Neuronal correlates of nestmate recognition in the carpenter ant, *Camponotus floridanus*

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Neuronale Korrelate der Nestgenossen-Erkennung bei der Rossameise, *Camponotus floridanus*

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Submitted by

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Chapter 2: Brandstaetter A.S., Endler A., and Kleineidam C.J. (2008) Nestmate recognition in ants is possible without tactile interaction. **Naturwissenschaften** 95(7):601-608. doi: 10.1007/s00114-008-0360-5

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Andreas Brandstaetter, _____

Name Kandidat(in), Datum, Unterschrift

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I. Summary

Cooperation is beneficial for social groups and is exemplified in its most sophisticated form in social insects. In particular, eusocial Hymenoptera, like ants and honey bees, exhibit a level of cooperation only rarely matched by other animals. To assure effective defense of group members, foes need to be recognized reliably. Ants use low-volatile, colony-specific profiles of cuticular hydrocarbons (colony odor) to discriminate colony members (nestmates) from foreign workers (non-nestmates). For colony recognition, it is assumed that multi-component colony odors are compared to a neuronal template, located in a so far unidentified part of the nervous system, where a mismatch results in aggression. Alternatively, a sensory filter in the periphery of the nervous system has been suggested to act as a template, causing specific anosmia to nestmate colony odor due to sensory adaptation and effectively blocking perception of nestmates. Colony odors are not stable, but change over time due to environmental influences. To adjust for this, the recognition system has to be constantly updated (template reformation).

In this thesis, I provide evidence that template reformation can be induced artificially, by modifying the sensory experience of carpenter ants (*Camponotus floridanus*; Chapter 1). The results of the experiments showed that template reformation is a relatively slow process taking several hours and this contradicts the adaptation-based sensory filter hypothesis. This finding is supported by first in-vivo measurements describing the neuronal processes underlying template reformation (Chapter 5).

Neurophysiological measurements were impeded at the beginning of this study by the lack of adequate technical means to present colony odors. In a behavioral assay, I showed that tactile interaction is not necessary for colony recognition, although colony odors are of very low volatility (Chapter 2). I developed a novel stimulation technique (dummy-delivered stimulation) and tested its suitability for neurophysiological experiments (Chapter 3). My experiments showed that dummy-delivered stimulation is especially advantageous for presentation of low-volatile odors.

Colony odor concentration in headspace was further increased by moderately heating the dummies, and this allowed me to measure neuronal correlates of colony odors in the peripheral and the central nervous system using electroantennography and calcium imaging, respectively (Chapter 4). Nestmate and non-nestmate colony odor elicited strong neuronal responses in olfactory receptor neurons of the antenna and in the functional units of the first olfactory neuropile of the ant brain, the glomeruli of the antennal lobe (AL). My results show that ants are not anosmic to nestmate colony odor and this clearly invalidates the previously suggested sensory filter hypothesis.

Advanced two-photon microscopy allowed me to investigate the neuronal representation of colony odors in different neuroanatomical compartments of the AL (Chapter 5). Although neuronal activity was distributed inhomogeneously, I did not find exclusive representation restricted to a single AL compartment. This result indicates that information about colony odors is processed in parallel, using the computational power of the whole AL network.

In the AL, the patterns of glomerular activity (spatial activity patterns) were variable, even in response to repeated stimulation with the same colony odor (Chapter 4&5). This finding is surprising, as earlier studies indicated that spatial activity patterns in the AL reflect how an odor is perceived by an animal (odor quality). Under natural conditions, multi-component odors constitute varying and fluctuating stimuli, and most probably animals are generally faced with the problem that these elicit variable neuronal responses. Two-photon microscopy revealed that variability was higher in response to nestmate than to non-nestmate colony odor (Chapter 5), possibly reflecting plasticity of the AL network, which allows template reformation.

Due to their high variability, spatial activity patterns in response to different colony odors were not sufficiently distinct to allow attribution of odor qualities like 'friend' or 'foe'. This finding challenges our current notion of how odor quality of complex, multi-component odors is coded. Additional neuronal parameters, e.g. precise timing of neuronal activity, are most likely necessary to allow discrimination. The lower variability of activity patterns elicited by non-nestmate compared to nestmate colony odor might facilitate recognition of non-nestmates at the next level of the olfactory pathway.

My research efforts made the colony recognition system accessible for direct neurophysiological investigations. My results show that ants can perceive their own nestmates. The neuronal representation of colony odors is distributed across AL compartments, indicating parallel processing. Surprisingly, the spatial activity patterns in response to colony are highly variable, raising the question how odor quality is coded in this system. The experimental advance presented in this thesis will be useful to gain further insights into how social insects discriminate friends and foes. Furthermore, my work will be beneficial for the research field of insect olfaction as colony recognition in social insects is an excellent model system to study the coding of odor quality and long-term memory mechanisms underlying recognition of complex, multi-component odors.

II. Zusammenfassung

Kooperation innerhalb sozialer Gruppen ist vorteilhaft und zeigt sich bei sozialen Insekten in seiner am höchsten entwickelten Form. Besonders eusoziale Hymenopteren, wie Ameisen und Honigbienen, zeigen ein Maß an Kooperation, das nur selten von anderen Tierarten erreicht wird. Um eine effektive Verteidigung der Gruppenmitglieder sicher zu stellen, ist die zuverlässige Erkennung von Feinden unerlässlich. Ameisen verwenden schwerflüchtige, koloniespezifische Profile kutikulärer Kohlenwasserstoffe (Kolonieduft) zur Unterscheidung zwischen Gruppenmitgliedern (Nestgenossen) und fremden Arbeiterinnen (Nestfremdlinge). Man geht davon aus, dass die aus einer Vielzahl von Komponenten bestehenden Koloniedüfte zum Zweck der Kolonieerkennung mit einer neuronalen Schablone, welche sich an bisher unbestimmter Stelle im Nervensystem befindet, abgeglichen werden. Dabei führt eine Diskrepanz zwischen Schablone und Kolonieduft zu Aggression. Eine alternative Hypothese besagt, dass ein sensorischer Filter in der Peripherie des Nervensystems die Aufgabe einer neuronalen Schablone übernimmt. Dies würde mittels sensorischer Adaptation zu spezifischer Anosmie gegenüber Nestgenossen-Kolonieduft führen, so dass die Wahrnehmung von Nestgenossen effektiv verhindert wäre. Allerdings sind Koloniedüfte nicht stabil, sondern verändern sich im Lauf der Zeit aufgrund von Umwelteinflüssen. Um dies zu kompensieren, muss das Erkennungssystem fortwährend aktualisiert werden (Schablonenerneuerung).

In dieser Arbeit erbringe ich den Nachweis, dass bei Rossameisen (*Camponotus floridanus*) die Schablonenerneuerung artifiziell durch Modifizierung der sensorischen Erfahrung induziert werden kann (Kapitel 1). Die Ergebnisse der in Kapitel 1 beschriebenen Experimente zeigen, dass die Schablonenerneuerung ein relativ langsamer Prozess ist, der mehrere Stunden in Anspruch nimmt. Dies widerspricht der Hypothese eines sensorischen Filters, welcher auf sensorischer Adaptation beruht. Dieser Befund konnte mittels erster in-vivo Messungen bestätigt werden, mit Hilfe derer die der Schablonenerneuerung zugrunde liegenden neuronalen Prozesse beschrieben wurden (Kapitel 5).

Die neurophysiologischen Messungen wurden zu Beginn dieser Studie durch das Fehlen eines adäquaten Mittels zur Präsentation von Koloniedüften erschwert. In einem Verhaltensversuch konnte ich zeigen, dass taktile Interaktionen für die Kolonieerkennung nicht notwendig sind (Kapitel 2). Ich entwickelte eine neuartige Stimulierungsmethode (Dummy-vermittelte Stimulierung) und testete deren Eignung für neurophysiologische Experimente (Kapitel 3). Meine Experimente zeigten, dass die Dummy-vermittelte Stimulierung besonders für die Präsentation von schwerflüchtigen Düften geeignet ist.

Die Konzentration von Koloniedüften im Gasraum konnte durch moderates Aufheizen der Dummys weiter gesteigert werden. Dies erlaubte mir, die neuronalen Korrelate von Koloniedüften im peripheren und im zentralen Nervensystem mittels Elektroantennographie bzw. funktionaler Bildgebung (*Calcium Imaging*) zu messen (Kapitel 4). Nestgenossen- und Nestfremdlings-Koloniedüfte riefen starke neuronale Antworten in den olfaktorischen Rezeptorneuronen der Antenne und in den funktionalen Einheiten des ersten olfaktorischen Neuropils des Ameisengehirns, den Glomeruli des Antennallobus (AL), hervor. Meine Ergebnisse

zeigen, dass Ameisen nicht anosmisch gegenüber Nestgenossen-Koloniedüften sind, womit die vorgeschlagene Hypothese eines sensorischen Filters eindeutig für ungültig erklärt werden kann.
Mittels fortschrittlicher Zwei-Photonen-Mikroskopie konnte ich die neuronale Repräsentation von Koloniedüften in verschiedenen neuroanatomischen Kompartimenten des AL messen (Kapitel 5). Obgleich die neuronale Aktivität inhomogen verteilt war, konnte ich keine exklusive Repräsentation finden, die auf ein einzelnes AL-Kompartiment beschränkt gewesen wäre. Dieses Ergebnis weist darauf hin, dass Informationen über Koloniedüfte parallel verarbeitet werden und dies erlaubt die Nutzung der Rechenleistung des kompletten AL-Netzwerkes.
Im AL waren die Muster glomerulärer Aktivität (räumliche Aktivitätsmuster) variabel, selbst wenn sie durch wiederholte Stimulierung mit dem gleichen Kolonieduft hervorgerufen wurden (Kapitel 4&5). Dieser Befund ist insofern überraschend, als frühere Studien darauf hinwiesen, dass die räumlichen Aktivitätsmuster im AL widerspiegeln, wie ein Duft von einem Tier wahrgenommen wird (Duftqualität). Unter natürlichen Bedingungen stellen Düfte, die aus einer Vielzahl von Komponenten bestehen, variable und fluktuierende Stimuli dar. Höchstwahrscheinlich sind Tiere generell mit dem Problem konfrontiert, dass solche Düfte variable neuronale Antworten hervorrufen. Mittels Zwei-Photonen-Mikroskopie konnte ich zeigen, dass die Variabilität in Antwort auf Nestgenossen-Kolonieduft höher war als in Antwort auf Nestfremdlings-Kolonieduft (Kapitel 5). Möglicherweise spiegelt dies jene Plastizität im AL-Netzwerk wider, welche die Schablonenerneuerung ermöglicht.
Aufgrund ihrer hohen Variabilität waren die von verschiedenen Koloniedüften hervorgerufenen räumlichen Aktivierungsmuster nicht hinreichend unterschiedlich, um eine Zuordnung von Duftqualitäten wie ‚Freund' oder ‚Feind' zu erlauben. Dieser Befund stellt unsere momentane Auffassung in Frage, wie die Duftqualität komplexer, aus vielen Komponenten bestehender Düfte kodiert wird. Höchstwahrscheinlich sind zusätzliche neuronale Parameter, wie z.B. die präzise, zeitliche Koordinierung neuronaler Aktivität, zur Diskriminierung notwendig. Die geringere Variabilität der von Nestfremdlings-Kolonieduft hervorgerufenen Aktivitätsmuster könnte die Erkennung von Nestfremdlingen auf der nächsten Ebene der olfaktorischen Bahn begünstigen.
Meine Forschungsarbeit hat das Kolonieerkennungssystem für direkte neurophysiologische Untersuchungen zugänglich gemacht. Meine Ergebnisse zeigen, dass Ameisen ihre eigenen Nestgenossen wahrnehmen können. Die neuronale Repräsentation von Koloniedüften ist über die AL-Kompartimente verteilt, was auf eine parallele Verarbeitung hinweist. Desweiteren könnte die geringere Variabilität der von Nestfremdlings-Kolonieduft hervorgerufenen Aktivitätsmuster die Erkennung von Nestfremdlingen auf der nächsten Ebene der olfaktorischen Bahn begünstigen. Erstaunlicherweise sind die räumlichen Aktivitätsmuster in Antwort auf Koloniedüfte hochvariabel. Die wirft die Frage auf, wie in diesem System die Duftqualität kodiert wird. Der experimentelle Fortschritt, den ich in dieser Doktorarbeit vorstelle, wird nützlich sein, um weitere Erkenntnisse zu gewinnen, wie soziale Insekten Freunde von Feinden unterscheiden. Desweiteren wird meine Arbeit dem Forschungsbereich Insektenolfaktion zuträglich sein, da die Kolonieerkennung bei sozialen Insekten ein hervorragendes Modelsystem darstellt, um die Kodierung von Duftqualität zu erforschen, sowie Langzeitmechanismen, die der Erkennung komplexer, aus vielen Komponenten bestehender Düfte zugrunde liegen.

III. General Introduction

Cooperation in social groups is beneficial for group members and has been described throughout the animal kingdom. Examples range from cooperative hunting in wolf packs and prides of lionesses, to cooperative defense and alarm signaling in honey bee colonies and groups of primates, and to cooperative care for young in Florida scrub jays or black-backed jackals, which assist their parents in rearing their siblings [1]. In all cases, individuals do better together than alone. The benefits are apparent when all group members profit without cost. In some cases, however, cooperative behavior may incur costs for an individual, whereas the rest of the group benefits, e.g. when alarm signaling attracts the interest of a predator, while chances of survival of other group members are increased due to the warning. How can such behavior evolve and become evolutionary stable? Ultimately, an animal's reproductive success is measured in how much of the individual's genetic repertoire is transmitted into the next generation. As relatives share parts of their genetic repertoire, animals may indirectly increase their reproductive success, if individuals in family groups assist each other, benefiting from so-called indirect fitness effects. Hamilton referred to this extended concept of fitness as inclusive fitness, and selection acting via indirect fitness has been termed kin selection [1-4]. Cooperative behavior may, thus, become an evolutionarily stable strategy, if it is facilitated via kin selection.

