liquid nitrogen at the end of the behavioural assay so that biogenic-amine levels could be linked to their individual behaviour (individual-level comparisons). In these two sets of experiments, 5HT levels were consistently higher in bees that were either exposed to the main component of the sting alarm pheromone (isoamyl acetate, IAA), that were aggressive, or both. DA levels were also higher in the central brain of bees exposed to IAA. To test if 5HT and DA mediate aggression, we manipulated these amines' levels by treating bees with a topical application of these molecules and then quantified their response to the rotating dummy. Agonist-treated bees stung the dummy significantly more often, while antagonist-treated bees exhibited reduced aggressiveness. This confirms the role of these amines in honeybee aggression, and we suggest that they are setting the threshold for aggressiveness. Our results are the first to confirm the long-

standing hypothesis that biogenic amines are important modulators of honeybee aggression.

## Materials and Methods

### *Honeybees*

Four hives housing unrelated Italian honeybee colonies (*Apis mellifera ligustica*) were used in the experiments investigating brain biogenic amine levels (Figure 1). These colonies were located at the Queensland Brain Institute, Brisbane, Australia. Honeybees involved in colony defense were selectively collected (using a black feather waived at the hive entrance) and tested in the behavioural assay for their aggressiveness as described below. In addition, some of the bees collected from the four colonies were snap-frozen without behavioural testing for the colony-level assessment. As a result, a total of 348 bees were collected for brain dissection.

The pharmacological experiments were performed at the Université Paul Sabatier, Toulouse, France. The honeybees were collected at the hive entrance with a black feather as before. To ensure that our observations were not specific to a single genotype, two to three unrelated colonies participated equally in each experiment, with a total of five colonies used. The colonies participating in the antagonistic treatments were chosen for being quite aggressive while those participating in the agonistic treatments were known to be gentler. This was done to increase our chances of measuring the expected effect of the amines and their antagonists.

### *Aggression assay*

The behavioural assay for testing honeybee aggression has been described in detail previously (Nouvian et al. 2015). In this assay, pairs of honeybees are confronted with a rotating dummy in a cylindrical arena, which they can choose to sting or not. The frequency at which at least one of the bees stung the dummy is recorded. Each pair of bees was exposed to either triethyl citrate (TEC, solvent) or IAA (10% vol/vol), carried through an air flow, during the whole length of the trial (3 min). Importantly, if a bee exhibited locomotor defects (clumsy walk or inability to hold upside down), the

whole pair was excluded from further analysis. The pharmalogical treatments did not affect the number of bees being excluded ($X^2$, p=0.787).

For the HPLC measurements, honeybees were classified into two groups according to their reaction: “aggressive” bees that engaged in defensive behaviour and stung the dummy, while “non-aggressive” bees did not. If the two bees displayed aggressive behaviour, only the first bee to act was used for further analysis.

***Brain collection and dissection***

All honeybees participating in the experiments were either caught directly at the hive entrance (colony-level experiment) or quickly re-caught in a 50 mL Falcon tube after the aggression test (individual-level experiment, see Figure 1), and snap-frozen in liquid nitrogen. The Falcon tube was drilled with four holes (3-4 mm) at its base and two holes (1-2 mm) on its lid to ensure the quick flow of liquid nitrogen inside. The honeybees were then put on dry ice until the end of this day’s trials and then stored at -80°C. The delay between the end of the trial and the bees being put on dry ice was 54.3 s on average thus ensuring only minimal changes in biogenic amine levels could occur after the end of the behavioural trial. The brains were then partially freeze-dried (55 min, 600 mTorr, -40°C) and dissected on dry ice. Intact brains were separated into 3 regions: central brain (CB), optic lobes (OL) and sub-esophageal zone (SEZ). Incomplete or thawed brains were discarded.

***Biogenic amines quantification***

Biogenic amine levels were measured using High Performance Liquid Chromatography (HPLC) at Macquarie University, Sydney, Australia. The HPLC system was an Agilent 1200 series (Agilent Technologies, Santa Clare, CA, USA) coupled to an electrochemical detector (ESA coulechem III) connected to a dual electrode analytical cell (ESA, Chelmsford, MA, USA).

Just before analysis, the samples were taken out of the -80°C freezer, slowly thawed on ice and sonicated in a solution of 0.2 mol/l perchloric acid and 10 pg/µl DHBA (internal standard). CBs and OLs were extracted in 40 µl of this solution, while SEZs were extracted in 20 µl. After sonication, the samples were incubated 20min on ice in the dark, centrifuged (14 min, 13.2 rpm, 0°C), and 20 µl of the supernatant was loaded in the autosampler. External standards of octopamine, dopamine, tyramine and serotonin were included before and after each run.

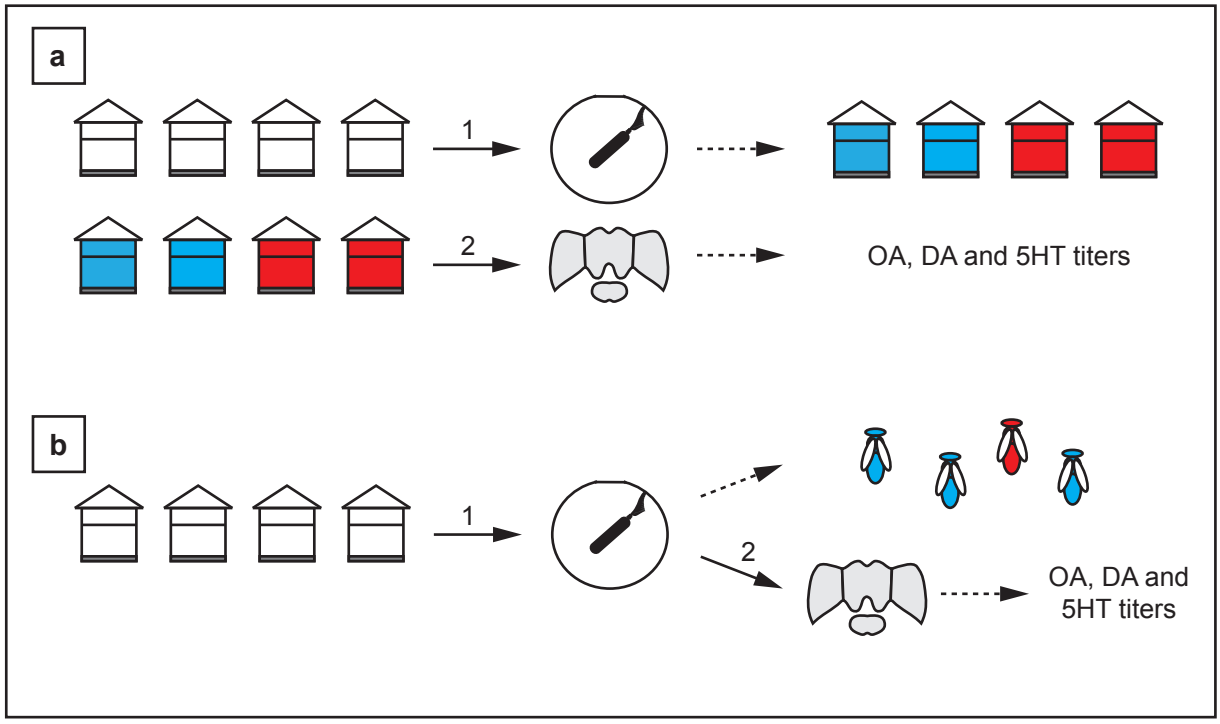



**Figure 1: Experimental design of the HPLC experiments.**
**a.** Colony-level experiment. Bees from four colonies were tested in the arena assay (arrow 1). As a result, some colonies were identified as “gentle” (blue), while others were “aggressive” (red). Brains were then dissected from naive bees from the same colonies, i.e. bees that were not tested in the behavioural assay, and HPLC titers of biogenic amines determined (arrow 2).
**b.** Individual-level experiment. After testing in the arena assay (arrow 1), individual bees were classified according to their behaviour (aggressive=red or not=blue). Their brains were dissected and brain biogenic amines were measured using HPLC (arrow 2).

### *Pharmacology*

The pharmacological treatments consisted of topical applications of 1 µl of solution on the thorax of the bees, 15 to 50 min before the aggression assay. This method and its timing were based on the work by Barron et al. (2007), which measured that approximately 1% of topically applied OA reached the brain of treated bees and that this amount was stable between 15 and 60 min. Four sets of experiments were performed during which the bees were treated with 5HT, DA, cyproheptadine hydrochloride sesquihydrate (Cyp, 5HT antagonist) or cis-(Z)-flupentixol dihydrochloride (Flu, DA antagonist) dissolved in dimethylformamide (dMF). All chemicals were obtained from Sigma-Aldrich. Control bees received pure dMF, while treated bees received 0.2 mg/ml, 2 mg/ml or 20 mg/ml of the active compounds. This highest concentration corresponds to 113 mM of 5HT, 130mM of DA, 62 mM of Cyp and 39 mM of Flu. The antagonists and the concentrations were chosen based on previous work on the modulation of aversive responsiveness in honeybees (Tedjakumala et al. 2014). Both bees in a test pair received the same treatment.

### *Statistical analysis*

Behavioural results were analysed using a Generalized Linear Model (GLM) set-up with a logit function appropriate for binomial data. The HPLC data was first checked for the presence of outliers using the method described in Hoaglin and Iglewicz (1987). Among 1941 data points (from 215 bees), a total of 17 outliers were removed, with a maximum of 2 in the same test group. To study the effect of the colony of origin, the results were analysed with Kruskal-Wallis tests. For the study at the individual level, the results were first analysed with a three-way ANOVA taking as factors odour, aggressiveness and colony. In all but two cases, discussed in the results section, this last factor did not appear to have an influence. Thus, a second analysis was performed without it. The effects of the two other main factors (odour and behaviour) were consistent across these two rounds of analysis.

## Results

The behavioural assay used to test honeybee aggression consisted of a small cylindrical arena in which the bees were confronted with a black, rotating dummy. Two bees were placed in the arena in order to potentiate aggression towards the dummy (Nouvian et al. 2015). A constant air flow allowed delivery of odours during the test: we always tested the bees either with a solvent control (triethylcitrate, TEC), or with the alarm pheromone IAA. Honeybees could choose to sting the dummy - perceived as a threat - or to ignore it, and the results are therefore expressed as the percentage of trials during which this stinging response was observed. For the subsequent HPLC analysis of brain biogenic amines content, the brain was always separated into 3 regions (see small insets on Figures 2 and 3): the optic lobes (OL), the sub-esophageal zone (SEZ) and the remaining central brain (CB). This last region contained all the structures involved in olfactory processing such as the antennal lobes, mushroom bodies and lateral horns.

### *Colony-level: honeybees from an aggressive background have higher serotonin levels in their central brain*

We first determined aggression levels of colonies. To this end, bees collected at the entrance of four unrelated colonies were tested in the laboratory for their aggressive response towards a rotating dummy in the presence of the main component of the alarm pheromone, IAA, or a solvent control TEC (n=26 pairs of bees in each of the 8 groups). This analysis revealed significant differences in the behaviour of workers from these colonies (Figure 2a, GLM, TEC: $p<0.001$, IAA: $p=0.017$). Post-hoc pairwise comparisons showed that bees issued from colonies 1 and 2 stung the dummy less often than bees from colony 3 or 4 both in the IAA and TEC conditions. Thus, colonies 1 and 2 can be described as “gentle” colonies, while colonies 3 and 4 are more “aggressive”. This classification reflects well the general defensiveness observed for these colonies during routine beekeeping inspections.