III.1 Eusociality

Cooperation in its most sophisticated form has evolved in eusocial species. Eusociality is characterized by cooperative brood care, an overlap of at least two generations, and most importantly, reproductive division of labor, where the majority of group members forego reproduction on their own and rather assist close relatives in raising their offspring [5]. Eusocial behavior has been mainly described in social insects (ants, bees, wasps, and termites) [6], but it also occurs in other insects, like aphids [7,8], beetles [9], and thrips [10], a crustacean species, the sponge-dwelling snapping shrimp [11], and in two mammal species, the Damaraland and the naked mole rat [12,13]. Eusociality is most prevalent in ants, bees, and wasps, and it seems that the haplo-diploid sex determination system of Hymenoptera favored the evolution of eusocial colony structures [5,6]. Males hatch from unfertilized, haploid eggs, while females originate from fertilized, diploid ones, and this results in a skewed relationship between sexes and generations within a colony. Sisters are related more closely to each other than to their mother and to their own offspring. Thus, it pays a female to rather assist her mother in producing more reproductive sisters (i.e. new queens) than to reproduce by her own directly, as the former behavior increases the female's inclusive fitness [2,3]. It is important to note that this inclusive fitness benefit only prevails to its full extent in colonies which are both monogynous and monoandrous, i.e. which

have only one single-mated queen. Recently, evidence was provided that monogyny and monoandry are ancestral traits of Hymenopteran species, and this allowed, and probably also promoted, the evolution of eusociality in the order Hymenoptera [14].

An important precondition for the evolution of eusociality is the ability of group members to discriminate kin from non-kin, in order to assist close relatives while avoiding exploitation by rivals. Kin discrimination does not necessarily require true kin recognition – by matter of fact evidence for the later is extremely rare [but see 15]; it may suffice to recognize individuals that belong to some category correlated with kinship. To this end, social Hymenoptera use colony membership as an indicator of kinship [6,16]. The importance of accurate and precise colony recognition (often referred to as nestmate recognition) is exemplified by aggressive defensive behavior, e.g. to defend common resources or reproductive relatives against rivals, where recognition errors swiftly become fatal mistakes.

III.2 Chemical basis of colony recognition

Colony recognition in social insects is mediated by chemical cues [6,16]. The insect cuticle is coated with a hydrophobic lipid layer, and long-chained and low-volatile hydrocarbons (HC), which prevent desiccation and act as a barrier against infection, often contribute to colony recognition [17-21]. In social insects, the profiles of cuticular hydrocarbons (CHC) are complex, multi-component mixtures: for a given species the components are identical, but the ratios differ for different colonies [22,23]. CHC profiles are, thus, colony-specific (colony odor). The chemical composition of colony odors has been investigated most thoroughly in ants and by now ample evidence has been gathered that CHCs are indeed necessary and sufficient to discriminate colony members (nestmates) from foreign workers (non-nestmates) [22,24-29]. HCs are endogenously produced by metabolic activity and the biochemical pathways may be genetically determined. After synthesis, HCs are transported through the hemolymph via lipophorins to the cuticle and the postpharyngeal glands (PPG), a highly specialized organ located in the head and uniquely found in ants [6,19,20]. HCs are stored in the PPGs and applied onto the cuticle via self- and allo-grooming. During trophallaxis, HCs are exchanged between nestmates, which aids to homogenize CHC profiles within a colony. By this the colony odor is more or less unified [30-34]. Colony odors are not totally uniform, though, as different castes (performing distinct tasks like nursing, foraging, and nest maintenance) and life stages within a colony show minor differences in CHC profiles. Furthermore, colony odors are influenced by environmental factors and may change over time in the range of weeks and months [25,35-47]. As a consequence the recognition system has to be plastic in order to adjust to a changing colony odor [21,48-51]. How colony recognition is achieved by the nervous system remains largely elusive, though.

III.3 Neuronal basis of colony recognition

Colony odors are detected and processed by olfaction and the insect olfactory system is well investigated [52]. Particularly, the Hymenopteran olfactory system is characterized by a neuroanatomical compartmentalization, yet the functional role of this organization in not fully understood [23,53].

III.3.1 The insect olfactory system

Insects receive odors via sensilla on their antennae [54]. The sensilla are the functional unit of the antenna and in Hymenoptera each sensillum houses multiple olfactory receptor neurons (ORN), ranging from 5 to about 50 neurons in pore plate and hair sensilla to more than 100 neurons in basiconic sensilla [55-61]. Odor specificity of ORNs is given by the expressed receptor molecule, however, receptor molecules often respond to a range of different odors and ORNs with different odor specificity may be housed in the same sensillum. ORNs are primary, bipolar receptor neurons and their axons project via the double-stranded antennal nerve to the first olfactory neuropil of the insect brain, the antennal lobe (AL). ORNs expressing the same receptor molecule converge onto the same functional units of the AL, spherical structures called glomeruli [62,63]. This organization results in odor-specific, spatial patterns of neuronal activity in the AL (spatial activity patterns), and there are indications that the spatial representation of odors in the AL reflects how an odor is perceived by an animal [odor quality; 64]. In honey bees the AL contains about 160 glomeruli in a peripheral layer around a non-glomerular core [65], while the ALs of all ant species investigated so far consist of several 100 glomeruli arranged in piles or clusters; the AL of e.g. the Florida carpenter ant *Camponotus floridanus* features approximately 460 glomeruli arranged in 7 distinct clusters [66-69]. Glomeruli are interconnected via local interneurons, which allow cross-talk between glomeruli and in this way odor information is reformatted by the antennal lobe network [70,71]. Odor information is further relayed from glomeruli via combinatorial activity of output (projection) neurons to higher integration centers of the insect brain, the mushroom bodies and the lateral horn of the protocerebrum [70,72]. In Hymenoptera, projection neurons innervate the mushroom bodies and the lateral horn via two parallel antenno-protocerebral tracts (APT) and the dual organization of the olfactory pathway may be an adaptation of the Hymenopteran olfactory system to (eu)social life [23,53,69,73]. The separation into a lateral and a medial APT (l- and m-APT) is already evident in the AL, which is separated into two respective hemilobes: The ventral-rostral hemilobe (VR-hemilobe) is innervated by the l-APT and the dorsal-caudal hemilobe (DC-hemilobe) by the m-APT. The temporal structure of odor-induced activity of projection neurons is often complex and we are just beginning to understand the informative value of the temporal pattern of neuronal activity. Synchronous activity of projection neurons may result in coincidental activity of successive

neurons in higher integration centers (e.g. Kenyon cells of the mushroom body), and this may be important for discrimination of behaviorally significant odor mixtures [74-80]. Experience and learning have been shown to cause neuronal plasticity at several levels along the olfactory pathway [23] and with respect to colony recognition such plasticity might be important for updating the recognition system to changing colony odors. However, until now, it is not known at which level of the olfactory system representations of colony odors are classified as being nestmate or non-nestmate specific and what parameters are used by the nervous system for discrimination.

III.3.2 The neuronal template

The question how colony odors are discriminated by social insects has been addressed almost exclusively by behavioral experiments [22]. The results of which led to the current notion that, during colony recognition, colony odors are compared to a neuronal template and a mismatch results in aggression [22,81]. As colony odors change over time such a template has to be constantly updated (template reformation) [48-51]. So far, it remains unclear in which part of the nervous system the neuronal template might be located. In the past, different mechanisms how a neuronal template might be realized in the nervous system have been suggested and they may even act in combination with each other. The classic idea is that sensory information about a colony odor (label) is compared to a neuronal template stored in long-term memory (label-template matching). Learning, long-term memory, and memory retrieval are probably involved in label-template matching and template reformation. Since the mushroom bodies have been shown to be important for learning and memory [82-85] higher integration centers of the insect brain are a possible site for this proposed recognition mechanism.

Another hypothesis is that colony odor information is specifically modified along the olfactory pathway and that these specific modifications act as a template. Several studies showed that learning results in changes of the neuronal representation of odors, e.g. in the AL [86-89]. Template reformation requires constant learning and as a result nestmate (or non-nestmate) colony odor information might be processed specifically in the nervous system to allow discrimination based on classification of colony odor representations.

Recently, a template in form of a sensory on-off filter was suggested. Ozaki et al. [90] described a sensillum on the antenna of *Camponotus japonicus*, which only responded to non-nestmate but not to nestmate colony odor (*sensilla basiconica*). The authors hypothesized that these sensilla are adapted to the constantly present nestmate colony odor and only information about non-nestmates is relayed to the brain (sensory filter). Here, template reformation would be simply resolved as sensory adaptation occurs within minutes [91], while the colony odors changes slowly in the range of weeks and months [25,35-47].

It is not known, which of these mechanisms are used for colony recognition and how template reformation is achieved. Within the framework of this project, a series of experiments were performed to address these questions as detailed below (see *III.5*). As a model system, I studied the Florida carpenter ant *Camponotus floridanus*.

III.4 Model system: the Florida carpenter ant

C. floridanus is an evolutionary-derived eusocial species with colonies consisting of more than 10,000 individuals but only one single-mated queen [92]. Hence, relationship between individuals is not complicated by different patrilines or matrilines within colonies. This is beneficial for studying basic principles of social organization as colony recognition effectively results in kin discrimination. Monoandry and monogyny further result in high genetic homogeneity within colonies and this is advantageous as heritable factors probably influence the composition of colony odor, and thus colony recognition, in this species [93,94]. Workers of *C. floridanus* show distinct colony recognition behavior, which has been studied in great detail [93-96], and are, hence, well suited for behavioral experiments. Furthermore, the nervous system of *C. floridanus* is well investigated and easily accessible for neurophysiological approaches like functional calcium imaging, which is a well established technique in this species [69]. As an additional advantage, *C. floridanus'* CHC profile is well known: it mainly consists of linear and methyl-branched alkanes of chain lengths between C29 and C32 [38,97]. Recently, the genome of *C. floridanus* has been sequenced [98], and this might allow for a whole new range of experimental and methodological approaches in the near future.

III.5 Thesis outline

Between 2007 and 2010, I conducted several behavioral and neurophysiological experiments to:

i) elucidate basic principles underlying the dynamics of template reformation (Chapter 1).

ii) develop an effective stimulation technique for low-volatile colony odors appropriate to study the olfactory system in neurophysiological approaches. To this end, I first investigated, whether colony odors can be detected on contact only or over short distances in a behavioral assay (Chapter 2), and second, tested a thereupon newly developed stimulation technique using neurophysiological approaches (Chapter 3).

iii) investigate how a neuronal template might be realized in the nervous system and what parameters of neuronal activity might be used for classification of colony odors. To address these questions, I measured neuronal correlates of colony odors in the peripheral (ORNs) and the central nervous system (AL) using electroantennography and calcium imaging, respectively (Chapter 4). Furthermore, I used calcium imaging with advanced two-photon microscopy to investigate, whether colony odors are represented

exclusively in single neuroanatomical compartments of the AL or distributed across different compartments (Chapter 5). Distributed activity across compartments would indicate parallel processing of colony odor information and this might be advantageous for discrimination of highly complex colony odors.

In detail, I focused on the following aspects:

1) *Dynamics of template reformation*

   The sensory input of tethered workers was artificially altered by masking their antennae with nestmate or non-nestmate colony odor and the behavioral response towards freely moving nestmates and non-nestmates was recorded after 2 h and after 15 h. In this way, it was tested, whether a change in sensory experience results in a changed acceptance range (indicating a change in the neuronal template) and in which time frame such a change is possible.

2) *Effective distance of colony odor detection*

   Here, dummies loaded with nestmate or non-nestmate colony odor were presented to free-moving, individual workers without allowing the ants to touch the dummies (distance ~1 cm) and the behavioral response was recorded to test, whether contact is necessary for colony recognition. Furthermore, a long-chained hydrocarbon (HC) that is not part of the natural *C. floridanus* CHC profile was added to nestmate colony odor to test, whether an additional low-volatile HC (cis-9-tricosene) would interfere with colony recognition even over a short distance.

3) *Effective stimulus delivery for low-volatile odors in neurophysiological approaches*

   In this study, a newly developed stimulus delivery apparatus was tested using two neurophysiological approaches: sensory responses of ORNs on the antenna were measured using electroantennography and neuronal activity of AL projection neurons was monitored using functional (calcium) imaging. Three odors of different volatility (undecane – highly volatile, nerolic acid – low-volatile, cis-9-tricosene – very low-volatile) were presented either via an air-stream (conventional air-delivered stimulation) or via a dummy positioned close to the antenna (newly developed dummy-delivered stimulation). Neuronal responses to the three odors using both stimulation techniques were compared to test, whether dummy-delivered stimulation is especially advantageous for stimulation with low-volatile odors.

4) *Neuronal correlates of colony odors*

Dummy-delivered stimulation was further improved by moderately heating the dummies and neuronal responses to colony odors were measured at ORN and AL level using electroantennography and calcium imaging, respectively. In this way, I tested whether nestmate and non-nestmate colony odors are represented in the peripheral and the central nervous system. Comparing the spatial activity patterns elicited in the AL by nestmate and non-nestmate colony odor allowed me to analyze whether spatial activity patterns can be used by the nervous system to discriminate complex, multi-component colony odors and classify them as 'friends' and 'foes'.

5) *Exclusive or distributed representation of colony odors in AL compartments*

Distinct AL clusters and the dual olfactory pathway constitute neuroanatomical compartments of the Hymenopteran olfactory system, yet the functional significance of this organization remains elusive. Calcium imaging with a two-photon microscope allowed me to record neuronal activity in response to colony odors in different AL compartments. By this, I tested whether colony odors are represented exclusively in single AL compartments or distributed across different compartments. A distributed representation would indicate parallel processing of colony odor information using the whole AL network. In a pilot experiment, I artificially induced a change in the neuronal template (as described in Chapter 1) and measured the neuronal correlates of different colony odors in a first step to investigate the neuronal processes underlying template reformation.

IV. Chapter 1: Reformation process of the neuronal template for nestmate-recognition cues in the carpenter ant *Camponotus floridanus*.

Abstract

Ants use cuticular hydrocarbons (CHC-profiles) as multicomponent recognition cues to identify colony members (nestmates). Recognition cues (label) are thought to be perceived during ant–ant encounters and compared to a neuronal template that represents the colony label. Over time, the CHC-profile may change, and the template is adjusted accordingly. A phenotype mismatch between label and template, as happens with CHC-profiles of foreign workers (non-nestmates), frequently leads to aggressive behavior. We investigated the template reformation in workers of the carpenter ant *Camponotus floridanus* by masking their antennae with postpharyngeal gland (PPG) extracts from nestmates or non-nestmates. The behavioral response of manipulated workers encountering unmanipulated workers was measured independently after 2 and after 15 h. After 2 h of incubation, workers treated with either of the two PPG-extracts showed low aggression towards nestmates and high aggression towards non-nestmates. In contrast, after 15 h of incubation, workers treated with non-nestmate PPG-extract showed low aggression towards both nestmates and non-nestmates. The slow (>2 h) adjustment of the template indicates a reformation localized in the central nervous system rather than in chemosensory neurons. In addition, our data show that template adjustment to a new CHC-profile does not impair the assessment of the old CHC-profile as nestmate label.

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Journal of Comparative Physiology A, 193, 2007, 993-1000. Reformation process of the neuronal template for nestmate-recognition cues in the carpenter ant *Camponotus floridanus*. Leonhardt S.D., Brandstaetter A.S., and Kleineidam C.J.

The originally published paper is available at:

http://www.springerlink.com/content/n11q2866642267g5/

and in the printed version of this thesis on pages 13-20.

V. Chapter 2: Nestmate recognition in ants is possible without tactile interaction.

Abstract

Ants of the genus Camponotus are able to discriminate recognition cues of colony members (nestmates) from recognition cues of workers of a different colony (non-nestmates) from a distance of 1 cm. Free moving, individual *Camponotus floridanus* workers encountered differently treated dummies on a T-bar and their behavior was recorded. Aggressive behavior was scored as mandibular threat towards dummies. Dummies were treated with hexane extracts of postpharyngeal glands (PPGs) from nestmates or non-nestmates which contain long-chain hydrocarbons in ratios comparable to what is found on the cuticle. The cuticular hydrocarbon profile bears cues which are essential for nestmate recognition. Although workers were prevented from antennating the dummies, they showed significantly less aggressive behavior towards dummies treated with nestmate PPG extracts than towards dummies treated with non-nestmate PPG extracts. In an additional experiment, we show that cis-9-tricosene, an alkene naturally not found in *C. floridanus'* cuticular profile, is behaviorally active and can interfere with nestmate recognition when presented together with a nestmate PPG extract. Our study demonstrates for the first time that the complex multi-component recognition cues can be perceived and discriminated by ants at close range. We conclude that contact chemosensilla are not crucial for nestmate recognition since tactile interaction is not necessary.

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Springer/Kluwer Academic Publishers, Naturwissenschaften, 95(7), 2008, 601-608. Nestmate recognition in ants is possible without tactile interaction. Brandstaetter A.S., Endler A., and Kleineidam C.J.

The originally published paper is available at:
http://www.springerlink.com/content/2305g5446h88xu38/
and in the printed version of this thesis on pages 22-29.

VI. Chapter 3: Dummies versus air puffs: efficient stimulus delivery for low-volatile odors.

Abstract

Aiming to unravel how animals perceive odors, a variety of neurophysiological techniques are used today. For olfactory stimulation, odors are commonly incorporated into a constant airstream that carries odor molecules to the receptor organ (air-delivered stimulation). Such odor delivery works well for odors of high volatility (naturally effective over long distances) but less or not at all for low-volatile odors (usually only received at short range). We developed a new odor stimulation technique especially suited for low-volatile odors and compared it with conventional air-delivered stimulation using 2 neurophysiological approaches. Odor-loaded dummies were moved into close vicinity of the receptor organs on the antenna of the Florida carpenter ant *Camponotus floridanus* (dummy-delivered stimulation). Neuronal activity was monitored either at receptor neuron level using electroantennography or in the first olfactory neuropile, the antennal lobes, using calcium imaging. We tested 3 odors of different volatility: *C. floridanus'* highly volatile alarm pheromone undecane, its low-volatile trail pheromone nerolic acid, and an even less volatile, behaviorally active C23 alkene, cis-9-tricosene. For low-volatile odors, dummy-delivered stimulation was particularly efficient. We conclude that dummy-delivered stimulation is advantageous compared to the commonly used air-delivered stimulation when studying an animal's detection and processing of low-volatile odors.

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Chemical Senses, 35(4), 2010, 323-333. Dummies versus air puffs: efficient stimulus delivery for low-volatile odors. Brandstaetter A.S., Rössler W., and Kleineidam C.J.

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http://chemse.oxfordjournals.org/content/35/4/323.abstract?keytype=ref&ijkey=saPQQYovYKBhMvH

and in the printed version of this thesis on pages 31-41.

VII. Chapter 4: Friends and foes from an ant brain's point of view – neuronal correlates of colony odors in a social insect.

Abstract

Successful cooperation depends on reliable identification of friends and foes. Social insects discriminate colony members (nestmates/friends) from foreign workers (non-nestmates/foes) by colony-specific, multi-component colony-odors. Traditionally, complex processing in the brain has been regarded as crucial for colony recognition. Odor information is represented as spatial patterns of activity and processed in the primary olfactory neuropile, the antennal lobe (AL) of insects, which is the analog to the vertebrate olfactory bulb. Correlative evidence indicates that the spatial activity patterns reflect odor quality, i.e. how an odor is perceived. For colony-odors, alternatively, a sensory filter in the peripheral nervous system was suggested, causing specific anosmia to nestmate colony-odors. Here, we investigate neuronal correlates of colony-odors in the brain of a social insect to directly test whether they are anosmic to nestmate colony-odors and whether spatial activity patterns in the AL can predict how odor qualities like 'friend' and 'foe' are attributed to colony-odors. Using ant-dummies that mimic natural conditions, we presented colony-odors and investigated their neuronal representation in the ant *Camponotus floridanus*. Nestmate and non-nestmate colony-odors elicited neuronal activity: in the periphery, we recorded sensory responses of olfactory receptor neurons (electroantennography), and in the brain, we measured colony-odor specific spatial activity patterns in the AL (calcium imaging). Surprisingly, upon repeated stimulation with the same colony-odor, spatial activity patterns were variable, and as variable as activity patterns elicited by different colony-odors. Ants are not anosmic to nestmate colony-odors. However, spatial activity patterns in the AL alone do not provide sufficient information for colony-odor discrimination and this finding challenges the current notion of how odor quality is coded. Our result illustrates the enormous challenge for the nervous system to classify multi-component odors and indicates that other neuronal parameters, e.g. precise timing of neuronal activity, are likely necessary for attribution of odor quality to multi-component odors.

This chapter is based on a pre-edited manuscript. The finally published paper is available at:
http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0021383

PLoS One, 6(6), 2011, e21383. Friends and foes from an ant brain's point of view – neuronal correlates of colony odors in a social insect. Brandstaetter A.S., Rössler W., and Kleineidam C.J.

Introduction

Eusocial insects live in complex societies, where the majority of individuals forego reproduction [6,99]. Instead, the colony benefits from cooperation, and ultimately, supporting the reproduction of closely related kin results in an indirect fitness gain for colony members [2,3,14]. In order to defend common resources and reproductive relatives against rivals, it is of paramount importance for social insects to discriminate members of their own colony (nestmates) from members of foreign colonies (non-nestmates). Colony recognition in social insects is mediated by chemical cues found on the cuticle [6]. The insect cuticle is coated with a hydrophobic layer of long-chained and low-volatile hydrocarbons, originally acting as a barrier against infection and desiccation [17,18]. In social insects, these cuticular hydrocarbons (CHC) are complex, multi-component mixtures. For a given species the components of the CHC profiles are identical, however, they differ in the ratios of components across colonies. Hence, CHC profiles are colony specific (colony odor). The chemical basis of colony recognition has been investigated most thoroughly in ants [22,24-29], yet the neuronal processes used to discriminate nestmates from non-nestmates remain elusive.

Ants detect and discriminate colony odors either by directly contacting another ant with their antennae or when antennating close-by [16,22,100]. The olfactory pathway of Hymenoptera is well investigated [68,69,73] and has been reviewed in great detail recently [23,53]. Odors are received by olfactory receptor neurons (ORNs) housed in olfactory sensilla of the antenna. From there, olfactory information is relayed to functional units (glomeruli) in the first olfactory neuropile of the insect brain, the antennal lobe (AL). The insect antennal lobe is analogous to the vertebrate olfactory bulb and similar information processing mechanisms seem to act in both [101,102]. Glomeruli are sites of synaptic interaction between ORNs, local interneurons, and output (projection) neurons. Ensembles of projection neurons relay olfactory information as a combinatorial code to higher integration centers of the insect brain (mushroom bodies and lateral horn). Since odors activate specific subsets of ORNs, this results in an odor specific glomerular activation patterns in the AL [spatial activity patterns; 103]. Earlier studies revealed that odors, which elicit similar spatial activity patterns in the AL, are perceived similarly, i.e. a similar odor quality is attributed [64,75]. This correlation led to the suggestion that the brain readily uses activity patterns in the AL to assess odor quality. It has never been investigated whether different colony odors are represented as distinct activity patterns in the AL, and it is not known at which level of the olfactory system the odor quality ‘nestmate’ or ‘non-nestmate’ is attributed to the neuronal representation.

Traditionally, it is assumed that colony odor is compared to a neuronal template located somewhere in the nervous system and any mismatch between colony odor and neuronal

template results in aggression [22,81]. Colony odors are a variable cue and may change over time in the range of weeks and months as they are influenced by environmental factors and vary with age, reproductive status, and/or caste [35-44]. As a consequence, a neuronal template has to be constantly updated [48-51]. Different mechanisms of how a neuronal template might be realized in the nervous system have been proposed and may even act in combination with each other. According to the classic idea, an internal representation of nestmate colony odor is stored as a template in higher integration centers of the insect brain, e.g. mushroom bodies and/or lateral horn [22,81]. Sensory information is compared to the internal representation and this eventually results in recognition. Another possible mechanism is that neuronal representation of nestmate or non-nestmate colony odor is specifically modified along the olfactory pathway, with the specific modifications acting as a template. It has been shown that learning results in changes of the neuronal representation of odors along the olfactory pathway, e.g. in the AL [86-89].

Alternatively, a sensory on-off filter in the periphery of the nervous system has been suggested to act as a template. Ozaki et al. [90] described an olfactory sensillum on the antenna of the ant *Camponotus japonicus* which only responded to non-nestmate, but not to nestmate colony odor. The authors suggested that the ORNs are "desensitized" to nestmates, e.g. by sensory adaptation to the constantly present nestmate colony odor. Hence, only non-nestmate specific information is relayed to the central nervous system (sensory filter), while ants are specifically anosmic to nestmate colony odor. This hypothesis is appealing due to its simplicity and it had a profound impact on the research field of colony recognition as it fundamentally challenges our current notion of how social insects identify nestmates and non-nestmates, namely by attributing the meaning 'friend' or 'foe' to a neuronal representation in the brain. However, the hypothesis of a template in form of a sensory filter fails to explain how social insects can discriminate between members of different castes and life stages within their colony under conditions in which nestmates were not detected [25,43,45-47]. Therefore, it is important to scrutinize the general validity of the suggested sensory filter hypothesis.

In a first step to understand how odor quality of colony odors is coded and how a neuronal template might be realized in the nervous system, we investigated the neuronal representation of colony odors at two levels of the olfactory system in the Florida carpenter ant *Camponotus floridanus* using a recently developed stimulation technique [104]. First, we measured neuronal responses of ORNs of the antenna to nestmate and non-nestmate colony odors by electroantennography. Second, we used calcium imaging to monitor spatial activity patterns of projection neurons of the AL and analyzed, whether different colony odors elicit distinct activity patterns. Our results show that both nestmate and non-nestmate colony odor elicit spatial

activity patterns in the AL. However, these spatial activity patterns alone are not sufficient for discrimination of nestmate and non-nestmate colony odor. Finally, we discuss which neuronal parameters of the combinatorial code of projection neurons are possibly used for quality coding of complex colony odors.

Results

Electroantennography

We used electroantennography (EAG) as a simple neurophysiological technique to test whether ORNs of the antenna respond to colony odors of nestmates and non-nestmates. For stimulation, we used heated dummies [dummy-delivered stimulation; see 104] loaded with NM, nNM1, nNM2 and control (see Tab. 1 for abbreviations). EAG revealed pronounced responses to colony odors in 8 antennal preparations. Within some preparations, repeated measurements were performed, which were pooled before the mean response curves for the 8 preparations were calculated (Fig. 1). NM, nNM1, and nNM2 elicited voltage responses with signal amplitudes in the range of around 0.7 mV. In contrast, control stimulation resulted in considerably weaker signal amplitude of around 0.25 mV, which might have been induced by solvent residues and/or an increased temperature at the antennae due to the heated dummy. The results demonstrate that dummy-delivered stimulation with both nestmate and non-nestmate colony odors evoked EAG amplitudes in a similar range.

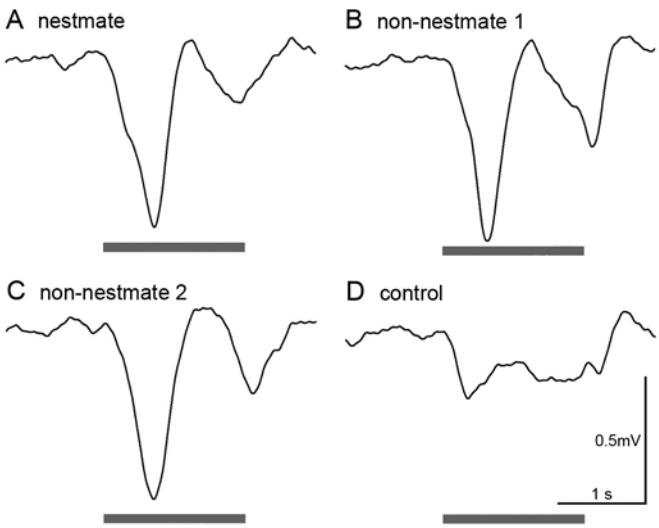

Figure 1. Electroantennography. Neuronal responses of olfactory receptor neurons (amplitude: ~ 0.7 mV) were measured upon stimulation with colony odor from nestmates (A), non-nestmates from the same population as nestmates (B; non-nestmate 1), and non-nestmates from a different population (C; non-nestmate 2). Presentation of a solvent-loaded and heated dummy (D; control) resulted in a comparably weak voltage response, probably induced by the solvent, and/or the increased temperature of the dummy. A grey bar indicates the stimulation period of 1.6 s. Repeated measurements within preparations were pooled and the mean responses of recordings from 8 antennal preparations were calculated.