To investigate whether biogenic amines are linked to colony aggressiveness, brain biogenic amine levels were measured in naïve (untested) bees collected from the same four colonies using HPLC (n=25-27 bees per colony). Significant variations in 5HT levels were detected in the CB and OL of these bees according to their

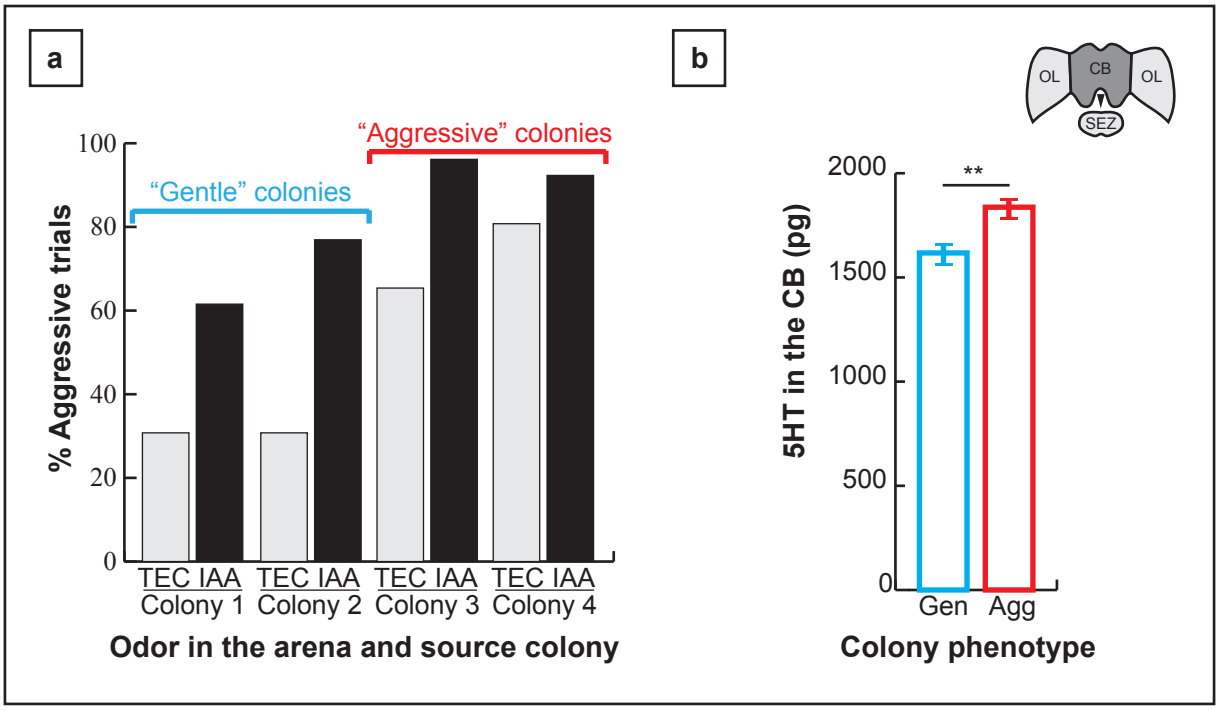



**Figure 2: Correlation between behavior at colony level and 5HT levels in the central brain.**

**a.** Percentage of trials in which at least one of the bees stung the dummy, recorded as a function of the odor present (TEC: solvent; IAA: alarm pheromone) and the source colony. Regardless of the odor condition, colonies 1 and 2 are significantly less aggressive than colonies 3 and 4.

**b.** The mean amount of 5HT measured in the brains of bees from aggressive colonies ("Agg", colonies 3 and 4) vs gentle colonies ("Gen", colonies 1 and 2) is significantly higher. **: $p<0.01$.

source colony (Kruskal-Wallis, CB: p=0.002, OL: p=0.035). OA and DA levels never varied significantly between colonies in any of the brain regions (Kruskal-Wallis, p>0.05 in all cases). However, when the data were pooled according to the classification established previously (aggressive vs. gentle colonies), only 5HT levels in the CB appeared to correlate with the aggressiveness: they were significantly higher in bees from the aggressive colonies (Figure 2b; Kruskal-Wallis, p=0.002). This result provides the first indication that 5HT levels in honeybee brains may be linked to aggressiveness at the colony level.

***Individual-level: correlation between high serotonin levels and exposure to IAA and aggressiveness***

Other bees, tested for their aggressive behaviour as described above, were used for biogenic amine analysis based on their individual responses. IAA and solvent-exposed bees (IAA-group and TEC-group, respectively) were separated into two subgroups each: aggressive bees, which stung the dummy, and non-aggressive bees, which did not, thus resulting in 4 groups in total. Bees were snap-frozen immediately after the assay, and the biogenic amine content was measured using HPLC (n=25-27 bees per group). The results (Figure 3) were first analysed using a three-way ANOVA design, taking as factors the odour to which the bees were exposed (IAA or TEC), their behavioural category (aggressive or not) and their source colony. This last factor was included in the analysis to check for potential bias in the dataset that could hinder the interpretation of the two other main effects. Indeed, while care was taken to balance the number of bees from each colony as much as possible, equal numbers were not always achieved. However, in all but one dataset (discussed below), this factor did not interact with the odour and behaviour effects. Thus, in a second round of analysis this factor was removed.

In the CB, during the first analysis a slight colony effect was detected in DA levels (ANOVA, $F(3,92)=2.816$, $p=0.044$), but it did not interact with the other main effects (ANOVA, $p>0.05$ in all two- and three-way interactions). The results of the second round of analysis (without this factor) are presented in Table 1. Levels of OA did not vary significantly between the groups tested (ANOVA, all $p>0.05$). On the contrary, the levels of DA and 5HT were significantly increased after exposure to IAA (Figure 3a; ANOVA, $F(1,104)=16.124$, $p<0.001$ for DA and $F(1,102)=10.345$,

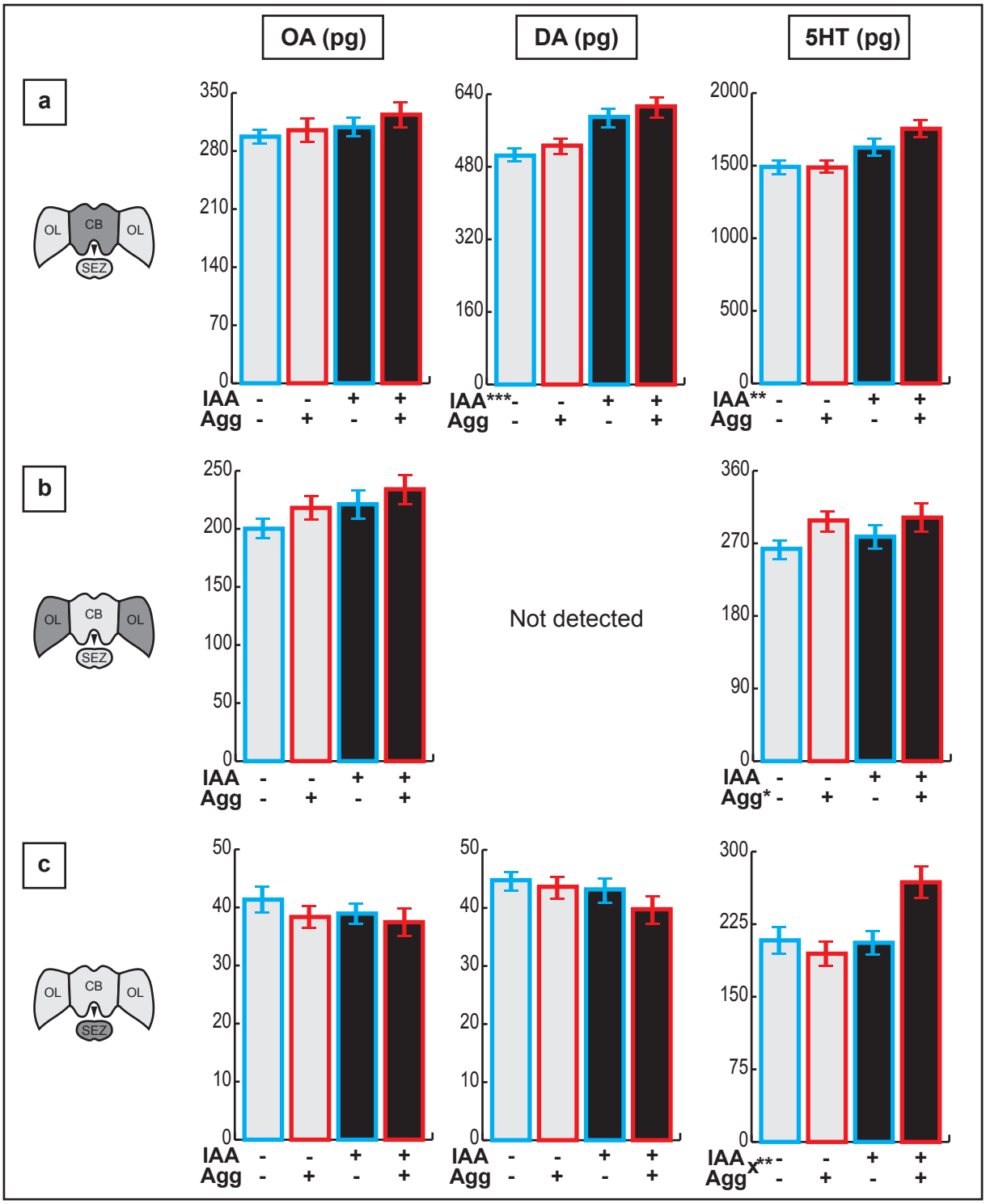



**Figure 3: Total amounts of OA, DA and 5HT in different brain regions of bees tested in the aggression assay.** Black fill indicates bees exposed to IAA (IAA+), grey fill bees exposed to TEC (IAA-) during testing. The bees were further classified according to their behavior: aggressive bees (Agg+) are represented by red outlines while non-aggressive bees (Agg-) are outlined in blue. *: factor significant at threshold 0.05, **: factor significant at threshold 0.01.

**a.** Central brain. Levels of DA and 5HT are significantly higher in bees exposed to the alarm pheromone.

**b.** Optic lobes. Levels of 5HT are significantly higher in bees that displayed aggression. DA is virtually undetectable in this region, thus no data is presented for this amine.

**c.** Sub-esophageal zone. 5HT levels are significantly higher in IAA-triggered aggressive bees (significant interaction).