Calcium imaging

Calcium imaging allows monitoring of neuronal activity by measuring changes in intracellular calcium levels using fluorescent calcium indicators, a technique that has been repeatedly used in ants [69,101,104-106]. As a test stimulus for functionality, we presented a general odor delivered via an air-stream (air-delivered octanol at a dilution of 10^{-1}) and measured neuronal activity in 22 animals. For colony odor stimulation we used NM, nNM1, nNM2, nNM3, and control (see Tab. 1). In 8 preparations all odors were tested at least twice.

Table 1. Abbreviations of colony odor stimuli presented on heated dummies.

Abbr.	colony odor extracts from
NM	nestmates, collected from the same colony
nNM1	non-nestmates of the same population as nestmates
nNM2	non-nestmates of a different population as nestmates
nNM3	non-nestmates of a different species (*C. rufipes*)
control	solvent only, no extract

NM and the three different non-nestmate colony odors (nNM) elicited neuronal activity in the AL with response intensities in a similar range (Fig. 2 A&C). No response was measured upon control stimulation (Fig. S1). Across animals, colony odor stimulation showed highly variable neuronal activity patterns (Fig. 2 C&D). This variability can be expected as colony odors change over time [42,49,50], and measurements were performed over the course of several months. Furthermore, activity patterns cannot be easily compared across individuals, as the AL of *C. floridanus* comprises ~450 small and densely-packed glomeruli [69] and, hence, calcium signals cannot be assigned to individual identified glomeruli. Therefore, in the following analyses neuronal activity patterns in response to different colony odors were compared exclusively within animals.

Within individual ants, NM and nNM activated similar AL regions (Fig. 2 A&C), i.e. spatial activity patterns were largely overlapping. In contrast, the spatial activity patterns in response to air-delivered octanol differed considerably from activity patterns elicited by colony odors (cp. Fig. 2 D&E). Repeated stimulation with octanol resulted in consistent activity patterns (Fig. 2 E&F), as shown earlier in another study [69], whereas repeated stimulation with colony odor resulted in surprisingly variable neuronal responses in terms of intensity ranges and activity patterns (Fig. 2 B&C). Octanol and colony odor were presented with different stimulation techniques (air- and dummy-delivered stimulation, respectively), and therefore we did not analyze octanol elicited activity patterns any further. It is important to note, though, that dummy-delivered stimulation with a single-component odor (nerolic acid) elicited stable activity patterns in an earlier study [104], and hence, the variability in activity patterns we measured in response to colony odors cannot be simply attributed to the stimulation technique we used.

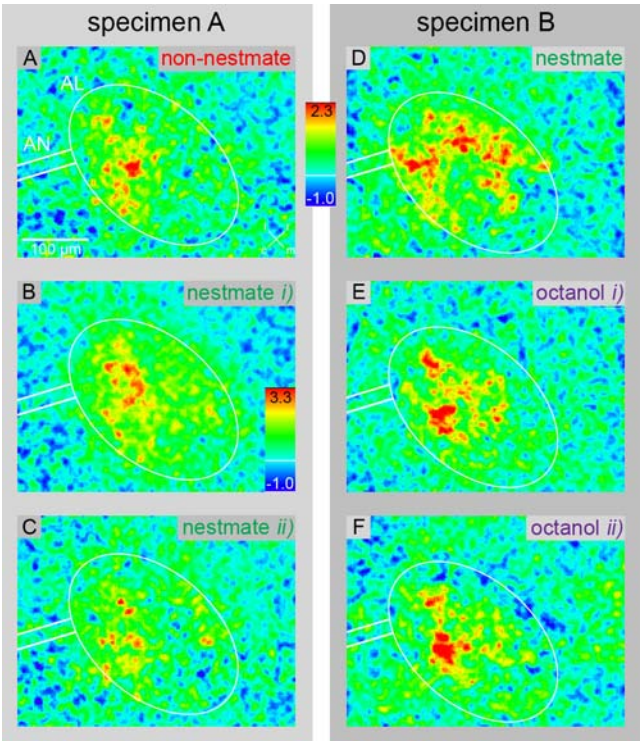

Figure 2. False-color coded neuronal activity (calcium imaging) in the antennal lobe (AL), in response to different odors. Examples of 2 different individuals (specimen A and B). Dummy-delivered stimulation with non-nestmate (A; different population as nestmate) and nestmate colony odor (C; NM) resulted in neuronal activity within the same region of the AL and in a similar range of intensities. Neuronal activity induced by NM was highly variable across animals (cp. C&D). Air-delivered octanol stimulation resulted in activity patterns that clearly differ from NM responses (cp. D&E). Repeated stimulation with octanol resulted in a consistent neuronal representation (cp. E&F), whereas spatial activity patterns and response intensity upon repeated NM stimulation were variable (cp. B&C). Time period between repeated stimulations was at least 24 min. Red indicates areas of high neuronal activity and a colored bar denotes the fluorescence change [ΔF/F]. To visualize the spatial activity pattern, intensity range of B is individually scaled as indicated by the individual scale bar.

In order to quantify variability between neuronal representations of NM and nNM, we performed a correlation analysis. The global intensity level of neuronal responses is not taken into account in a correlation analysis, and this allowed us to directly compare the spatial activity patterns elicited by different colony odors. We reduced the spatial resolution of the calcium image stacks to reduce noise level. Low-resolution activity patterns in response to NM and nNM looked very similar, but activity patterns still depicted distinct differences between activity patterns of e.g. NM and octanol (Fig. S2). Within animals, we calculated the coefficients of correlation over time by pair-wise comparing i) neuronal responses upon repeated stimulation with the same odor (equal odor pairs) and ii) responses upon NM stimulation to responses upon stimulation with another odor (unequal odor pairs).

For visualization, we pooled the coefficients of correlation of corresponding odor pairs of all 8 animals by calculating the median and plotted those of NM-NM and unequal odor pairs (Fig. 3). Prior to stimulation, correlation was close to 0. During stimulus presentation, correlation increased considerably for NM-NM and NM-nNM1/2/3, and decreased back to baseline after the end of stimulation. For NM-control, correlation remained low during the whole recording.

Coefficients of correlation of equal odor pairs (repeated stimulation with the same odor) were all in the same range upon stimulation (Tab. S1). To test whether the plotted coefficients of correlation for NM-NM and unequal odor pairs differed significantly during the stimulation period, we used a Friedman test and found a significant difference (Friedman rank sum test; chi^2 = 16.6, DF = 4, p = 0.0023). To test whether differences are due to the low coefficient of correlation for NM-control, we excluded this pair and performed a second Friedman test. No significant difference was found (chi^2 = 3.75, DF = 3, p = 0.2898).

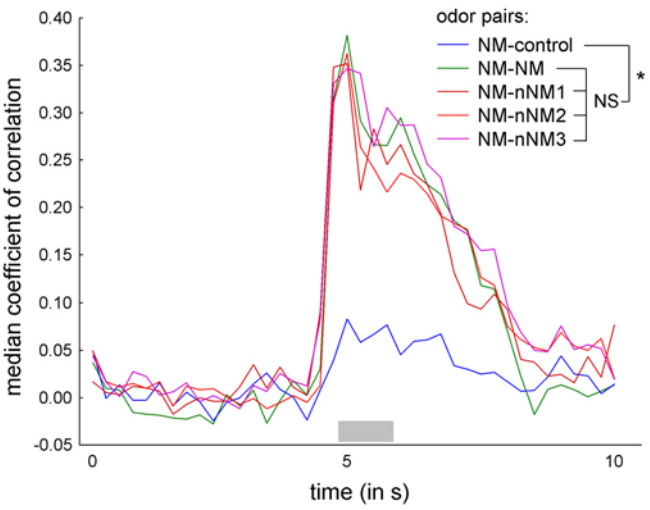

Figure 3. Correlation analysis of neuronal responses to different colony odors. In order to compare the variability in activity patterns elicited by different colony odors, coefficients of correlation were calculated comparing repeated stimulation with nestmate colony odor (NM-NM; see Tab. 1 for abbreviations), stimulation with nestmate to different non-nestmate colony odors (NM-nNM1/2/3), and nestmate colony odor to control stimulation (NM-control) within 8 animals. Prior to stimulation, coefficients of correlation are close to 0 for all odor pairs. Upon stimulation (a grey bar indicates the stimulation period of 1 s), coefficients of correlation increase considerably for NM-NM and NM-nNM1/2/3, whereas they remain low for NM-control. After stimulation, coefficients of correlation return to baseline. A Friedman test revealed a significant difference in the coefficients of correlation during stimulation, whereas a second Friedman test where NM-control was excluded showed that the coefficients of correlation for NM-NM and NM-nNM1/2/3 are not significantly different.

In summary, we find that the correlation of activity patterns elicited by repeated NM stimulation was not significantly different from the correlation of activity patterns elicited by stimulation with different colony odors (i.e. unequal colony odor pairs: NM-nNM1/2/3). Based on our correlation analysis, we conclude that on a large scale colony odors elicit similar spatial activity patterns in the AL. Within this large scale of colony odor representations, both, the activity patterns for nestmate and non-nestmate colony odor are variable to a similar extent. Thus, the spatial representation of nestmate- and non-nestmate is not specific enough to provide the nervous system with sufficient information for discrimination.

Discussion

In this study, we measured neuronal correlates of colony odors at two levels of the olfactory system of the carpenter ant *Camponotus floridanus*. Our results provide neurophysiological evidence that ants can perceive colony odors from both, nestmates and non-nestmates, contradicting the sensory filter hypothesis for colony recognition. At the level of the antennal lobe (AL; projection neurons) spatial activity patterns in response to colony odors were variable – even upon repeated stimulation with the same colony odor – and we did not find any significant differences in activity patterns upon stimulation with different colony odors. Thus, spatial activity patterns alone are not sufficient to classify colony odors as nestmate or non-nestmate specific. Nevertheless, behavioral experiments show that the nervous system must be able to classify nestmate and non-nestmate colony odors [93-96,100,107], despite the variable neuronal representation of complex, multi-component odors that we found in this study. Our results raise the question which parameters of neuronal activity are used besides spatial activity patterns to assess odor quality.

Both, electroantennography and calcium imaging, revealed neuronal activity in response to stimulation with nestmate colony odor in olfactory receptor neurons (ORNs) of the antenna and in projection neurons of the AL. There were no pronounced differences in the summed voltage responses of ORNs and in the spatial activity patterns in the AL elicited by nestmate and non-nestmate colony odor. This finding clearly contradicts the model proposed by Ozaki et al. on the closely related ant species *C. japonicus* [90] that complete adaptation to the nestmate specific ratios of cuticular hydrocarbons blocks perception of nestmate odor at the level of the antennal sensilla (nestmate specific anosmia). As the olfactory system in both *Camponotus* species is similarly organized [68,69], we conclude that a neuronal template for colony recognition is extremely unlikely to be implemented in form of a sensory on-off filter at the level of ORNs in the antenna of ants. Our conclusion is also supported by other studies, which consistently showed that template reformation, i.e. the process of updating the neuronal template to a changing colony odor is a relatively slow process, taking several hours [107,108], which is much longer than the time period expected for sensory adaptation at antennal ORN level.

What causes the high variability of spatial activity patterns within individuals as measured in response to repeated stimulation with the same colony odor? We obtained colony odors from extracts of postpharyngeal glands, which contain the same components at equivalent ratios as the CHC profile [24,30,33]. These extracts were readily discriminated by ants even without physical contact to the extract-loaded dummies [100]. Compared to an earlier study [104], we improved stimulus application by moderately heating the dummies to increase colony odor concentration in the headspace of dummies. Recently, a number of temperature-sensitive

glomeruli have been reported for the dorsal region of the AL in leaf-cutting ants [109]. However, we did not measure any unspecific temperature responses, probably because we were investigating the anterior part of the AL. In a behavioral experiment conducted in parallel to this study, we assured that the quality of the stimulus was not changed by the increased temperature, and workers significantly discriminated between heated dummies loaded with nestmate and non-nestmate colony odors without the need for tactile interaction. Whereas dummy-delivered stimulation with multi-component colony odors resulted in variable neuronal responses within animals, an earlier study showed that dummy-delivered stimulation with a single component, namely nerolic acid, the releaser component of *C. floridanus*' trail pheromone, resulted in stable spatial activity patterns across individuals and trials [104]. The same was true for air-delivered stimulation with nerolic acid [69]. We conclude that the variable neuronal responses to colony odors cannot originate from our dummy-delivered stimulation *per se*.

Individual components of colony odors have different chemo-physical properties. Depending on their vapor pressure, temperature, and humidity they evaporate into headspace at different rates. Thus, the multi-component odor stimulus arriving at the antenna of an ant not only depends on the chemical composition of the colony odor, but may also vary depending on external physical factors like temperature, humidity as well as the distance and diffusion rate between colony odor source and receiver. A recent study in moth showed that the ratios of odor components can vary to some degree without reducing its behavioral effect [110]. Likewise, ants accurately discriminate nestmates from non-nestmates despite the highly variable nature of the colony odor stimulus, be it on direct contact or over short distances and in a wide range of environmental conditions [16,22]. We propose that the variable activity patterns that we measured in response to repeated stimulation with the same colony odor within individuals reflects the natural variability of the multi-component colony odor stimulus. For presentation of colony odors, we used a stimulation technique resembling the natural situation by simulating close-range colony odor detection from a nearby nestmate or non-nestmate. Although experimental conditions were kept as constant as possible, the variable nature of the colony odor stimulus cannot be impeded as even minute differences in external factors may influence the composition of the low-volatile, multi-component colony odor stimulus. The high variability of the colony odor stimulus under controlled experimental conditions suggests that even higher variability occurs in the natural habitat. Olfactory information is integrated and processed in the AL network by interactions between glomeruli via local interneurons [103,111]. Detection and discrimination of complex, multi-component odors probably involves extensive neuronal processing, and even subtle differences of the odor stimuli may affect the resulting spatial

activity patterns [110,112]. However, to allow accurate colony recognition, the nervous system needs to classify colony odors as nestmate and non-nestmate specific despite their variable neuronal representation.