**Table 1: Summary of ANOVA analysis of the brain biogenic-amine content of individually tested bees.**

| Region | Amine | Factor | Df | F | p value |
|---|---|---|---|---|---|
| CB | OA | Odour | 1 | 0.596 | 0.442 |
| | | Behaviour | 1 | 0.276 | 0.600 |
| | | Od*Beh | 1 | 0.228 | 0.634 |
| | DA | **Odour** | 1 | 18.932 | **<0.001*****|
| | | Behaviour | 1 | 0.689 | 0.409 |
| | | Od*Beh | 1 | 0.077 | 0.783 |
| | 5HT | **Odour** | 1 | 16.529 | **<0.001*****|
| | | Behaviour | 1 | 3.084 | 0.082 |
| | | Od*Beh | 1 | 0.162 | 0.688 |
| OL | OA | Odour | 1 | 0.333 | 0.565 |
| | | Behaviour | 1 | 0.903 | 0.344 |
| | | Od*Beh | 1 | 0.525 | 0.470 |
| | 5HT | Odour | 1 | 0.274 | 0.602 |
| | | **Behaviour** | 1 | 5.616 | **0.020*** |
| | | Od*Beh | 1 | 0.056 | 0.814 |
| SEZ | OA | Odour | 1 | 0.882 | 0.350 |
| | | Behaviour | 1 | 1.094 | 0.298 |
| | | Od*Beh | 1 | 0.057 | 0.812 |
| | DA | Odour | 1 | 1.550 | 0.216 |
| | | Behaviour | 1 | 1.076 | 0.302 |
| | | Od*Beh | 1 | 0.269 | 0.605 |
| | 5HT | **Odour** | 1 | 6.910 | **0.010*** |
| | | **Behaviour** | 1 | 6.931 | **0.010*** |
| | | **Od*Beh** | 1 | 10.616 | **0.002**** |

p=0.002 for 5HT). These findings suggest that 5HT and DA signalling could mediate the aggressive response triggered by the alarm pheromone.

In the OL, again OA levels did not show any significant variation between the groups tested. 5HT levels, however, clearly reflected the bee's level of aggressiveness (ANOVA, $F(1,104)=5.616$, $p=0.020$): 5HT levels were higher in aggressive bees, irrespectively of the odour they were exposed to (Figure 3b). DA levels were very low, and seldom reached the detection threshold of the HPLC. As a consequence, this specific data set could not be analysed. These results further confirm the role of 5HT in honeybee aggression.

Finally, in the SEZ, OA levels did not vary significantly according to any of the factors tested, nor did DA levels (Table 1). The first analysis of the 5HT data set revealed a slightly significant three-way interaction between odour, behaviour and colony (ANOVA, $F(3,91)=2.834$, $p=0.043$). Careful examination of the dataset revealed that this effect was driven by a single bee in a subgroup with a particularly low sample size (n=3). Removing this bee caused the three-way interaction to become non-significant (ANOVA, $F(3,90)=2.060$, $p=0.111$). Furthermore, all the analyses performed (with or without colony as a factor) detected a significant two-way interaction between odour and behaviour (ANOVA, $F(1,103)=10.616$, $p=0.004$). This robust effect appears to be driven by an increase in 5HT levels in the SEZ of bees which have been exposed to IAA and displayed aggressive behaviour (Figure 3c), again supporting the notion of 5HT playing a crucial role in honeybee aggression. While each factor also appears significant on its own in this last analysis (Table 1), this was likely driven by the effect of the two-way interaction.

### *Among aggressive bees, high serotonin levels also correlate with fast reaction times*

The stinging response is an absolute measure of aggression, but it is possible to transform it into a more continuous variable by measuring the time between the introduction of the bee in the arena and the stinging response. The major drawback of this method is that it is restricted to bees that did sting. Nonetheless, this measure has often been used in studies of honey bee defensive behaviour (Kolmes and Fergusson-Kolmes 1989; Guzman-Novoa et al. 2003) where it was shown that bees react faster in presence of alarm pheromone. This effect was detected in our dataset (Figure 4a), as bees exposed to IAA attacked on average 19 s after the beginning of

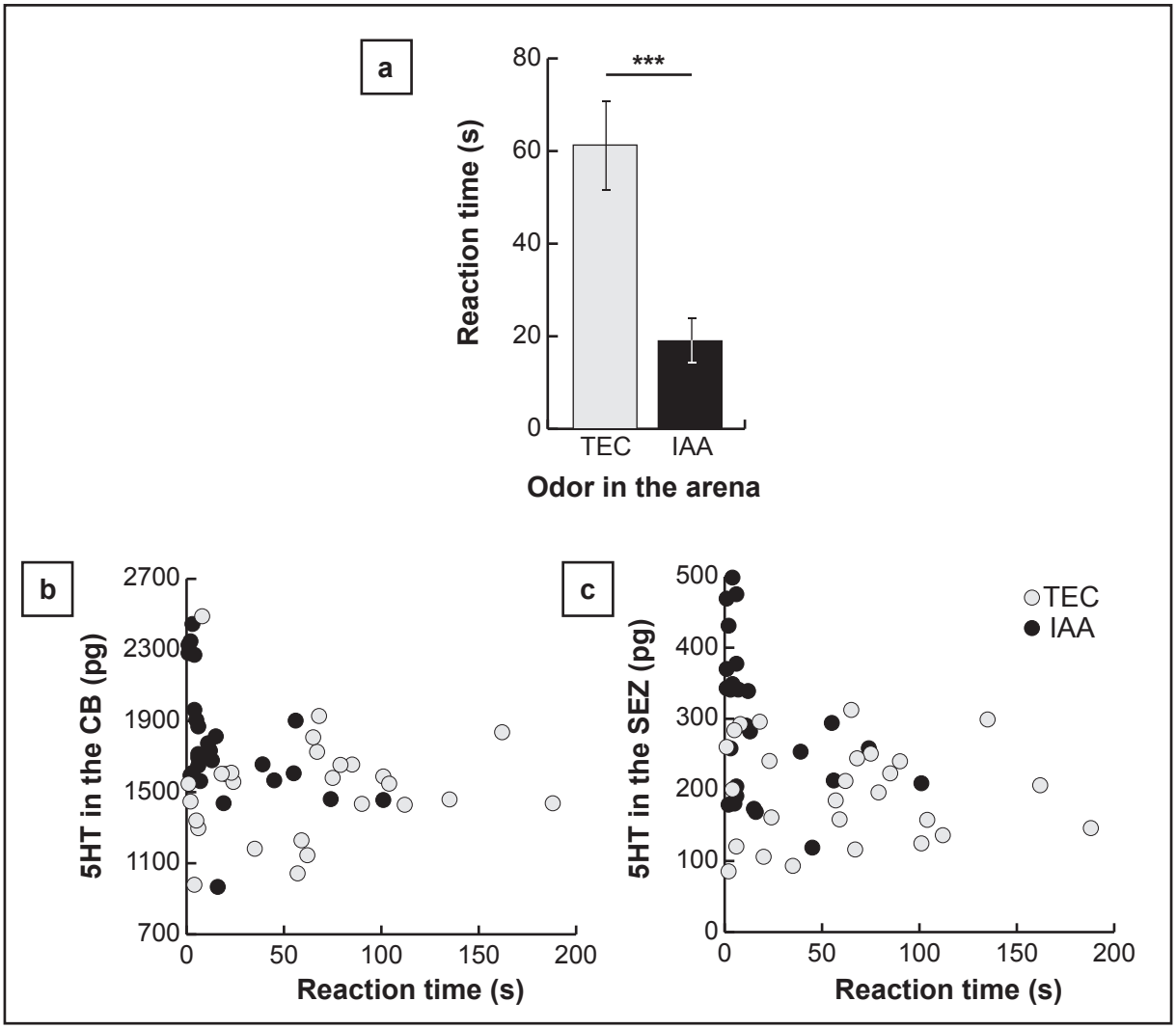


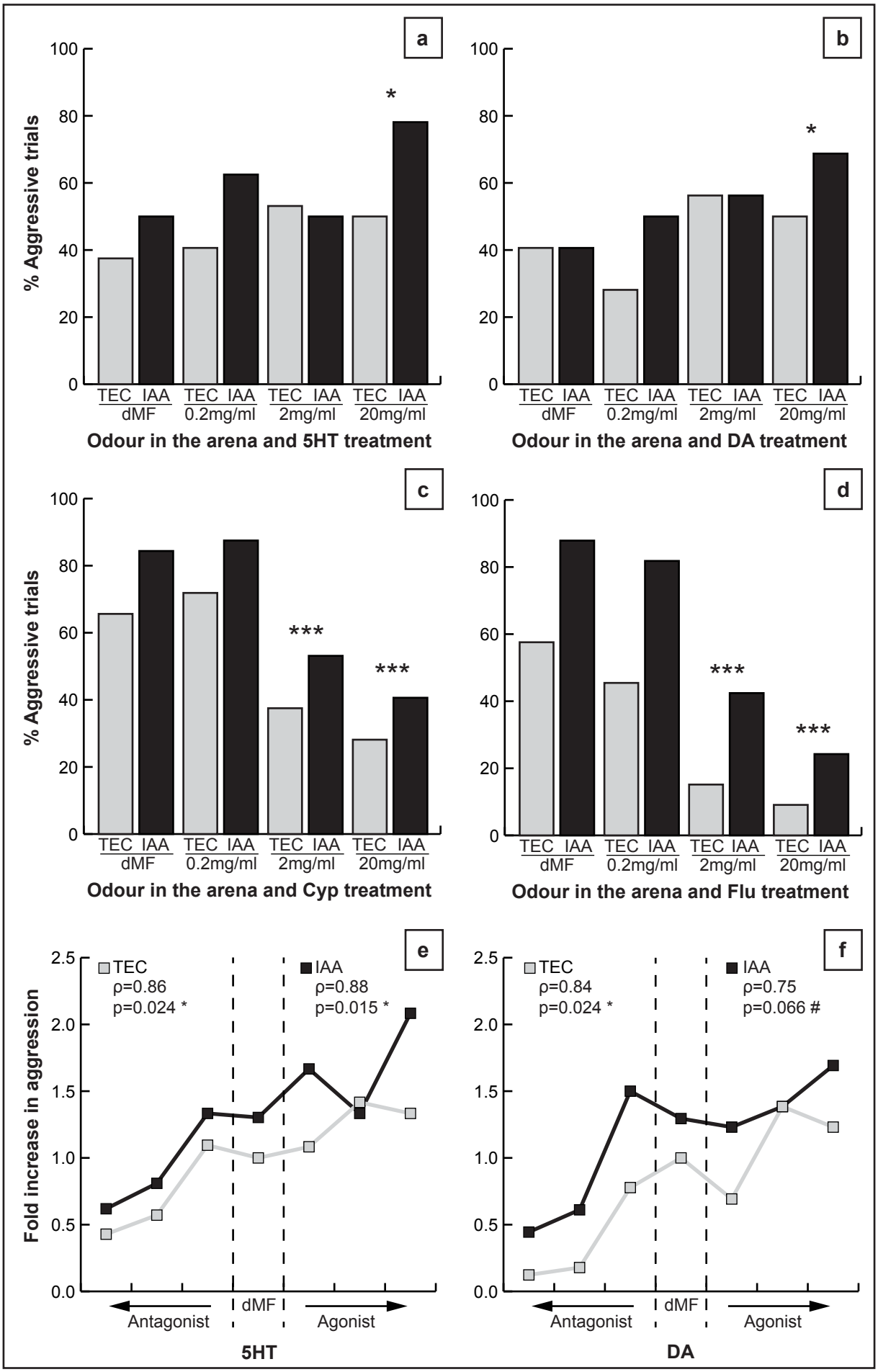

**Figure 4: High 5HT levels correlate with fast reaction times.**
**a.** Honeybees react faster in the presence of IAA. ***: Mann-Whitney, $p<0.001$.
**b.** The reaction time of each bee plotted against the amount of 5HT measured in its CB. All bees with over 2000pg of 5HT reacted extremely fast.
**c.** The reaction time of each bee plotted against the amount of 5HT measured in its SEZ. All bees with over 300pg of 5HT reacted extremely fast.

the assay while control bees took approximately 1 min to sting (Mann-Whitney, $p<0.001$, n=27 bees per condition). Individually plotting the 5HT content of each aggressive bee against its latency to sting revealed a striking pattern (Figures 4b (CB) and 4c (SEZ)): when 5HT levels varied within a certain range (1000 to 2000 pg in the CB, and 100 to 300 pg in the SEZ), there was no correlation between this measure and the latency to sting (Spearman, CB: $r=-0.074$, $p=0.595$; SEZ: $r=-0.070$, $p=0.662$). Above these levels, however, all bees reacted extremely fast to the dummy, usually within the first 10 s. A similar although less obvious trend could be seen for 5HT in the OL, but not for any of the other amine/region combinations (Supplementary Figure 1). These results suggest that high 5HT levels may be sufficient to induce high aggression in honeybees.

***5HT and DA levels regulate aggressiveness in bees***

To confirm that high 5HT and DA levels do not only correlate with but also cause high aggressiveness in honeybees, we treated bees with a topical thoracic application of either a control (dMF) or a solution (0.2, 2 or 20 mg/ml in dMF) containing one of four molecules: 5HT, Cyp (5HT antagonist), DA or Flu (DA antagonist). This method was chosen for being non-invasive and thus not affecting behavioural outcomes. Based on measurements made after OA treatments, approximately 1% of the deposited substance is expected to be transferred to the brain (Barron et al. 2007). After this treatment, the bees were tested for their aggressiveness in the arena assay, either in the presence of the alarm pheromone (IAA) or of a solvent control (TEC).

No interaction could be detected between the presence/absence of alarm pheromone and the pharmacological treatment in any of the four datasets (GLM, all $p>0.2$). Therefore, the data was analysed using an additive model. As expected, the presence of alarm pheromone always induced higher aggression levels, except in the DA dataset where it was only close to significance (Figure 5; GLM, Odour, 5HT: $p=0.017$, DA: $p=0.104$, Cyp: $p=0.011$, Flu: $p<0.001$). The pharmacological treatment was also a significant factor influencing the bees' aggressiveness in all experiments but the one with 5HT treatments (Figure 5; GLM, Treatment, 5HT: $p=0.132$, DA: $p=0.036$, Cyp: $p<0.001$, Flu: $p<0.001$). However, pairwise comparisons revealed that bees treated with the highest concentration of 5HT were more aggressive than the controls (Figure 5a; GLM, dMF vs 20mg/ml, $p=0.022$). Similar results were obtained


a
b
c
d
e
f
% Aggressive trials
Fold increase in aggression
TEC IAA
dMF
0.2mg/ml
2mg/ml
20mg/ml
Odour in the arena and 5HT treatment
Odour in the arena and DA treatment
Odour in the arena and Cyp treatment
Odour in the arena and Flu treatment
TEC
ρ=0.86
p=0.024 *
IAA
ρ=0.88
p=0.015 *
ρ=0.84
p=0.024 *
ρ=0.75
p=0.066 #
Antagonist
Agonist
5HT
DA

on the group of bees treated with DA (Figure 5b; GLM, dMF vs 20mg/ml, p=0.034), while the two highest concentrations of both antagonists significantly decreased the aggressiveness of the bees (Figures 5c, 5d; GLM, p<0.001 for all four comparisons). Normalizing the controls and ordering the bee groups from the lowest to the highest expected brain level of 5HT (Figure 5e) or DA (Figure 5f) highlights the monotonic relationships between these levels and the aggressiveness displayed by the bees during the aggression test, regardless of the odour they were exposed to (Spearman, 5HT: ρ=0.86, p=0.024 for bees exposed to TEC and ρ=0.88, p=0.015 for bees exposed to IAA, DA: ρ=0.84, p=0.024 for TEC-exposed groups and ρ=0.75, p=0.066 for IAA-exposed groups). Thus, manipulating the brain content in 5HT and DA directly affect the aggressiveness of honeybees.

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**Figure 5: Pharmacological manipulations of 5HT and DA brain levels**
Overall, bees exposed to IAA (in black) attacked more than those exposed to the solvent control TEC (in grey), and there was no interaction between odour and pharmacological treatment. *: treatment significant at threshold 0.05; ***: treatment significant at threshold 0.001.
a. Honeybee aggressiveness after 5HT treatments. The bees treated with the highest concentration of 5HT attacked the dummy significantly more often than the control bees.
b. Honeybee aggressiveness after DA treatments. The bees treated with the highest concentration of DA attacked the dummy significantly more often than the control bees.
c. Honeybee aggressiveness after 5HT blockade through Cyp treatments. The bees treated with the two highest concentrations of Cyp exhibited reduced aggressiveness.
d. Honeybee aggressiveness after DA blockade through Flu treatments. The bees treated with the two highest concentrations of Flu exhibited reduced aggressiveness.
e. Overall effect of manipulating 5HT levels. The data has been normalized with respect to the dMF-TEC control groups, and the two dMF-IAA groups averaged after normalization. The more the amount of 5HT in the bee brain was expected to be high, the more the bees were aggressive.
f. Overall effect of manipulating DA levels. The data has been normalized with respect to the dMF-TEC control groups, and the two dMF-IAA groups averaged after normalization. The more the amount of DA in the bee brain was expected to be high, the more the bees were aggressive.

## Discussion

While biogenic amines are known to play an important role in aggression in a wide range of invertebrate species (Kravitz and Huber 2003; Zhou et al. 2008; Alekseyenko et al. 2013; Szczuka et al. 2013), their involvement in honeybee aggression had not been confirmed until now. Here, we show for the first time that high 5HT brain levels correlate with high aggression both at the individual and colony level, and/or with exposure to alarm pheromone in honeybees. DA levels were also increased after exposure to IAA in the CB. Furthermore, pharmacological manipulations of 5HT and DA levels confirmed that these molecules control the occurrence of aggressive behaviour. OA was not involved in aggression in our experimental framework, although it plays a crucial role during male flies fighting (Dierick 2008; Alekseyenko et al. 2013). This may be a technical artefact due to the poor spatial resolution of the HPLC procedure, or reflect differences in the behaviours studied. Indeed, in our assay worker honeybees were confronted to a potential threat, while male flies fight each other to gain access to mates. This behaviour would be more similar to the fights that happen between virgin queen bees when they emerge at the same time. In this respect, it would be interesting to check if OA would play a role in honeybees in this specific context.

The finding that exposure to IAA resulted in high levels of DA and 5HT in the CB, correlates with the findings from studies that investigated either the effect of IAA or the effect of biogenic amines on a range of honeybee behaviours. To start with, dopaminergic neurons are believed to code for aversive stimuli in honeybees (Vergoz et al. 2007; Tedjakumala and Giurfa 2013). Thus, our finding that the alarm pheromone, which is released in the presence of potentially noxious stimuli, mediates an increase in DA is not surprising. In addition, it is interesting to note that a previous work found that long term exposure to IAA provokes analgesia in honeybees (Núñez et al. 1998). Thus if our conclusion that the effects of IAA are mediated through an increase in DA and 5HT levels is correct, one would expect that injecting these amines into the brain of honeybees would also decrease their shock responsiveness. This is exactly what was demonstrated in a recent study measuring the response threshold to electric shocks after injections of biogenic amine antagonists (Tedjakumala et al. 2014). Finally, exposure to IAA has been reported to decrease the performance of bees during a subsequent appetitive learning task in

which bees learn to associate an odorant with sucrose reward (Urlacher et al. 2010). In parallel, it was demonstrated that both 5HT and DA injections inhibit the retrieval of the resulting appetitive memory (Mercer and Menzel 1982). While the effects of IAA exposure and DA/5HT injections are strikingly similar in all these papers, our results are the first to provide direct evidence that IAA exposure increases DA and 5HT levels, and thus to create a link between these two kinds of treatment. Thus, we suggest that the long-term physiological responses to IAA, such as analgesia and impairment of appetitive learning, are mediated through both DA and 5HT signalling in the central brain.

Bees that stung the dummy had higher levels of 5HT in the optic lobes. The fact that this modulation is located in the optic lobes is interesting in itself considering that the stimulus, in our assay, was mostly visual (black moving dummy). In the case of the SEZ, high levels of 5HT are observed only if the bees were aggressive as a response to the alarm pheromone. This singular pattern suggests that two converging serotoninergic pathways may be mediating the aggressive response of honeybees: a visual, SEZ-independent pathway and an olfactory, SEZ-dependent pathway. Nonetheless, our work did not identify the exact cells or networks implicated. Interestingly the primary olfactory centers, the antennal lobes, are innervated by a single serotoninergic interneuron, the deutocerebral giant cell (DCG) (Bicker 1999). This neuron is an extremely good candidate for supporting this modulation of aggression as it connects the antennal lobes, the lateral protocerebrum (which is believed to play an important role in linking odorants to their biological values (Galizia 2014; Roussel et al. 2014)) and the SEZ before descending along the nerve cord, possibly towards motor centers. As such, in follow-up studies it would be interesting to investigate whether the activation of this neuron could be sufficient to trigger elements of the aggressive response (for example the sting extension reflex). The SEZ further contains three pairs of serotonin-immunoreactive neurons in each hemisphere, while each OL is densely innervated by 20 to 30 serotoninergic cells (Mercer et al. 1983; Bicker 1999). Serotonin injections in the OLs reduce the spontaneous activity and the specificity of motion-sensitive neurons (Scheiner et al. 2006), suggesting a possible function of this amine in the detection of moving targets.

Overall, these results provide new insights into the neurophysiological mechanisms underlying honeybee aggression. 5HT, like in other species (Edwards

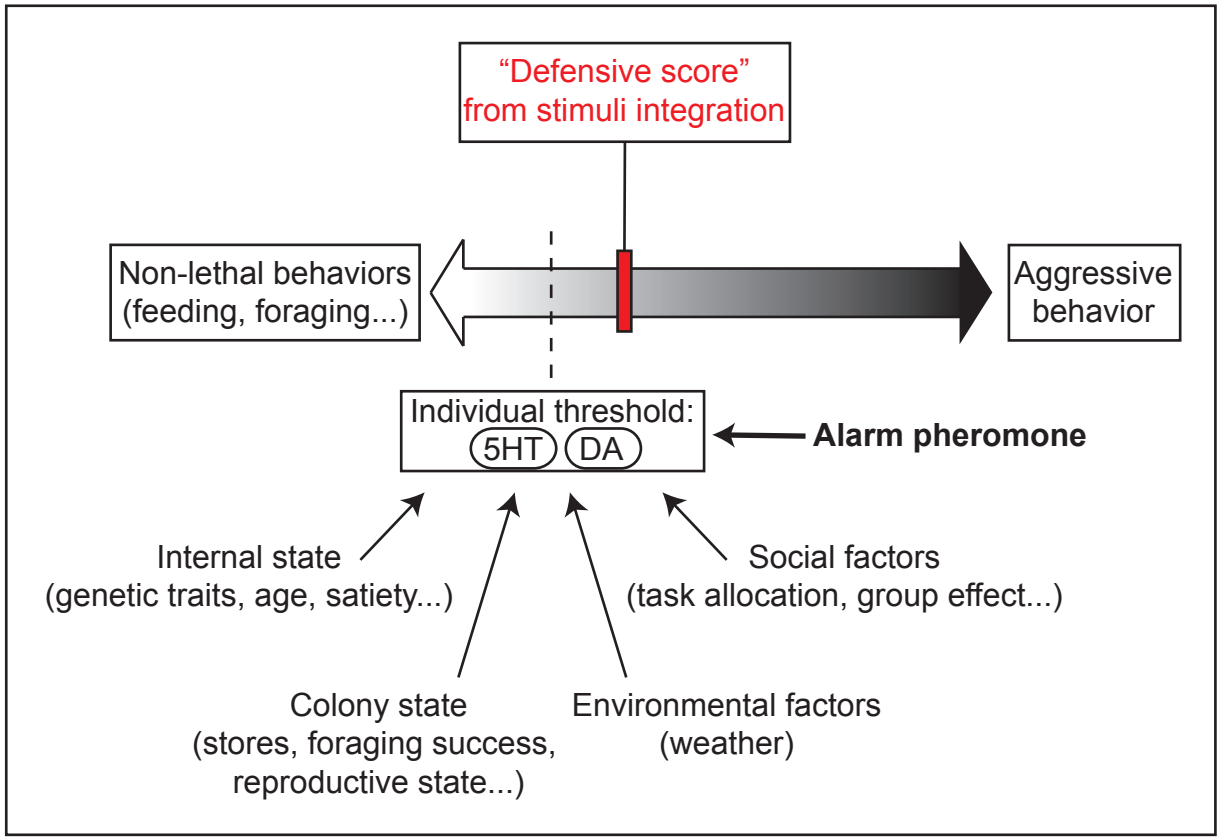


**Figure 6: Refined model for the decision making process underlying honeybee aggression.** Adapted from Nouvian et al., 2015.
This model postulates that the honeybee brain computes a defensive score from stimuli integration, which is compared to an individual threshold in order to determine the behavioural outcome. In agreement with our new results, this threshold may be represented by the levels of 5HT and DA in the bee brain. Furthermore, we suggest that the alarm pheromone is not integrated as a stimulus, but rather acts on this individual threshold. This new interpretation reconciles the results presented here, in our previous work (Nouvian et al. 2015) and in other studies (Urlacher et al. 2010, Tedjakumala et al. 2014).