Which parameters are used by the nervous system to classify colony odors? It has been shown that spatial activity patterns highly correlate with perceived odor quality [64,75]. However, here we show that different colony odors activated largely overlapping AL areas. Overlapping and equally variable spatial activity patterns for different colony odors may be expected, given that the chemical profiles of nestmate and non-nestmate colony odor contain the same chemical components, only at differing ratios. Interestingly, spatial activity patterns upon stimulation with colony odor of another *Camponotus* species (*C. rufipes*) were also not significantly different from activity patterns elicited by colony odors of *C. floridanus*. Both, *C. rufipes* and *C. floridanus'* colony odors probably contain linear and methyl-branched alkanes within the same range of chain length, and a large overlap of chemical profiles would explain the similarity of neuronal responses elicited by *C. floridanus* and *C. rufipes* colony odors. We suggest that the overlapping spatial activity patterns we measured may code for the general odor quality 'colony odor'. If the spatial activity patterns are variable like shown here for the case of colony odors, either many patterns have to be learned in order to discriminate nestmates from non-nestmates or other parameters besides the spatial activity pattern are used for colony odor classification. Several studies emphasize the importance of precise timing of neuronal activity for discrimination of chemically similar odors and odor blends [74-79]. The complex interplay between glomeruli via local interneurons results in distinct temporal firing patterns of projection neurons of the AL, which may be specifically modified (e.g. as a result of template reformation, i.e. learning). Specific colony odors may then result in synchronous activity in ensembles of projection neurons leading to patterns of coincidence in postsynaptic neurons at the next levels of the olfactory pathway, i.e. the mushroom bodies or the lateral horn. Thus, temporal activity patterns of AL projection neurons may suffice to code for nestmate or non-nestmate specificity. Furthermore, distinct spatio-temporal activity patterns in higher integration centers of the insect brain (e.g. Kenyon cells in the mushroom bodies) may be compared to a template stored in long-term memory, which then results in recognition. Memory consolidation is accompanied by a calcium induced long-term structural rearrangement of mushroom body synapses [85,113] and this may be important for template reformation.

As our present study clearly shows that ants are not anosmic to nestmate colony odors and that information about different colony odors are transferred equally to olfactory centers in the brain, the future challenge is to unveil what kind of information is used to classify nestmate and non-nestmate colony odors, and in general, how insects assess the quality of multi-component

odors. Natural, multi-component odors constitute varying and fluctuating stimuli, and most probably animals are generally faced with the problem that these elicit variable neuronal responses which have to be classified correctly by the nervous system to allow accurate odor recognition. Colony recognition in social insects is an excellent model system to study the coding of odor quality and long-term memory mechanisms underlying recognition of complex, multi-component odors, as it allows investigating the neuronal representation of the same odor stimulus with potentially opposing attributes: friend or foe.

Materials and Methods

Ethics Statement

The performed experiments comply with the current laws of the Federal Republic of Germany and collection of founding queens for laboratory colonies conformed to the laws of the United States of America and the Oriental Republic of Uruguay effective at time of collection.

Animals

C. floridanus is an evolutionary-derived eusocial species with colonies consisting of more than 10,000 individuals but only one single-mated queen [92]. Genetic homogeneity within colonies is high and heritable components of the colony odor are probably important for colony recognition in this species [93,94]. Workers show distinct colony recognition behavior, which has been studied in great detail [93-96]. Their cuticular hydrocarbon profiles mainly consist of linear and methyl-branched alkanes of chain lengths between C29 and C32 [38,97].

Experimental colonies were raised from founding queens collected by A. Endler and S. Diedering in Florida (USA) at Florida Keys after mating flight. Colonies were kept in the laboratory in artificial plaster nests at a constant temperature of 25 °C and 50% humidity (12h/12h photoperiod) and provided with artificial diet [114], honey-water, and dead cockroaches (*Nauphoeta cinerea*) twice a week and water ad libitum. Colony size was approximately 4000 ants. Neurophysiological experiments were conducted with large workers (head width > 3 mm) from a colony, with a founding queen collected at Sugarloaf Shores in July 2003 and nestmate colony odor was obtained from small workers (head width < 3 mm) of the same colony (NM). Non-nestmate colony odors were obtained from small workers, whose founding queens had been collected at Sugarloaf Shores in July 2002 and 2003 (same population as nestmates; nNM1), and Orchid Island in August 2001 (different population than nestmates; nNM2), respectively. Non-nestmate colony odor of a different species was obtained from small workers of a *Camponotus rufipes* colony, with a founding queen collected in La Pedreras (Uruguay) by O. Geissler in December 2002 (nNM3). Rearing conditions were identical to those of *C. floridanus* colonies. Abbreviations for colony odor stimuli are described in Tab. 1.

Colony odor extraction

Colony odors were obtained from postpharyngeal glands (PPG), which contain the same components as the colony odor found on the cuticle in equivalent ratios [24,30,33]. PPGs were dissected and extracted in hexane for at least 2 h before loading them onto dummies as described in detail previously [100]. As colony odors change over time in the range of weeks and months [42,49,50], we used only PPG extracts which had been prepared maximally 5 days in advance. PPG extracts contain remarkably less short-chain components (which do not belong to the hydrocarbons constituting the colony odor) than hexane cuticle washes [100].

Stimulus delivery

For stimulation with colony odors, we used a recently developed stimulus delivery technique, which closely mimics the natural situation of odor dispersal from solid surfaces like e.g. an insect cuticle: a dummy is loaded with an odor and moved into close vicinity of the antenna. This has been shown to be advantageous for stimulation with low-volatile odors [104]. In order to further increase colony odor concentration in headspace, dummies were heated to a temperature of 40 °C before applying the colony odor (EAG: KTY temperature sensor heated by a constant current power source, Conrad Electronic SE; calcium imaging: Firerod Cartridge Heater operated by a F4SL ramping temperature controller, Watlow GmbH). Prior to stimulation, hexane-rinsed dummies were loaded with 20 µl of colony odor using hexane-rinsed Hamilton syringes (Hamilton Company), and the solvent was allowed to evaporate for 2 min. Room temperature was kept constant at 25 °C.

For EAG recordings, a colony odor was presented 2 to 3 times with an inter-stimulus-interval of ~1 min. Subsequently, a different colony odor was presented. The overall sequence of colony odors was pseudo-random. For calcium imaging, colony odors were presented in a fixed sequence with an inter-stimulus-interval of 4 min as follows: nNM2 – NM – control – nNM1 – nNM3 – control. Again, this stimulation sequence was repeated 2 to 3 times, and the inter-stimulus-interval between repeated stimulation with the same colony odor was at least 24 min.

Electroantennography

A cut antenna of a worker was mounted between 2 chlorinated silver electrodes and the sum potential of ORNs in response to NM, nNM1, and nNM2 during a stimulation period of 1.6 s was measured. For each odor, sensory responses to repeated stimulation within preparations were pooled and the mean response curves for 8 antennae were calculated. Details on the experimental setup and data processing have been described earlier [104].

Calcium imaging and data evaluation

Projection neurons of the AL were retrogradely loaded with Fura2-dextran (potassium salt, 10 000 MW, F3029, Molecular Probes), and ratio-metric recordings at 340 and 380 nm excitation wavelength were obtained at a frame rate of 4 Hz as detailed previously [104]. We prepared 172 workers of which 82 (47.7 %) showed bright staining of projection neurons in the AL. Dummy-delivered stimulation with NM, nNM1, nNM2, and nNM3 started 5 s after start of recording for a stimulation period of 1 s.

Imaging data were analyzed using custom software written in Interactive Data Language (IDL 6.0; ITT Visual Information Solutions, Boulder, CO, USA) by Giovanni Galizia and Mathias Ditzen (University of Konstanz, Germany). We calculated the ratio of fluorescence intensity of the images taken at 340 and 380 nm excitation wavelength for each pair as: $R = F_{340}/F_{380}$ and corrected manually for possible movement of the AL between measurements. To visualize neuronal responses to the different colony odors as false-color coded images, we subtracted the average of 3 frames prior to stimulation from the average of 3 frames during stimulation.

In order to quantify variability in neuronal responses to different colony odors, we compared neuronal activity patterns using a pixel-based Pearson's product-moment correlation analysis over time (MS Office Excel 2007 SP2). We reduced noise by reducing the spatial resolution of image stacks by a factor of 8. This resulted in a pixel size of 20 x 20 µm, which approximately corresponds to the size of one glomerulus in *C. floridanus* and suffices to discriminate distinct spatial activity patterns (cp. Fig. S2 E&F). To compensate for different onset of neuronal responses, we calculated the coefficients of correlation for a floating time window of 4 frames (1 s), which moved frame-by-frame through the whole recording time of 40 frames (10 s). Because of the high number of glomeruli in the AL of *C. floridanus* [69], calcium signals could not be assigned to identified glomeruli, as it is possible e.g. in *Apis mellifera* [65]. For this reason, neuronal activation patterns were only compared within individual animals. Pearson's coefficient of correlation was calculated pairwise, i) for equal odor pairs, i.e. for repeated stimulation with the same odor, comparing 1st stimulation with odor A to 2nd stimulation with odor A (A1-A2) and ii) for unequal odor pairs, i.e. for stimulation with two different odors (see Tab.1 for abbreviations). In order to correct for possible effects of stimulation sequence, we calculated 2 coefficients of correlation for unequal odor pairs, comparing 1st stimulation with odor A to 2nd stimulation with odor B and vice versa (A1-B2 and A2-B1), and used their median for further analysis. For repeated odor stimulations within each individual, we calculated the median of the coefficients of correlation for all possible odor pairs, and used these medians for further analysis. For visualization, coefficients of correlation for NM-NM and unequal odor pairs of all 8 animals were pooled (by calculating median curves) and plotted (Statistica 9.1, Statsoft).

We tested for significant differences in coefficients of correlation of the equal odor pair NM-NM and unequal odor pairs within individual animals during stimulus presentation using a Friedman test (R statistic software 2.10.1, The R Foundation for Statistical Computing). To test the coefficients of correlation across colony odor stimulation only, we performed a second Friedman test excluding NM-control. To correct for multiple testing, we adjusted the significance level to $\alpha = 0.025$, based on a Bonferroni correction.

Acknowledgements

We thank Katrin Vogt for experimental support in electroantennography and Giovanni Galizia for valuable assistance in IDL data analysis.

Authors' contributions

A.S.B. and C.J.K. devised and designed improved dummy-delivered stimulation. A.S.B., W.R., and C.J.K. designed the experimental procedure. A.S.B. collected calcium imaging data and did the data analysis. A.S.B. and C.J.K. wrote and W.R. edited the manuscript. All authors read and approved the final manuscript.

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Figure S1. False-color coded neuronal activity (calcium imaging) in response to control stimulation. Presentation of a heated dummy loaded with solvent only did not result in changes of neuronal activity within the AL.

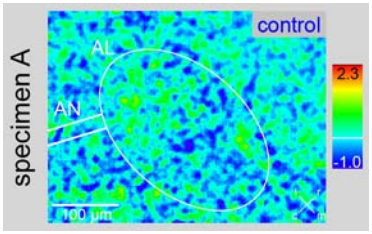

Figure S2. Low-resolution, false-color coded images of neuronal activity (calcium imaging) in the AL of 2 individuals (specimen A&B, see Fig. 2). For the correlation analysis, spatial resolution of the recorded image stacks was reduced to reduce noise and trimmed to an area corresponding to the AL. Spatial activity patterns in response to colony odors appear similar (A-D), whereas the pattern in response to octanol is different from that to nestmate colony odor (E&F; intensity ranges are individually scaled for visualization). Nestmate and non-nestmate 1/2/3 correspond to the abbreviations described in Tab. 1 (NM and nNM1/2/3, respectively).

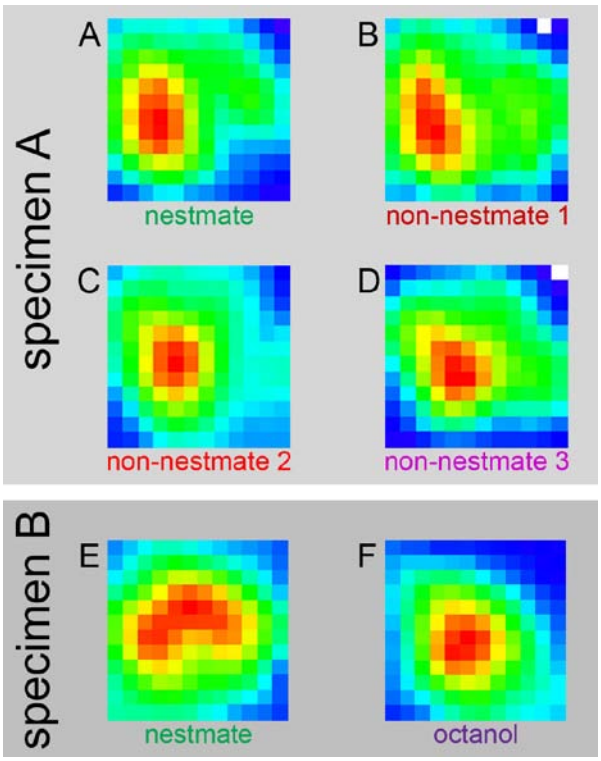

Table S1. Coefficients of correlation of neuronal responses to colony odors.

correlated odor pair	coefficient of correlation		
	minimum	maximum	median
NM-NM	0.090	0.671	0.382
nNM1-nNM1	0.037	0.468	0.347
nNM2-nNM2	0.030	0.631	0.400
nNM3-nNM3	-0.038	0.589	0.297
NM-nNM1	0.046	0.545	0.352
NM-nNM2	0.062	0.454	0.362
NM-nNM3	-0.029	0.610	0.347
NM-control	-0.030	0.329	0.083

Minimal, maximal, and median coefficients of correlation of odor pairs during stimulation are listed (see Tab. 1 for abbreviations). Coefficients of correlation of equal odor pairs (i.e. repeated stimulation with the same colony odor) are all in the same range.

VIII. Chapter 5: Distributed representation of social odors indicates parallel processing in the antennal lobe of ants.

Abstract

Social Hymenoptera, like ants and honey bees, live in complex societies, which are mainly regulated through social odors. Odor information is first processed and represented in spatial activity patterns in the primary olfactory neuropile of the insect brain, the antennal lobe (AL), which is analog to the vertebrate olfactory bulb. The olfactory system is characterized by neuroanatomical compartmentalization, yet the functional significance of this organization is unclear. We investigated the neuronal representation of multi-component colony-odors, which are used to discriminate friends (nestmates) from foes (non-nestmates), in the carpenter ant *Camponotus floridanus*, using two-photon calcium imaging. We measured colony-odor elicited spatial activity patterns, which were distributed across different AL compartments. Activity patterns in response to nestmate and non-nestmate colony-odor were overlapping, and this was expected since both odor cues consist of the same components at differing ratios. Colony-odors change over time and the nervous system has to constantly adjust for this. Measured activity patterns were variable, and variability was higher in response to nestmate than to non-nestmate colony-odor. This finding might indicate that repeated stimulation with colony-odor resulted in plasticity within the olfactory system, particularly in response to nestmate colony-odor stimulation. Furthermore, the lower variability of non-nestmate colony-odor elicited activity patterns might facilitate recognition of non-nestmates at the next level of the olfactory pathway. Our results indicate that information about colony-odors is processed in parallel in different neuroanatomical compartments, using the computational power of the whole AL network. Parallel processing might be advantageous, allowing reliable discrimination of highly complex social odors.

This chapter is based on a pre-edited manuscript. The finally published paper is available at:
http://jn.physiology.org/content/106/5/2437

Journal of Neurophysiology, 106, 2011, 2437-2449. Distributed representation of social odors indicates parallel processing in the antennal lobe of ants. Brandstaetter A.S. and Kleineidam C.J.

Introduction

Social insects live in complex societies, where cooperation ultimately results in a fitness benefit for colony members [6]. In colonies of eusocial Hymenoptera, like ants or honey bees, cooperation is organized through social odors, and particularly ants rely on a sophisticated odor (pheromone) communication system [16]. Pheromones are used to facilitate recruitment, to mark trails to profitable food sources, and to signal alarm when the colony is under attack [115]. Furthermore, low-volatile substances on the cuticle (cuticular hydrocarbons, CHC) serve as intra- and inter-specific recognition cues: CHCs are used to assess fertility status and inform about caste and colony membership [26,43,45,116-118].

The chemical and behavioral basis of colony recognition has been described in great detail for ants [21,22]. For a given species, CHC profiles consist of the same multiple components, yet different ratios of the components provide colony-specificity. Ants use these colony-specific CHC profiles (colony odor) to discriminate between colony members (nestmates) and foreign workers (non-nestmates) [24,26,27,29]. Despite their very low-volatility, colony odors can be detected by olfactory sensilla over short distances [100].

According to the common notion, a detected colony odor (label) is compared to a neuronal template that is located in a so far unidentified region of the nervous system (label-template matching). Any mismatch between label and template results in aggression [81]. Colony odors are not stable, but change over time in the course of weeks and months as they are influenced by environmental factors and vary with age, reproductive status, and/or caste membership of the bearer [39,41-43]. Consequently, the neuronal template needs to be continuously updated, a process called template reformation [48-51]. It has been shown that template reformation is a relatively slow process requiring several hours if induced artificially, and during this learning process social interaction is not required [107,108,119].

Alternatively, a sensory filter in the periphery of the nervous system has been suggested to act as a template [90]. Ozaki et al. described a sensillum (*sensilla basiconica*) on the antennae of *Camponotus japonicus*, which is CHC-sensitive. The authors reported *S. basiconica* to be selectively activated by non-nestmate colony odor only. According to their hypothesis, sensory adaptation causes specific anosmia to nestmate colony odor and only information about non-nestmates is relayed to the brain. It remains elusive how colony odors are processed by the nervous system and the mechanism by which the olfactory system allows reliable recognition of nestmates and non-nestmates is unknown.

The insect olfactory system is well investigated [52]. Odors are received at olfactory receptor neurons (ORN) housed in olfactory sensilla on the antenna. ORN axons are bundled in two antennal nerves that reach the antennal lobe (AL), the first olfactory neuropile of the insect

brain. The insect AL is the analog to the vertebrate olfactory bulb and similar odor processing mechanisms seem to act in both [101,102]. Axons of ORNs of similar type terminate in single glomeruli, which constitute the functional units of the AL. [63,120]. Odor-induced activation of ORNs results in glomerular patterns of activity in the AL (spatial activity pattern). Glomeruli are densely interconnected via local interneurons and olfactory information is processed within the antennal lobe network [112,121]. Processed odor information is further relayed by AL output neurons (projection neurons, PN), which project to higher integration centers of the insect brain (mushroom bodies and lateral protocerebrum) through segregated pathways [70,72].

The olfactory system is characterized by neuroanatomical compartmentalization along the olfactory pathway, and neuronal compartments may have an important functional role for odor processing. Compartmentalization is particularly prominent in Hymenoptera. The first compartments are the olfactory sensilla, with their multiple ORNs [56,60,90]. In ants, two types of sensilla are important for odor detection. The most abundant olfactory sensilla, the hair-shaped *sensilla trichodea curvata* contain up to 50 ORNs. Even more ORNs (more than 130) are associated with the peg-shaped *sensilla basiconica*. The high number of ORNs and the many corresponding functional units within the AL suggests that both sensilla types are sensitive to a wide range of different odors [59,122]. Interaction between ORNs within olfactory sensilla has been reported on in honey bees [123-126].

Second, the antennal nerves split into several sensory tracts before entering the AL. In carpenter ants, each of the 7 sensory tracts (T1-T7) innervates a distinct sub-region (glomerular cluster) of the AL, which contains a total of approximately 460 glomeruli [68,69]. Different clusters in the AL have been suggested to act as processing centers, e.g. for alarm pheromones or CHC profiles [68,90,127]. However, several studies in honey bees and ants indicate that social odors (e.g. alarm pheromone) are represented as distributed activity patterns at the level of the AL [69,104,105,128,129].

Third, in some species prominently large glomeruli (macroglomeruli) have been found in the AL. Macroglomeruli are often male-specific and sensitive to sex pheromones [65,130-132]. Remarkably, large workers of leaf-cutting ants possess a non-sex pheromone sensitive macroglomerulus that processes information about the species-specific trail pheromone [67,106,133-135].

Fourth, the Hymenopteran AL is separated by its output tracts into two hemilobes. PNs project to higher integration centers either via a lateral or a medial antenno-protocerebral tract (l- and m-APT, respectively) and this organization results in a dual olfactory pathway. The hemilobe located in the ventral-rostral part of the AL (VR-hemilobe) is innervated by l-APT PNs, while the dorsal-caudally located hemilobe (DC-hemilobe) is innervated by m-APT PNs [69,70,72,73].

Parallel processing of odor information has been suggested in the dual pathway and different odor processing mechanisms seem to operate in the VR- and the DC-hemilobe [78,136,137].

Exclusive representation of odors in single compartments suggests a functional segregation of odor information. In contrast, distributed representation across multiple compartments indicates parallel processing of odor information, taking advantage of the computational power of the whole AL network. Depending on the requirements on discrimination or detection of an odor, one or the other processing mechanism might be advantageous.

In this study, we investigated whether colony odor is represented exclusively in single AL compartments or whether distributed representations indicate parallel processing in the AL. Calcium imaging with advanced two-photon microscopy allowed us to monitor neuronal activity in response to colony odors in different AL compartments of the carpenter ant *Camponotus floridanus*. We analyzed the spatial activity patterns elicited by nestmate and non-nestmate colony odor, in order to further our understanding on how ants discriminate friends from foes.

Materials and Methods

Ethics statement

The performed experiments comply with the current laws of the Federal Republic of Germany and collection of founding queens for laboratory colonies conformed to the laws of the United States of America effective at time of collection.

Animals

C. floridanus is an evolutionary-derived eusocial species with colonies consisting of more than 10,000 individuals but only one single-mated queen [92]. Genetic homogeneity within colonies is high and heritable components of the colony odor are probably important for colony recognition in this species [93,94]. Workers show distinct colony recognition behavior, which has been studied in great detail [93-96]. Their cuticular hydrocarbon profiles mainly consist of linear and methyl-branched alkanes of chain lengths between C29 and C32 [38,97].

Experimental colonies were raised from founding queens collected by A. Endler and Ch. Strehl at Florida Keys (Florida, USA), after mating flight. Colonies were kept in the laboratory in artificial plaster nests at a constant temperature of 25 °C and 50% humidity (12h/12h photoperiod) and provided with artificial diet [114], honey-water, and dead cockroaches (*Nauphoeta cinerea*) twice a week and water ad libitum. Colony size was approximately 4000 ants. Neurophysiological experiments were conducted with large workers (head width > 3 mm) from a colony, with a founding queen collected at Sugarloaf Shores in July 2002 and nestmate colony odor was obtained from small workers (head width < 3 mm) of the same colony. Non-nestmate colony

odors were obtained from small workers, whose founding queens had been collected at Orchid Island in September 2001.

Colony odor extraction

Colony odors were obtained from postpharyngeal glands (PPG), which contain the same components as the colony odor found on the cuticle in equivalent ratios [24,30,33]. In order to obtain nestmate (NM) and non-nestmate (nNM) colony odor, a small worker was immobilized on ice, the gaster removed, and the thorax pinned upside down onto a silicone elastomer (Sylgard 182, Dow Corning, USA) in a Petri dish. The head was covered with distilled water, the maxillo-labial apparatus was removed and the PPGs were taken out by pulling out the pharynx. In order to compensate for differences in content quantity between glands, three PPGs were collected in 500 µl of distilled hexane and the glandular content was extracted for at least 2 h. Prior to experiments, hexane was evaporated under a constant stream of pure N_2 (Sauerstoffwerk Friedrichshafen GmbH, Germany) to a volume of ~75 µl. As colony odors change over time in the range of weeks and months [42,49,50], we used only PPG extracts which had been prepared no more than 5 days in advance. A previous study showed that PPG extracts contain remarkably less short-chain components, which do not belong to the hydrocarbons constituting the colony odor, than hexane cuticle washes and are readily discriminated by ants [100].

Stimulus delivery

For stimulation with colony odors, we used a recently developed stimulus delivery technique, which simulates a nearby nestmate or non-nestmate: a dummy loaded with colony odor is moved into close vicinity of the antenna. In order to further increase colony odor concentration in headspace, dummies were heated to a temperature of 40 °C before applying the colony odor (Firerod Cartridge Heater [power rating: 23 W, diameter: 3 mm] operated by a F4SL controller, Watlow GmbH, Germany). Dummy-delivered simulation has been shown to be advantageous for stimulation with low-volatile odors [104]. A custom-built positioning system with a solid rod moved via a crank shaft by a computer-controlled servo-motor allowed for precise positioning of the dummy. Prior to stimulation, hexane-rinsed dummies were loaded with 25 µl of colony odor using hexane-rinsed Hamilton syringes (Hamilton Company, Switzerland), and the solvent was allowed to evaporate for 2 min. Room temperature was kept constant at 21 °C. The loading of a dummy with colony odor corresponded to 1 PPG equivalent, and this has been shown to elicit adequate behavioral responses [100].

Calcium imaging

Neuronal activity was monitored via calcium imaging by measuring changes in intracellular calcium levels using fluorescent calcium indicators. This technique has been repeatedly used in ants [69,101,104-106] and was recently combined with two-photon microscopy to measure neuronal activity in response to thermal stimuli in leaf-cutting ants [109].

Large workers were immobilized by briefly cooling them on ice for a few minutes and then tethered in a custom-made Plexiglas stage using soft dental wax (surgident periphery wax, Heraeus Kulzer, Germany). A small window was cut in the head capsule with a piece of razorblade attached to a blade holder (Fine Science Tools GmbH, Germany) to access the brain and the site of dye application. Tracheae and glands were carefully moved aside with Dumont tweezers and a sharp glass electrode was used to penetrate the tissue of the lateral protocerebrum, dorsolaterally to the vertical lobe of the right mushroom body. Subsequently, another sharp glass electrode coated with Fura-2 dextran (potassium salt, 10,000 MW, F3029, Molecular Probes, USA) dissolved in 2% bovine serum albumin solution was inserted at the same region, aiming for the projection neurons of the l- and the m-APT. The window in the head capsule was closed with the cut piece of cuticle and the animals were kept in darkness and moistened air for a staining period of six to eight hours. Prior to imaging, antennae and mandibles were fixated with wax and the window in the head capsule was enlarged to access the right AL. Glands and trachea were carefully removed and the esophagus was pulled out of the head capsule to prevent movement of the brain during data acquisition. Hemolymph above the brain was removed and substituted by two-component adhesive (KWIK-SIL, World Precision Instruments, Germany) to further prevent movement and desiccation of the brain. During experiments the preparation was kept at constant temperature of 25 °C using a heat lamp (IOT 100, Elstein, Germany).

Calcium imaging experiments were performed using an upright microscope (LSM 510 Meta, Carl Zeiss GmbH, Germany) equipped with a 20x water-immersion lens (Apochromat 20x, NA 1, VIS-IR, Carl Zeiss GmbH). For excitation, a two-photon laser was used at an excitation wavelength of 810 nm (Chameleon® Titan:Sapphire LASER, Coherent Deutschland GmbH, Germany; beam splitters: MBS: HFT KP 650, NDD MBS: NDD KP685, NDD Refl.: none; filters: NDD2: HC680/SP) and laser power was adjusted depending on preparation and focal plane. The focal plane within the AL was adjusted to 40 µm, 160 µm, and 200 µm below the ventral AL surface using a focusing system integrated in the microscope. We describe the orientation of neuropiles according to the nomenclature used for the honey bee [138]. The ventral AL surface corresponds to the anterior AL surface in Drosophila literature [139]. For each stimulus a series of 40 frames

was recorded at a sampling rate of 4 Hz at a resolution of 64 x 64 pixels and an image pixel size of 3.4 x 3.4 µm to 5.4 x 5.4 µm. Pixel exposure time was ~25 µs.

We prepared 144 workers of which 38 (26.4 %) showed bright staining of projection neurons and glomeruli across the whole AL and in 18 preparations we measured spontaneous activity of glomeruli. As a test stimulus for functionality, we presented a general odor (octanol at a dilution of 10^{-2}) incorporated into a constant and moistened air-stream by a computer-controlled solenoid valve as a 1 s odor puff. We measured neuronal activity in response to air-delivered octanol in 13 animals. For colony odor stimulation we used dummies loaded with a PPG extract of nestmates (NM), a PPG extract of non-nestmates (nNM), and solvent only on a heated dummy (control). Dummy-delivered stimulation was triggered by the imaging software and started 5 s after recording onset, for a stimulation period of 1 s. Colony odors were presented in a stereotyped stimulation sequence. Each stimulation sequence consisted of 3 stimulation cycles. Within a stimulation cycle we presented odors in the following sequence: NM – nNM – control (i.e. 3 odor trials). Within each odor trial, we recorded successively neuronal responses at three different focal planes (at 40 µm, 160 µm, and 200 µm below AL surface), hence, stimulating 3 times with the same odor at an inter-stimulus-interval of 1 min. Inter-trial-interval between different odors was at least 6 min The whole stimulation sequence at three different focal planes was recorded in 9 animals; however in 2 animals recordings at 200 µm could not be analyzed.