and Kravitz 1997), appears to be a key modulator of the honeybee aggressive behaviour. While DA was not as preeminent in our HPLC results, the pharmacological experiments suggest that it also has an important role. Interestingly, some DA populations have been postulated to act as a gain control system, generally suppressing responsiveness to a large variety of stimuli (Tedjakumala et al. 2014). In doing so, such a network would allow selective attention processes to take place, so that the insect can focus on the relevant stimuli in a given situation and ignore the others (Van Swinderen and Andretic 2011). We recently proposed a new model for the decision-making process underlying honeybee aggression (Nouvian et al. 2015). Given our new results and the literature discussed above, we would like to hereby refine this model by suggesting that 5HT and DA levels may be setting the threshold for aggressiveness in honeybees, through the modulation of their responsiveness to relevant stimuli (Figure 6). Furthermore, since exposure to IAA increases the amount of 5HT and DA contained in the CB (Figure 3a), we propose that the alarm pheromone may not be integrated as a stimulus in itself, but rather acts on this same threshold. This hypothesis fits well with both our current knowledge of honeybee aggression and the known effects of IAA on other behaviours, such as the decreased sensitivity to nociceptive and appetitive stimuli (Urlacher et al. 2010; Tedjakumala et al. 2014). This important conceptual change gives a new light to previous studies on honeybee aggression, and is fundamental for future studies aiming at understanding the neural mechanisms underlying this conspicuous behaviour. Overall, we believe that our work represents an important step for the study of honeybee aggression as it hints at possible substrates (e.g. the DGC) and mechanisms, and opens new questions for future investigations.

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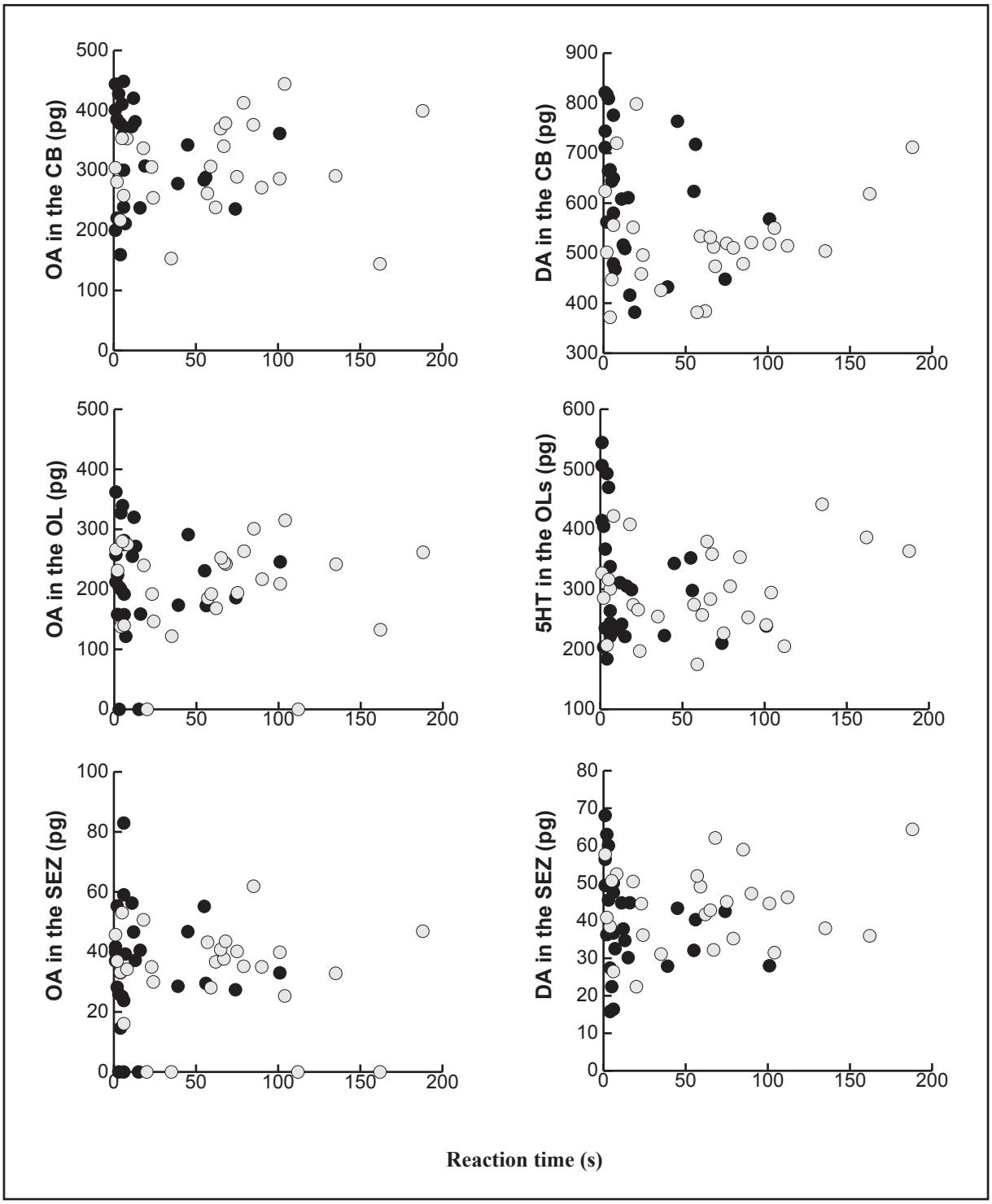


**Supplementary Figure 1. Reaction times of individual bees plotted against the amount of biogenic amines measured in different regions of their brains.** Except for 5HT, no clear relationship appears.

# Chapter 6.

# General Discussion

# Introduction

The first monograph describing the anatomy of the honeybee brain was published in 1896 by Kenyon (Kenyon 1896). Since then a wealth of information about the honeybee brain has been gathered, in an effort to link the anatomy and physiology of the central nervous system with its behavioural output. Considerable attention has been given to visual (Avarguès-Weber et al. 2012) and olfactory (Sandoz 2011; Galizia 2014) processing, and how this sensory information is integrated and modified during learning (Menzel and Giurfa 2001; Giurfa and Sandoz 2012), which is a crucial part of the honeybee's most prominent behaviour, namely foraging. In contrast, little is known about the neural basis of honeybee aggression in spite of this being a similarly conspicuous behaviour of bees. The focus of this thesis was therefore to elucidate some of the mechanisms at play regulating honeybee aggression.

First, I conducted a behavioural study to determine if olfactory cues other than the alarm pheromone modulate the aggressive behaviour of honeybees (Chapter 3). This study revealed that honeybees presented with some of the most common floral odours exhibited reduced responsiveness to the alarm pheromone. These compounds also elicited spontaneous appetitive responses, even in bees that never encountered them during adulthood. These two properties of the floral odours were strongly correlated: the most appetitive compounds were also the most effective in blocking the aggressive response to IAA. While this study focussed exclusively on behaviour, the results provide an important theoretical framework about what the honeybee brain is capable of doing: in this case, integrating contradictory sensory information before making the decision to engage into (potentially lethal) defense.

The second part of this thesis (Chapter 4) aimed at linking this theoretical framework to its potential neural substrate. The primary olfactory center of the bee brain is the antennal lobe, thus we decided to start the search in this region. However, specific interactions between the alarm pheromone and appetitive odours were not detected by our optophysiological analyses of neural activity, neither at the periphery (input of the antennal lobe) nor after processing within this structure (output). This suggests that the olfactory modulation of aggression observed in behavioural assays likely arises in higher-order brain centers. A particularly good

candidate structure would be the lateral horn, which is thought to evaluate the valence of odours (Fisek and Wilson 2014; Galizia 2014).

The third part of this thesis (Chapter 5) took a slightly different approach. Its aim was to determine if brain biogenic amines have a role in honeybee aggression, like in many other invertebrate species (Kravitz and Huber 2003). The results revealed that indeed biogenic amines, more specifically serotonin and dopamine, do regulate aggression in bees. Exposure to the alarm pheromone induced higher levels of both amines in the central brain region, and high serotonin levels were sufficient to induce high aggressivenes. By providing additional information on the characteristics and location of the neuronal populations controlling honeybee aggression, this study thus opens the door for a more detailed neuro-anatomical analysis of this important behaviour.

These behavioural, physiological and molecular findings are discussed in detail in the respective chapters. In this last chapter of my thesis I will discuss the open questions arising from the above findings as well as from additional side observations made throughout my thesis. The aim is to put the discoveries of my thesis into a biological framework, present hypotheses regarding their adaptive significance, and provide an outlook for future research.

## Variability in the response to the alarm pheromone: environmental factors

The defensive behaviour of honeybees against large predators is comprised of two main steps: the initial response of one or a few individuals, and an amplification phase during which a large number of additional defenders are recruited through the release of a potent alarm pheromone (Chapter 2). A major contribution of this thesis was the development of a novel behavioural assay which allows a precise dissection of the responsiveness of individual bees during each of these steps. To measure the initial aggressiveness of honeybees, all the experiments performed throughout this thesis included a group that was only exposed to the odourless solvent control. Comparison of the aggressiveness of this group with a second one exposed to the alarm pheromone allowed quantifying the responsiveness of the bees to the second regulatory step of aggression, the recruitment phase. Consistent with the literature

(Boch et al. 1962; Ghent and Gary 1962; Maschwitz 1964), most of the time the presence of the alarm pheromone provoked more attacks in our set-up (see for example Figure 2 in Chapter 3). However, intriguing deviations from this pattern were also observed, with season and weather both significantly affecting the aggressive response.

*Seasonal effect.* The most striking evidence that the response to the alarm pheromone depends on environmental factors is presented in Chapter 5. Because of time constraints, in this study we tested the aggressiveness of honeybees during the winter months, and found a complete disappearance of the response to IAA. In this experiment the control bees were treated with the solvent dMF, which served as a control for topical applications of serotonin (Figure 4 of Chapter 5). We replicated the experiment with untreated bees and found exactly the same results (Figure 1a). In addition when re-testing bees from the same two colonies in spring, we found that the responsiveness to IAA was restored, and that the initial response had not been affected (Figure 1a). These results were a confirmation of observations made at the very beginning of my thesis, when I was performing the experiments presented in Chapter 3. This time, honeybees from four colonies were first tested during autumn and exhibited heightened aggressiveness in presence of IAA, but when they were tested again in winter this response was not significant anymore (Figure 1b). These results are all the more compelling for they have been obtained in two very different environments: Figure 1a presents data from colonies housed in Toulouse, France, which has a temperate climate, while the data from Figure 1b was collected in Brisbane, Australia, where the climate is subtropical. Thus, “French bees” experienced a cold winter with complete cessation of foraging activity, while “Australian bees” were subjected to the cool temperatures and sunny days of Brisbane’s dry season, during which the hive activity is strongly reduced but never completely stopped.

*Effect of weather conditions*. Throughout my thesis, I also observed a similar phenomenon on a much smaller time scale: under poor weather conditions. Unfortunately, rigorous documentation of this observation was difficult because no single meteorological variable seemed to correlate well with this response. Rather, this seemed to arise from any condition impeding flight and foraging activity: strong wind, rain or particularly heavy overcast conditions. During Brisbane’s storm season I also made similar, anecdotal observations during the hours preceding a storm.

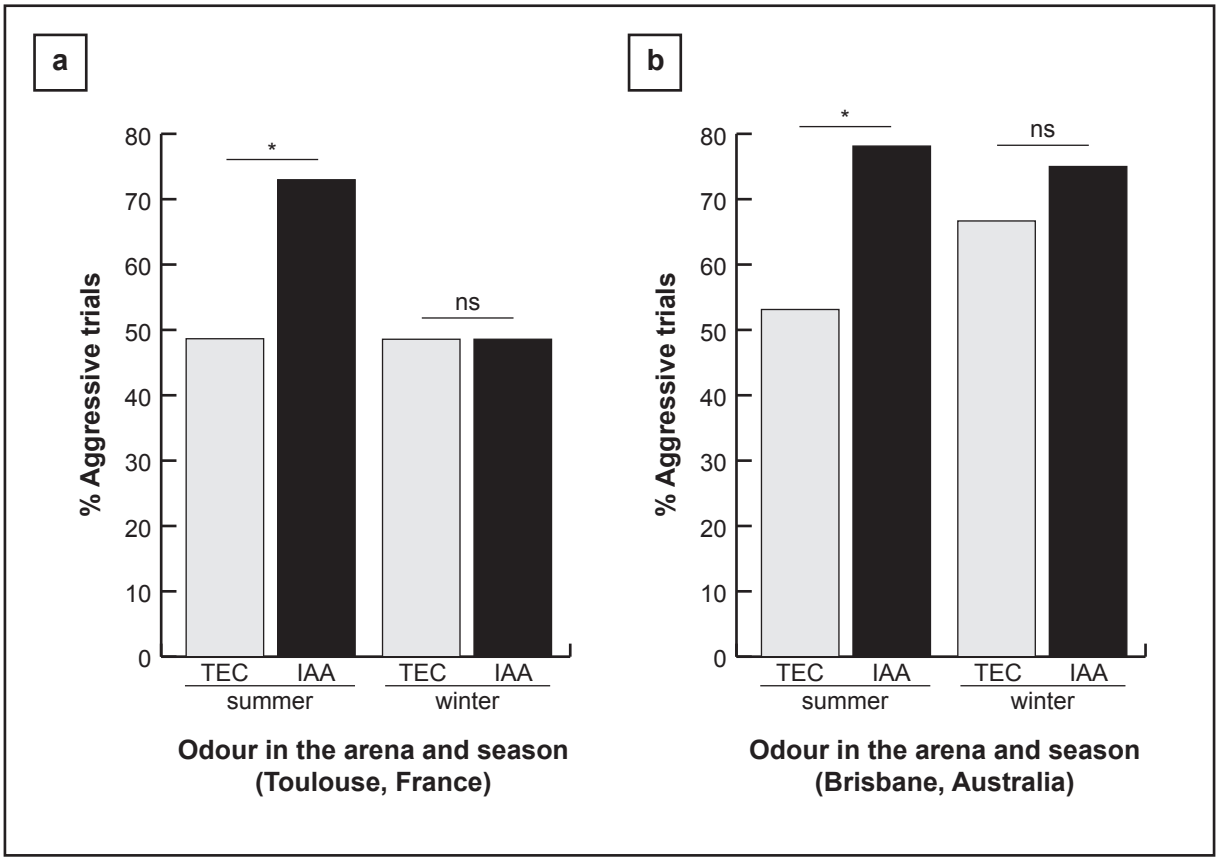


**Figure 1: Seasonal variations in the response to the alarm pheromone.**
Percentage of trials in which at least one of the bees stung the dummy, recorded as a function of the odor present (TEC: solvent; IAA: alarm pheromone) and the season. Winter bees did not respond to the presence of alarm pheromone. Fisher Exact test, *:$p<0.05$, ns: $p>0.05$.
**a.** Data from two colonies housed in Toulouse, France.
**b.** Data from four colonies housed in Brisbane, Australia.

There are some published studies that corroborate these observations: Southwick and Moritz tested the defensive behaviour of whole colonies under different meteorological conditions, using a field test (Southwick and Moritz 1987). They found that honeybees were more defensive on sunny, hot and relatively humid days. In contrast, wind velocity was negatively correlated with aggressiveness. While overall these results support my observations, their protocol included the simultaneous presentation of both a moving target and the alarm pheromone, thus they did not disentangle exactly which phase of the defensive behaviour was affected. Another study (Collins 1981) tested the response of newly emerged bees to alarm pheromones under different temperatures and humidity conditions, with similar results: the bees reacted more strongly at high temperature and high humidity. However, this might have been because of changes in the volatility of the alarm pheromone, whereas all my experiments were conducted under controlled laboratory conditions with a constant temperature of 25°C (see methods in Chapter 3).

As a direct, practical consequence of these early observations, I avoided as much as possible performing experiments in poor weather or in winter. However due to the time constraints of a PhD project, the calcium-imaging data had to be recorded in winter bees. The activity maps elicited by all odours, and especially by IAA, were checked against those reported in previous studies and found to be consistent (Joerges et al. 1997; Deisig et al. 2006; Wang et al. 2008), and the principles of mixture processing also seemed unaffected (Deisig et al. 2006; Deisig et al. 2010). Thus, it seems unlikely that the general organization and the mechanisms underlying olfactory processing in the antennal lobe are modified in winter bees. Nonetheless, to exclude any seasonal or weather effect, the physiological studies would need to be replicated using summer bees.

The question remains of the mechanism underlying the reduced responsiveness to the alarm pheromone in winter bees. Since IAA elicited strong patterns of activation in the antennal lobe of winter bees (Chapter 4), it seems highly unlikely that they were not able to smell the alarm pheromone. Moreover, a striking feature of our results is that the visual response triggered by the presence of a dark, moving object was not affected (Figure 1). Some bees attacked the dummy in the control TEC group throughout the year, thus ruling out the hypothesis that winter bees do not maintain any kind of defense. Two kinds of mechanisms could explain such variability in the response to the alarm pheromone. The first one would be that

some physiological modification of all individual bees (for example a shift in biogenic amine levels) intervenes in poor weather/winter conditions and causes reduced responsiveness to the alarm pheromone. The second one relates to the structure of the honeybee population inside the hive. Indeed, as we mentioned previously the one factor that seemingly correlates best with this phenomenon is the absence of foraging activity. It may be that by their presence inside the colony, grounded foragers bias the population of bees sampled during aggression tests. This hypothesis could easily be tested in our arena assay by measuring the responsiveness to IAA of bees from different castes (nurses, guards and foragers). If this second mechanism is valid, foragers should exhibit little responsiveness to IAA.

## The appetitive value of floral odours modulating aggression

Being their unique food source, flowers are absolutely central to the biology of honeybees. Thus, it is maybe not surprising that we found that some of the most common floral compounds (Knudsen et al. 2006) act on honeybee aggression (Chapter 3). These odours were also appetitive to honeybees sampled from the defensive population and to naïve bees that never experienced these odours during adulthood. This last result raises the question: are these odour preferences innate or acquired prior to emergence? In terms of adaptive fitness, both hypotheses could be valid. On the one hand, innate preferences for some of the most common floral compounds could help naïve foragers or scouts searching for flowers. On the other hand, learning of the floral compounds during the larval or pupal stage could be advantageous because it would prime the bees specifically for the scents of the rewarding flowers present in their environment and being visited by their nestmates. Early olfactory experience during the first week of adult life is known to produce long-term changes in the structure and function of the antennal lobes (Masson and Arnold 1984; Arenas et al. 2012), however no experiment has been performed on larval bees. As already discussed in Chapter 3, an innate preference seems the most likely scenario as innate colour preferences have already been reported (Giurfa et al. 1995), while there is no evidence that honeybees can learn at the larval stage and retain this information through metamorphosis (albeit there is in the fruitfly: Tully et al. 1994). Furthermore, the same preferences were found in experiments conducted

in Australia (aggression assays presented in Figure 2a of Chapter 3) and in France (field test presented in Figure 2d of Chapter 3) despite the very different vegetation found at these locations. Nonetheless, rigorous experiments would be required in order to answer this question experimentally. Recent advances in techniques for *in vitro* rearing of honeybee larvae could allow such experiments (Crailsheim et al. 2013). An interesting experimental design would be to rear three groups of larvae: one without any odour, a second with a designated odour A with no suspected innate appetitive value (e.g. limonene) in the atmosphere and a third with the odour in the food. After emergence, testing the spontaneous proboscis extension response elicited by odour A, a control odour B with no appetitive value (e.g. linalyl acetate if limonene is odour A) and a putative innately appetitive odour (e.g. linalool) in honeybees from these three groups should answer the questions whether and how honeybee larvae can learn to associate some floral odours to a reward prior to emergence.

The other obvious question arising from our results is: can associations learned during adulthood influence aggression? In other words, if we train a bee to associate a reward to a given odour, does this odour subsequently block the aggressive response triggered by the alarm pheromone? And inversely, would an odour trained in an aversive paradigm cause higher aggressiveness? Considering the large array of robust conditioning protocols that are available in honeybees, it is easy to imagine experiments that would answer these questions. Bees trained and captured at a scented feeder or conditioned using the classical PER (or SER) protocol could be tested in the aggression assay in the presence of IAA and with or without the odour with an acquired appetitive valence. The results of such experiments would provide valuable information about sensory processing in the honeybee brain, especially if they were combined with those of the experiments described above. Indeed if conditioned odours block aggression, it would mean that honeybees are able to transfer and use the information about an acquired appetitive value in a completely different context. Furthermore, if we postulate that the odour preferences we observed were innate, then this second set of experiments would provide information about how the properties of innate and acquired values compare in the bee brain.