Imaging data were analyzed using custom software written in Interactive Data Language (IDL 6.0; ITT Visual Information Solutions, USA) by Giovanni Galizia and Mathias Ditzen (University of Konstanz, Germany). We calculated the change in fluorescence intensity of the images (ΔF/F) and corrected manually for possible movement of the AL between measurements. Furthermore, intensity value signs were inverted, and as a result an increase in brightness indicates an increase in neuronal activity. In order to visualize neuronal responses to the different colony odors as false-color coded images, we subtracted the average of 3 frames prior to stimulation from the average of 3 frames during stimulation.

Anatomy of the AL

After calcium imaging experiments, a high-resolution image stack was recorded for visual inspection of the AL anatomy (256 x 256 pixel, 1 µm step size). We assured that similar AL areas were recorded in the different specimens, by comparing identified landmarks in the AL. In 1 specimen an image stack with a resolution of 512 x 512 pixel (1 µm step size) was recorded and used to reconstruct the glomeruli of the AL using 3D-reconstruction software (AMIRA 3.1, Mercury Computer Systems, Germany). Based on landmarks within the AL, glomeruli were assigned to the VR- or the DC-hemilobe at each focal plane by comparison to earlier anatomical studies in Camponotus species [68,69]. The AL volume we measured was larger than reported in

other publications, probably because in our study were recorded in-vivo, hence, the ALs were not subject to shrinking. At 40 µm, glomeruli belonging to the VR-hemilobe (l-APT innervated) are apparent, and the brightly stained ventral-lateral somata cluster is visible (Fig. 1A, white arrow heads). The focal plane at 160 µm is at the dorsal border of the antennal nerve entrance point (Fig. 1D) and the ventral border of the medial-dorsal somata cluster [69] is visible (Fig. 1B). At 200 µm, the medial-dorsal somata cluster and the "lateral passage" [73,140,141] are clearly visible (Fig. 1C). From this, we conclude that the brightly stained glomeruli at 160 µm and the glomeruli at 200 µm below ventral AL surface belong to the DC-hemilobe of the AL (m-APT innervated).

Data evaluation

First, we selected regions of interest (ROIs) within animals at each recorded focal plane (at 40 µm, 160 µm, and 200 µm below the ventral AL surface). ROIs were selected where glomeruli were morphologically visible and/or where their location was revealed by spontaneous activity. In order to separate spontaneous activity from colony odor elicited activity, we pooled the fluorescence changes over time of repeated stimulations with the same colony odor. By this, random spontaneous activity is averaged out, while stimulus correlated activity remained high. A glomerulus was defined as activated by colony odor if its fluorescence change upon stimulation was at least 3 times higher than the standard deviation of variation in fluorescence changes prior to stimulation. Glomeruli activated by control stimulation were excluded from further analyses. To test whether there are differences in the strength of neuronal responses to colony odors at different focal planes, we determined the proportion of colony odor activated glomeruli at each focal plane and tested for differences using Wilcoxon-tests (matched-pair comparison within animals). Furthermore, we identified the glomeruli activated most strongly (i.e. with the highest fluorescence change) by colony odors at each focal plane and tested for differences in maximal fluorescence change between focal planes using paired t-tests (comparison within animals). Significance levels for both tests were adjusted to correct for multiple testing using the Bonferroni-Holm method, setting α_1 to 0.017, α_2 to 0.025, and α_3 to 0.05.

In order to compare spatial activity patterns elicited by nestmate and non-nestmate colony odors, we calculated principal component analyses (PCA) over time (ranging from 1 s prior to stimulation to 2 s after stimulation). We prepared one matrix for each animal containing all ROIs as rows and all stimuli in consecutive time frames as columns as described by Niessing and Friedrich [142] and calculated covariance-based principal components (PC). We included all ROIs in the PCAs and this yielded qualitatively similar results as PCAs containing only ROIs of colony odor activated glomeruli or glomeruli located 160 µm below the ventral AL surface. For each odor trial (i.e. stimulation at all 3 focal planes), we pooled i) eigenvector loadings of 1 s prior to

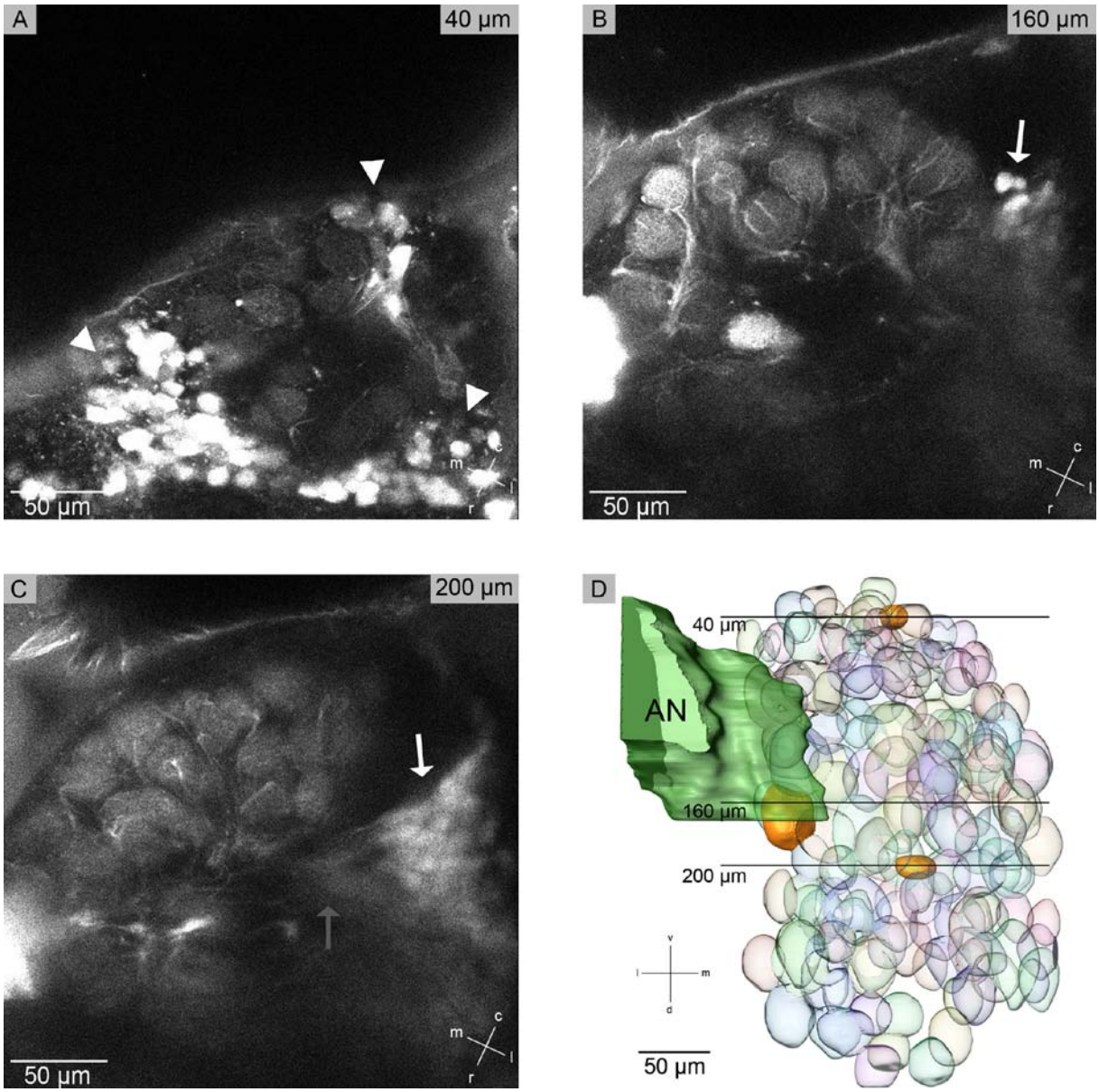

Figure 1: Antennal lobe (AL) anatomy: two-photon images and 3D-reconstruction. A-C: High-resolution two-photon microscope images of an in-vivo recorded AL (ventral view) at three different focal planes (40 µm, 160 µm, and 200 µm below ventral AL surface). Glomeruli are visible as spherical structures with a diameter of 20 – 50 µm, which are innervated by projection neuron dendrites (bright branches). Projection neuron somata are brightly stained and the somata clusters can be used as landmarks for orientation. At 40 µm (A) the ventral-lateral somata cluster is visible (white arrow heads). At 160 µm (B) the ventral border of the medial-dorsal somata cluster is visible (white arrow). At 200 µm (C) the medial-dorsal somata cluster can be clearly seen (white arrow) in addition to the "lateral passage" (grey arrow). Based on the landmarks, glomeruli at 40 µm below AL surface belong to VR- hemilobe (l-APT-innervated), while the brightly stained glomeruli at 160 µm and glomeruli at 200 µm belong to the DC-hemilobe (m-APT-innervated). **D:** 3D-Reconstruction of a vital AL (caudal view). A high-resolution image stack allowed reconstructing the AL to illustrate the location of the focal planes at 40 µm, 160 µm, and 200 µm below ventral AL surface. For orientation, the antennal nerve (AN) is shown in transparent green. For each focal plane the glomerulus responding most strongly to colony odor stimulation has been marked in yellow. c caudal, r rostral, m medial, l lateral, v ventral, d dorsal.

stimulation (*pre*) and ii) eigenvector loadings during stimulation (*stim*) in order to describe how the spatial activity patterns evolve upon stimulation (*odor* trajectories). For visualization of trajectories, we pooled within animals the eigenvector loadings of repeated odor trials of the same colony odor (pooled trajectories) and plotted the first three PCs in a three-dimensional space (3D-Plot). For statistical analysis, we calculated Euclidean distances of eigenvector loadings (ED) between odor pairs of nestmate and non-nestmate trials (NM-nNM) prior to stimulation (*pre*) and during stimulation (*stim*). According to Scree tests, we included 8-14 PCs, describing 33.3 – 69.6 % of the variance. To test whether the activity patterns change upon stimulation, we compared EDs of the odor pair NM-nNM in *pre* and in *stim* conditions using paired t-tests.

In order to describe the variability of spatial activity patterns of repeated odor trials of the same colony odor, we plotted the trajectories representing individual odor trials within animals. To quantify variability, we calculated EDs between odor pairs of consecutive repeated odor trials with the same colony odor (nestmate: NM-NM; non-nestmate nNM-nNM) and between odor pairs of consecutive odor trials with nestmate and non-nestmate colony odor (NM-nNM). We tested these distances statistically using paired t-tests. First, we compared EDs of similar odor pairs in *pre* and *stim* conditions; second, we compared EDs of the odor pairs NM-NM, nNM-nNM, and NM-nNM in the *stim* condition. Again, significance levels were adjusted to correct for multiple testing using the Bonferroni-Holm method, setting α_1 to 0.008, α_2 to 0.01, α_3 to 0.0125, α_4 to 0.017, α_5 to 0.025, and α_6 to 0.05. Euclidean distances were calculated with table calculation software (MS Office Excel 2007 SP2, Microsoft Deutschland GmbH, Germany). For statistical testing and plotting of graphs we used Statistica 9.1 (StatSoft Europe GmbH, Germany).

Artificially induced template reformation

In a pilot experiment, we artificially induced a change in the neuronal template of 2 workers by modifying their sensory experience prior to calcium imaging experiments. To this end, PPGs of small non-nestmate workers of a colony with the founding queen collected at Sugarloaf Shores in July 2003 were dissected, transferred to a cleaned microscope slide, and carefully squeezed with tweezers to open the glands and disperse the content. Immediately afterwards, the antennae of an immobilized (on ice) large nestmate worker were uniformly coated with spread PPG content and the worker was tethered in a custom-made Plexiglas stage preventing it from cleaning its antennae. Antennae were masked at least 15 h before calcium imaging experiments. Extracts of the colony odor used to mask the antennae (nNM-masked) were obtained for stimulation as described. We measured neuronal responses to NM, nNM, nNM-masked, and control stimulation and analyzed the elicited spatial activity patterns using PCAs as described.

Results

We investigated the neuronal representation of colony odors in glomeruli of the AL in 9 animals. We recorded neuronal activity of VR-hemilobe glomeruli at 40 µm and of DC-hemilobe glomeruli at 160 µm and 200 µm below the ventral AL surface. Glomeruli were clearly visible at all recorded focal planes of the AL and the high spatial resolution of two-photon microscopy allowed for collecting high-resolution image stacks right after recording neuronal activity (Fig. 1). Spontaneous activity was very high throughout the recordings ($\Delta F/F > 2.5$ %), and this allowed identification of less clearly visible glomeruli. In the 9 investigated animals between 102 and 264 regions of interest (ROIs) corresponding to glomeruli were selected for further analysis (mean: 197 ROIs). We measured both activation and inhibition of glomeruli in response to nestmate and non-nestmate colony odor stimulation at all recorded focal planes (see Fig. 2 as an example at 160 µm) and between 13.5 % and 32.4 % of all ROIs responded to stimulation with colony odors (mean: 21.7 %). Spatial activity patterns in response to nestmate and non-nestmate colony odors were overlapping (cp. Fig. 2B&C): between 12.0 % and 44.4 % of the ROIs responding to colony odor stimulation responded to both nestmate and non-nestmate colony odor (mean: 27.3 %).