## Adaptive significance of floral odours modulating aggression

Most studies tend to classify behavioural responses to odours along a single axis: from strong attraction to strong repulsion (Khan et al. 2007; Knaden et al. 2012; Galizia 2014; Saha and Raman 2015). Our results, however, show an interesting deviation from this view. Indeed, while it could be tempting to say that the appetitive value of floral odours is equivalent to attraction, the sting alarm pheromone itself is also an attractant to bees. Furthermore citral, a pheromonal compound produced by the Nasanov gland and known to be a strong attractant to honeybees (Pickett et al. 1980), did not reduce the response to IAA in our assay. Citral is often released by honeybees at a rewarding food source to attract other foragers (see for example Fernandez and Farina 2001) and can therefore be encountered by bees in the same context as appetitive floral compounds. It is however not restricted to this context, since it can also be released at the hive entrance or during swarming (Trhlin and Rajchard 2011). Stimulations with alarm pheromones have also been reported to trigger exposure of the Nasanov gland and fanning in young bees (Collins 1980; Collins 1981), a behaviour that I regularly observed during the aggression assay. Interestingly, Nasanov scenting was reported to play a role during defensive events in *Apis dorsata* (Kastberger et al. 1998). Could the Nasanov pheromone have a secondary role as alarm pheromone in *Apis mellifera* too? In the results presented earlier (Figure 2a of Chapter 3), the presence of citral alone induced a very slight, non-significant increase in aggressiveness. To double check this trend, I replicated this experiment and included other compounds from the Nasanov gland (Figure 2). They did not induce any change in the aggressive response of honeybees, making it unlikely that the Nasanov pheromone has a role in modulating aggression in *Apis mellifera*.

Thus, our results have shown that only odours that are strict markers of foraging or feeding contexts block aggression in honeybees. A detail of importance here is that our experimental protocol included feeding of the bees just before testing them in the aggression assay (see Methods in Chapter 3, the bees had access to unscented sugar water when recovering from the anaesthesia and until they were inserted in the arena, and were often seen drinking). Satiation levels are therefore not likely to play a role in the blocking effect of appetitive floral compounds, ruling out

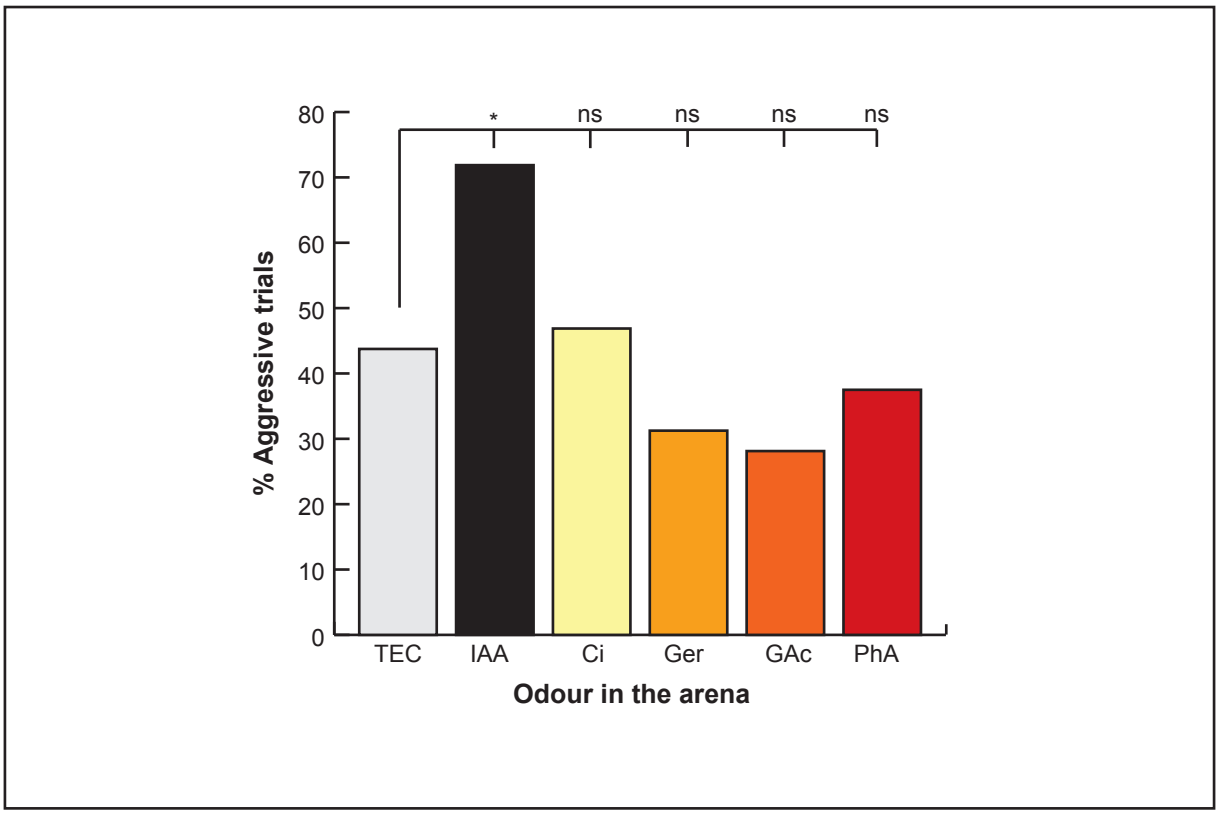


**Figure 2: Compounds from the Nasanov pheromone do not increase aggression.** Percentage of trials in which at least one of the bees stung the dummy, recorded as a function of the odor present. TEC: solvent; IAA: alarm pheromone; Ci: citral; Ger: geraniol; GAc: geranic acid; PhA: phenylacetaldehyde. Ci, Ger and GAc are the main components of the Nasanov pheromone. PhA is a floral compound known to strongly attract bees (attractant control). GLM, *: $p<0.05$, ns: not significant.

the hypothesis that honeybees are detracted from defense by the direct expectation of food. This leaves us with foraging as the main alternative context. What could be the link between foraging and inhibition of defense? Two possible mechanisms have already been evoked in this thesis, and they are not mutually exclusive. Firstly, since honeybees usually encounter floral odours when they are far from the nest, these odours could act as a signal that the presence of alarm pheromone is irrelevant. Such a modulation of aggression according to the distance from the nest was already demonstrated in another central place foraging species, the ant *Cataglyphis fortis* (Knaden and Wehner 2004). Interestingly IAA is produced by a number of flowering species (El-Sayed 2016), so it may not be that unusual for honeybees to encounter this pheromonal compound on foraging grounds, where defensive responses would not be appropriate. Large numbers of workers unnecessarily dying after stinging would deplete the colony of its workforce, thus creating a selection pressure to suppress these responses in an irrelevant context. The second possible adaptive mechanism (put forward in Chapter 2) is based on the observation that social bees avoid dangerous foraging sites (Nieh 2010; Llandres et al. 2013). More specifically, honeybees exposed to the alarm pheromone when foraging prevent other bees from advertising this particular location by head-butting them when they perform the waggle dance, causing them to stop (Nieh 2010). Thus, inhibition of the stinging behaviour in a foraging context would also ensure that the bee returns to the colony and signals the presence of a threat. By efficiently shaping its foraging strategy, the colony may gain increased fitness from this mechanism.

## Biogenic amines in honeybee aggression: perspectives

Using HPLC, we detected higher DA and 5HT levels in the central brain region of honeybees exposed to the alarm pheromone compared to bees exposed to a control solvent. These biogenic amines are thus likely to play a role in the olfactory regulation of aggression. Could they be involved in the modulation of aggression by floral odours as well? To answer this question, we performed similar titrations on bees that were exposed to LoI during the aggression test (Figure 3). Both the similarities and the differences between these results and the ones presented previously (Figure 3 of Chapter 5) are interesting. To start with, the measurements in

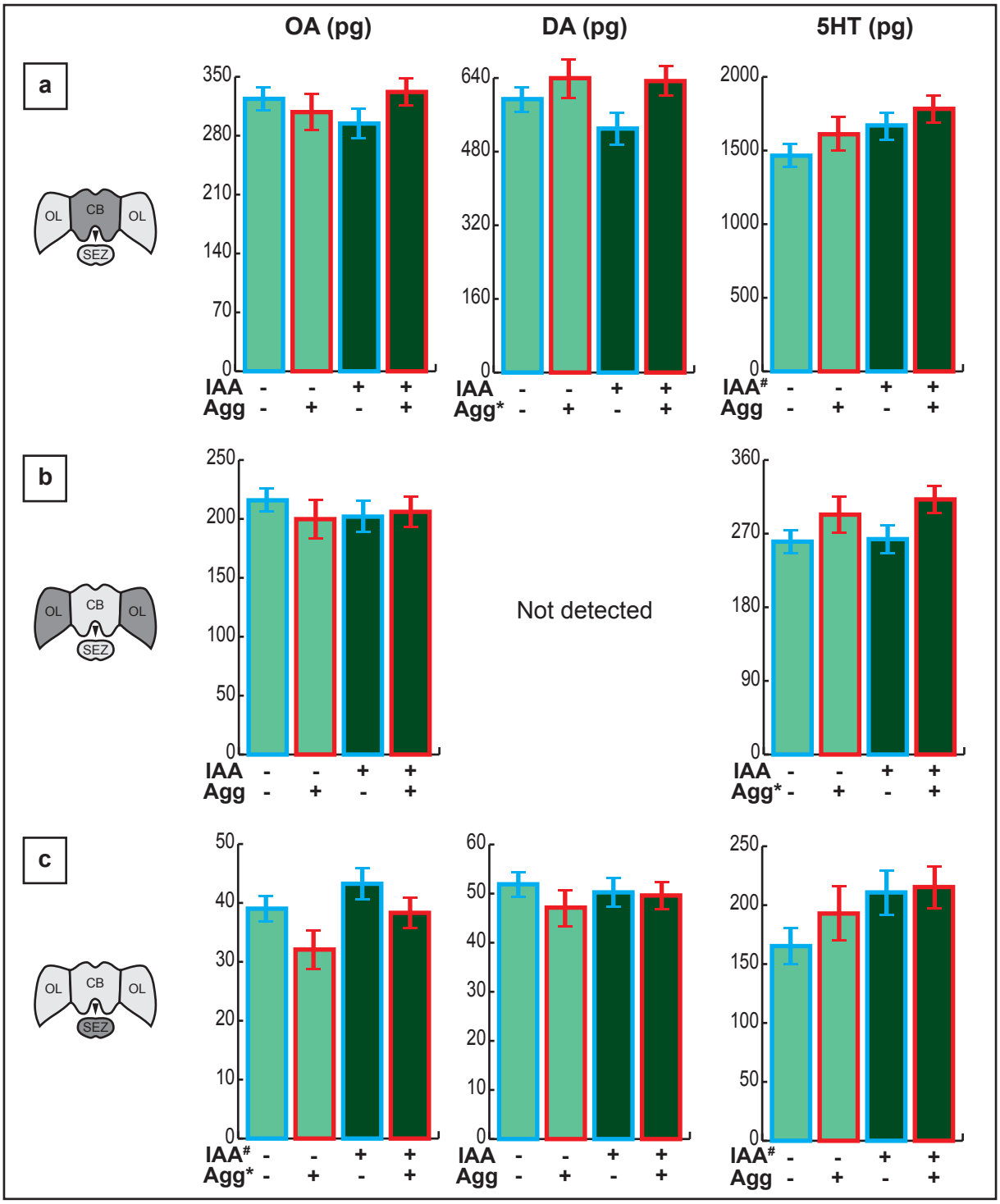


**Figure 3: Total amounts of OA, DA and 5HT in different brain regions of bees exposed to the appetitive floral compound LoI during the aggression assay.** Dark green fills indicates bees exposed to IAA+LoI (IAA+), light green fills bees exposed to TEC+LoI (IAA-) during testing. Aggressive bees (Agg+) are represented by red outlines while non-aggressive bees (Agg-) are outlined in blue. GLM, #: factor close to significance ($p<0.1$); *: factor significant at threshold 0.05.

**a.** Central brain. Levels of DA are significantly higher in aggressive bees ($p<0.05$), and levels of 5HT are slightly higher in bees exposed to the alarm pheromone ($p=0.063$).

**b.** Optic lobes. Levels of 5HT are significantly higher in bees that displayed aggression.

**c.** Sub-esophageal zone. OA levels are significantly higher in non-aggressive bees. There is a trend for IAA exposed bees to have higher OA ($p=0.070$) and 5HT ($p=0.089$) levels.

the regions that are not involved in olfaction, the optic lobes, yield the same conclusion as in the control bees, i.e. that 5HT levels are higher in aggressive bees. On the contrary in the central brain, which comprises the main olfactory centers (antennal lobes, lateral horn and mushroom bodies), the results obtained are slightly different. Levels of DA were not increased after exposure to the alarm pheromone, but were higher in aggressive bees. 5HT levels were still slightly, albeit not significantly, higher in bees exposed to IAA+LoI (GLM, p=0.063 for this factor). Aggressive bees triggered by IAA had higher 5HT levels in the SEZ, but this increase was completely abolished in presence of LoI: rather, there was a non-significant increase of 5HT levels in bees exposed to IAA+LoI (GLM, p=0.089). We also found that within this group of LoI-exposed bees, non-aggressive bees had more OA in their SEZ than aggressive ones. This last result is particularly intriguing because OA is also known to potentiate foraging (Barron et al. 2002) and appetitive learning (Mercer and Menzel 1982; Hammer and Menzel 1998). Hence, overall the presence of LoI seems to decrease the biogenic amine "signature" of aggression while increasing a foraging-related marker. It is tempting to conclude from this observation that biogenic amines are indeed involved in the olfactory modulation of aggression, through a complex interplay between the different amines. However, these results have to be considered with caution since this dataset was obtained in parallel to a control one using the bees exposed to LiA (data not shown) which did not replicate the results from the study presented in Chapter 5 - contrary to what we were expecting. This is likely because the sample sizes were much lower (10 to 26 bees per group instead of 27 in all groups) and the groups more heterogeneous in terms of colony of origin than in the previous study. Hence, these results are promising but more robust experiments are necessary before making a decisive conclusion on the role of biogenic amines in the olfactory modulation of aggression.

## Global circuitry of aggression

This thesis provided crucial new insights into the neural and molecular mechanisms underlying the defensive behaviour of honeybees, however we are still far from precisely identifying the populations of neurons controlling this behaviour. Nonetheless, our studies give some insights about the location of these populations,

and more data is available from the *Drosophila* literature (Zwarts et al. 2012). The behavioural and imaging studies (Chapters 3 and 4) suggest that the signal provided by the alarm pheromone is integrated in higher-order brain centers, downstream of the antennal lobes which are the primary olfactory center in the insect brain. This is an important result because it rules out the possibility that the detection of this pheromone by the antennal lobes is the start of a hard-wired, reflex-like circuit. We also found physiological correlates with elements of aggression in all the brain regions defined in our biogenic amine study: the optic lobes and the subesophageal zone (SEZ) received a serotoninergic modulation, while the central brain contained both dopaminergic and serotoninergic information. Furthermore, these aminergic inputs were differently associated with the behaviour displayed and/or the presence of alarm pheromone, thus suggesting that they are produced by different neuronal populations.

In our arena assay, aggression was triggered through two main sensory channels: a visual stimulus consisting of the movement of the black dummy, and the olfactory signal of the alarm pheromone. As the primary centers processing this information are the optic lobes and the antennal lobes respectively, it is highly likely that these structures are the starting points of the circuit underlying aggressive responses. Furthermore, processing of the alarm pheromone likely involves the lateral horn, which is thought to be important in the evaluation of odours with innate meaning (Galizia 2014; Roussel et al. 2014). Where in the brain is all this information integrated? The mushroom bodies would be good candidates since they are well-known to integrate multisensory information (Menzel 2014). Aggression was completely abolished in mutant flies with no synaptic output from these structures (Baier et al. 2002), giving weight to this hypothesis. In *Drosophila*, aggressive responses are also modulated by dopaminergic neurons arborizing in the central complex (Alekseyenko et al. 2013). Numerous studies have demonstrated that the central complex is crucial for proper orientation, navigation and locomotion (rewieved in Pfeiffer and Homberg 2014). Thus, I suggest that this structure might be required for the insect to localize and target the rival/intruder during aggressive events. Finally, our findings suggest an important role of serotoninergic neurons localized in the SEZ, especially when aggression is triggered by the alarm pheromone. Admittedly, the SEZ is mostly a gustatory center, processing the information from and controlling the movements of the mouthparts (Miyazaki and Ito 2010).

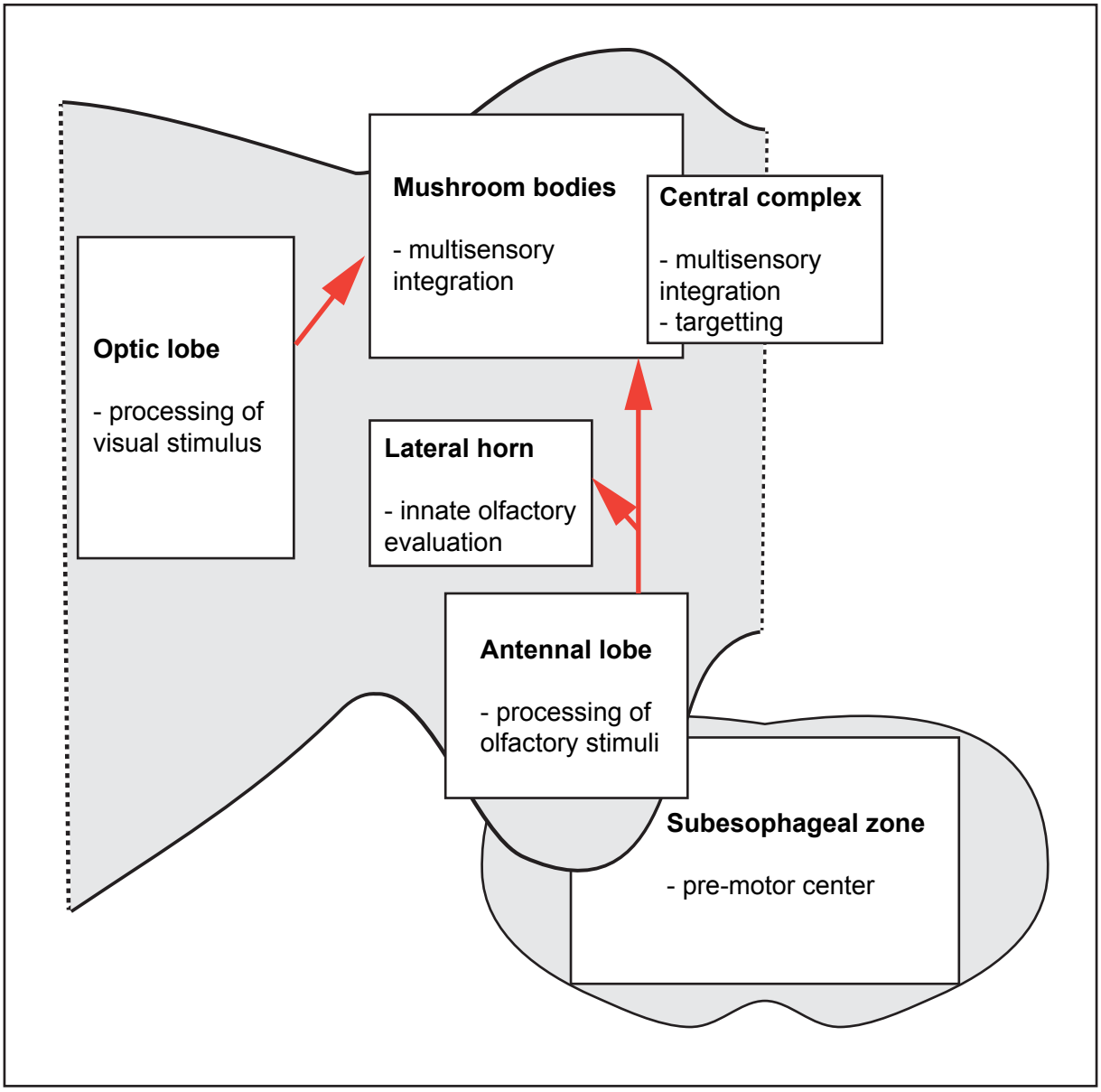


**Figure 4: Putative roles of different brain regions in producing the aggressive response.** The red arrows represent known connective tracts.
This schematic presents the hypothetical role of some brain regions in aggression in honeybees. Note that virtually all of these suggestions still require experimental validation.

Nevertheless, this thesis as well as previous studies (Rehder 1988; Maeda et al. 2014) suggest that this region also receives other inputs. Two small clusters of octopaminergic neurons within this region control the fighting behaviour of male flies: when these neurons are silenced the flies do not fight, whereas when their activity is artificially increased the flies become more aggressive (Zhou et al. 2008). Because of these results, its bridging position between the cerebral ganglia and the ventral nerve cord and its importance in the generation of simple motor patterns such as ventilation (Otto et al. 1990), walking or flying in other insects (Kien and Altman 1984; Rehder 1988; Roth et al. 1994; Gal and Libersat 2006), I suggest that the SEZ may also be an important pre-motor center for stereotyped movements in honeybees, including those pertaining to the aggressive panel (e.g. sting extrusion). Figure 4 presents a putative overview of the neuronal circuit underlying aggression. Detailed experiments investigating the role of each of these regions in honeybee aggression and their neuronal connections will be necessary to validate, correct and refine this scheme. Importantly, since honeybees have a dedicated defensive structure, namely the sting innervated by the 7$^{th}$ ganglion, retrograde staining of neurons projecting to this ganglion might prove informative to start unravelling the neural circuitry underlying aggressive behaviour in bees.

## Conclusion

The defensive behaviour of honeybees is a complex and finely regulated behaviour. As this thesis has shown, it requires multisensory integration of both the aggression-related stimuli and contextual information. Furthermore, this thesis has uncovered some of the neural and molecular mechanisms at play during aggression in honeybees, especially those related to its olfactory modulation, providing significant advances in this field of research. Firstly, the results presented here suggest that higher-order olfactory centers such as the lateral horn and/or mushroom bodies are likely key players in regulating honeybee aggression. Secondly, we have shown for the first time that the biogenic amines dopamine and serotonin play an important role in honeybee aggression. Most importantly, these findings highlight that comprehensive, integrated studies of honeybee aggression like this thesis are

required to fully understand the neurobiology of this important behaviour, and to provide effective management tools to handle aggressive colonies.

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# Appendix 1.

# The defensive response of the honeybee *Apis mellifera*

Nouvian M, Reinhard J, Giurfa M. (2016) The defensive response of the honeybee *Apis mellifera*. *Journal of Experimental Biology* 219: 3505-3517, doi: 10.1242/jeb.143016

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# Appendix 2.

# Appetitive floral odours prevent aggression in honeybees

Nouvian M, Hotier L, Claudianos C, Giurfa M, Reinhard J. (2015) Appetitive floral odours prevent aggression in honeybees. *Nature Communications* 6, doi:10.1038/ncomms10247

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