In order to quantify neuronal responses to colony odors in different AL compartments, we investigated the strength of response to colony odors across focal planes. As a measure of response strength we counted how many ROIs responded at each focal plane and assessed the maximal signal amplitude (i.e. fluorescence change; Fig. 3). A higher number of ROIs responded to colony odors at 160 µm and 200 µm than at 40 µm (Tab. 1). In order to avoid bias from a possibly higher number of selected ROIs at the two more dorsally located levels, we compared the proportion of responding ROIs for each focal plane within each animal. A significantly higher proportion of ROIs responded to colony odors at 160 µm than at 40 µm (Fig. 3A; Bonferroni-Holm corrected Wilcoxon-test, $Z = 2.55$, $p = 0.011$), whereas no significant differences were found between 40 µm and 200 µm ($Z = 0.169$, $p = 0.866$) and between 160 µm and 200 µm ($Z = 1.69$, $p = 0.091$). To compare signal amplitudes across focal planes, we selected the ROI most strongly activated by colony odor (i.e. with the highest fluorescence change) on each focal plane and in each animal and tested them statistically (Fig. 3B). Maximal signal amplitudes were significantly higher at 160 µm than at 40 µm (Bonferroni-Holm corrected paired t-test, $t = -3.55$, degrees of freedom [DF] = 8, $p = 0.0075$). Maximal signal amplitudes at 200 µm were between those at 40 µm and 160 µm, yet no significant differences were found (40 µm vs. 200 µm: $t = -1.57$, DF = 6, $p = 0.167$; 160 µm vs. 200 µm: $t = 1.21$, DF = 6, $p = 0.271$).

Table 1: Number of ROIs responding to colony odors across focal planes.

focal plane below ventral AL surface	median	range	total
40 µm	9	2 - 12	75
160 µm	21	14 - 38	197
200 µm	16	5 - 23	101

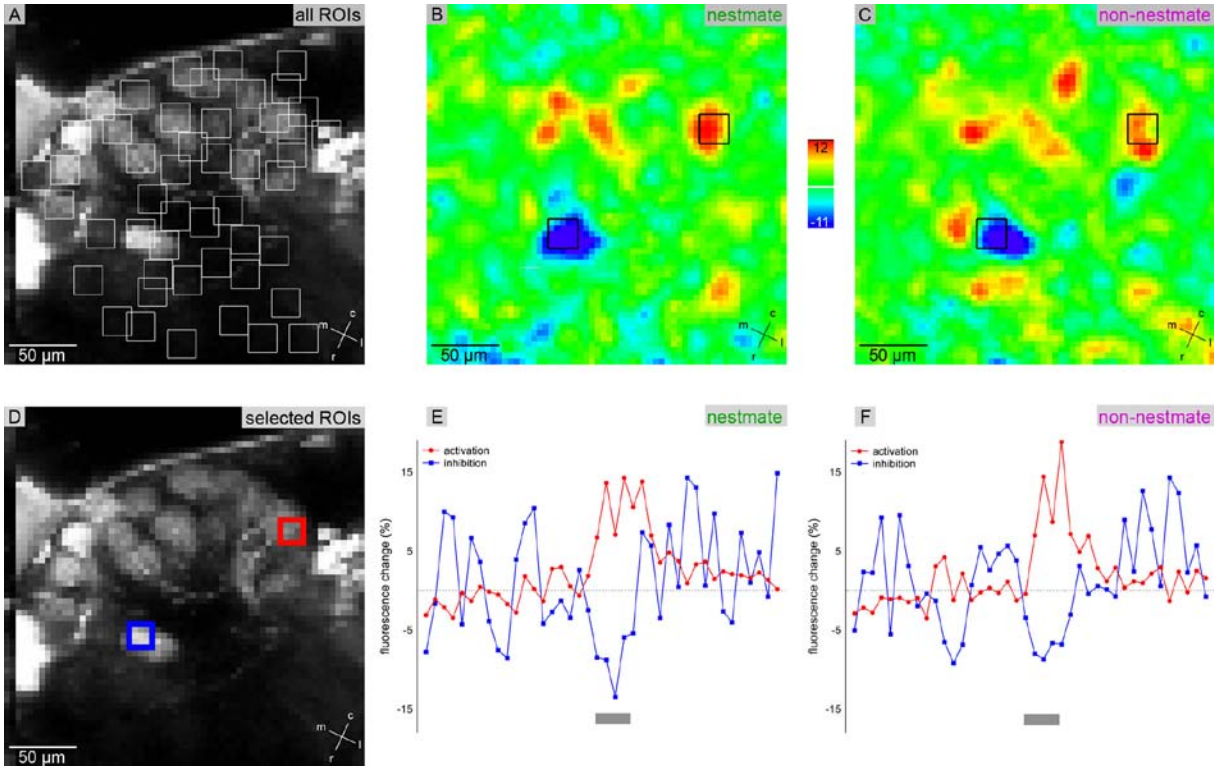

Figure 2: Calcium imaging: Neuronal activity in response to colony odor stimulation. A: Low-resolution image of the AL at a focal plane of 160 µm below the ventral surface of the antennal lobe (AL) with marked regions of interest (ROI; white boxes). Note that the resolution for calcium imaging was reduced to reduce exposure time. ROIs were selected were glomeruli were clearly visibly or where their location was revealed by spontaneous activity. **B&C:** False-color-coded images of neuronal activity in response to nestmate (B) and non-nestmate colony odor (C). Red indicates areas of increased neuronal activity (activation), while blue indicates regions of reduced activity (inhibition). A colored bar indicates the fluorescence changes in %. Spatial activity patterns elicited by nestmate and non-nestmate colony odors were overlapping. **D-F:** Kinetics of two selected ROIs (D), each representing one activated glomerulus (red line) and one inhibited glomerulus (blue line) upon stimulation with nestmate (E) and non-nestmate colony odor (F). Selected ROIs are marked in B&C as black boxes. A grey bar indicates the stimulation period of 1 s. The red glomerulus is only activated upon stimulation. In contrast, the blue glomerulus is spontaneously active throughout the recording, being only briefly inhibited during stimulation.

Table 2: Odor identity of ROIs most strongly activated by colony odors across focal planes.

focal plane below ventral AL surface	N	activated by NM	nNM	NM&nNM
40 µm	9	7	4	2 (22 %)
160 µm	9	9	9	9 (100 %)
200 µm	7	5	6	4 (57 %)

For each focal plane below ventral AL surface, the ROI activated most strongly (with the highest fluorescence change) by colony odor was selected in each animal. The table shows how many ROIs responded most strongly to which colony odor in each focal plane: nestmate (NM), non-nestmate (nNM), or both (NM&nNM). The proportion of how many ROIs were activated by both, nestmate and non-nestmate colony odor, at each focal plane is given in brackets. N: number of tested animals per focal plane.

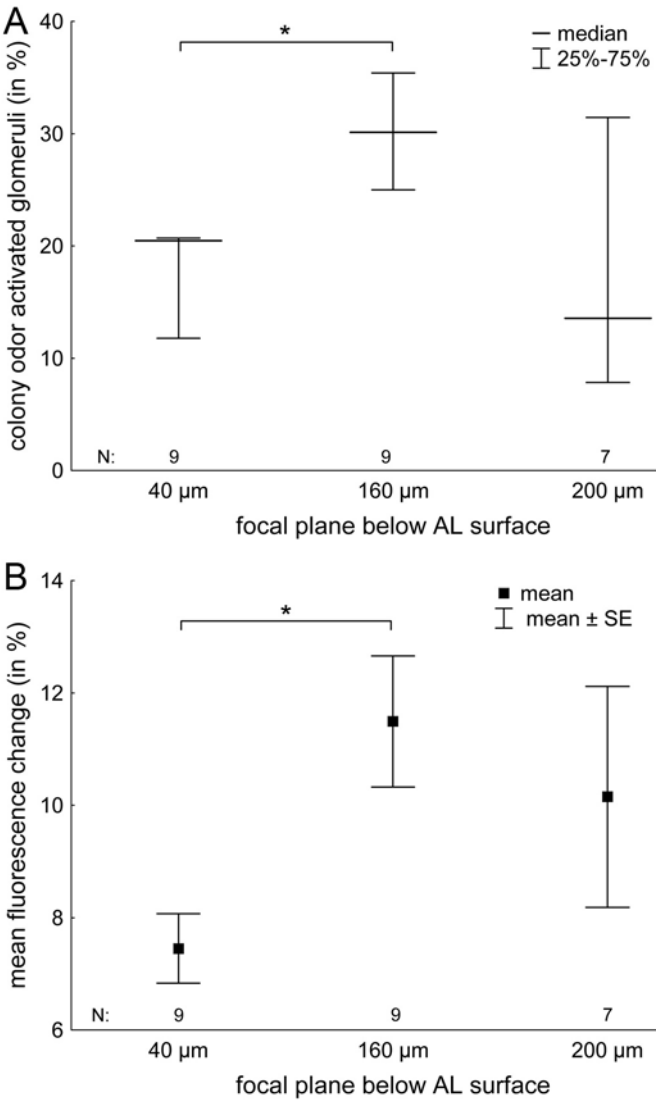

Figure 3: Comparison of neuronal response strength to colony odors across focal planes. **A:** Proportion of regions of interest (ROI, corresponding to glomeruli) activated by colony odors across focal planes below ventral AL surface. An asterisk marks a significant difference in a Bonferroni-Holm corrected Wilcoxon-test. A significantly higher proportion of glomeruli was activated by colony odors at 160 µm than at 40 µm. **B:** Mean signal amplitude (fluorescence change) of the most strongly activated glomeruli in each focal plane. For each focal plane in each animal the glomerulus activated most strongly by colony odor was selected and the measured fluorescence changes tested across focal planes. An asterisk marks a significant difference in a Bonferroni-Holm corrected paired t-test. Glomeruli located 160 µm below ventral AL surface responded with a significantly higher signal amplitude to stimulation with colony odors than glomeruli located at 40 µm. Thus, colony odors are represented stronger in the DC-hemilobe (at 160 µm) than in VR-hemilobe (at 40 µm), i.e. more glomeruli responded with higher signal amplitudes. N: Number of tested animals per focal plane.

Interestingly, all ROIs responding most strongly to colony odors at 160 µm responded to both nestmate and non-nestmate colony odor (Tab. 2). In summary, neuronal responses to colony odors were stronger in the DC-hemilobe (at 160 µm) than in the VR-hemilobe (at 40 µm), i.e. more glomeruli responded with higher signal amplitudes. Importantly, however, neuronal activity elicited by colony odors was not restricted to specific AL compartments.

Next, we compared the spatial activity patterns elicited by nestmate and non-nestmate colony odor. We performed principal component analyses within animals. First, we plotted pooled *NM* and *nNM* trajectories (Fig. 4). Upon stimulation (*stim*) the *NM* and *nNM* trajectories evolved into the same direction, which was different from the *control* trajectory (Fig. 4A&B). We compared EDs of the odor pair NM-nNM in *pre* and in *stim* conditions and found a significant difference (Fig. 4C; paired t-test, t = -4.64, DF = 8, p = 0.0017). This result shows that the spatial activity patterns in the AL become more different in response to nestmate and non-nestmate colony

odor stimulation, indicating a change from spontaneous activity prior to stimulation to colony odor specific activity patterns upon stimulation.

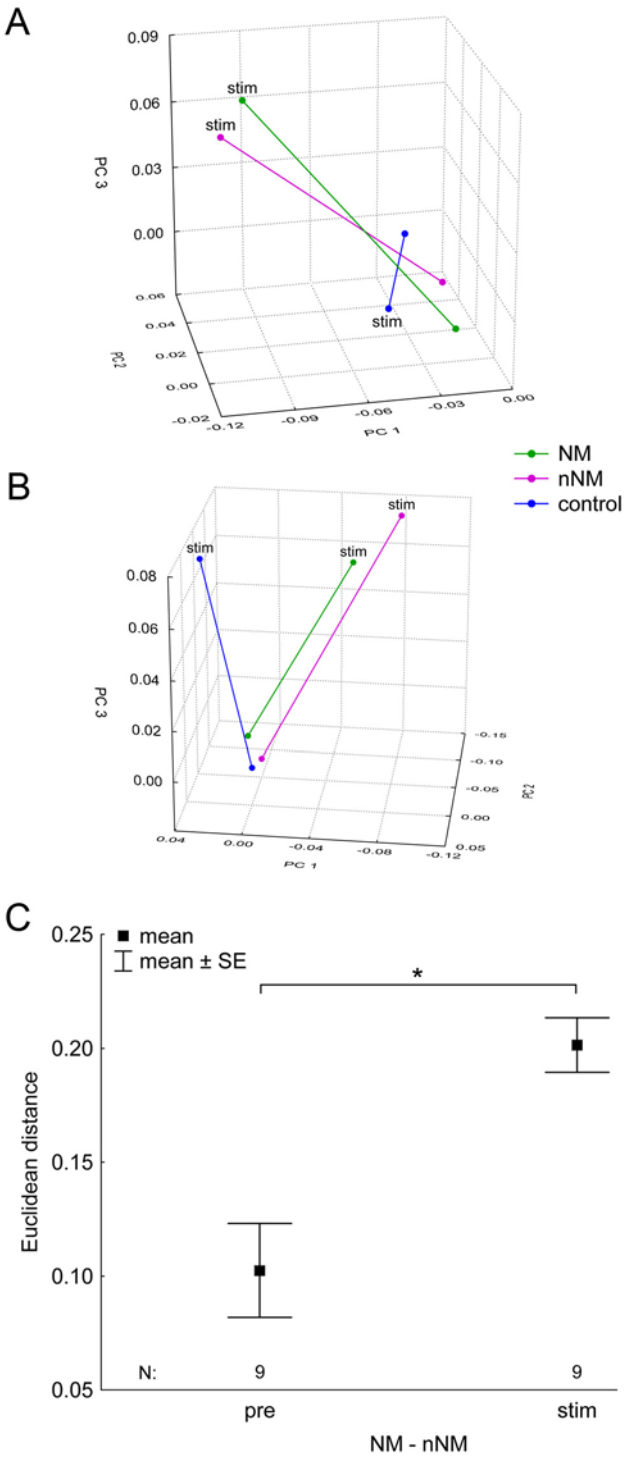

Figure 4: Principal component analysis: pooled 3D-trajectories upon colony odor stimulation. A&B: Exemplary 3-D trajectories representing the evolution of spatial activity patterns from pre-stimulus condition to stimulation (stim) in 2 animals. Repeated stimulations with nestmate (NM, green) and non-nestmate colony odor (nNM, magenta) were pooled. In addition, a trajectory describing the control stimulation is shown (control, blue). Colony odor trajectories evolve into the same direction, which is different from the control stimulation trajectory. **C:** Mean Euclidean distances of eigenvecotrs between nestmate and non-nestmate trajectories (NM-nNM) of 9 animals before (pre) and during simulation (stim). An asterisk denotes a significant difference in a paired t-test. This result shows that the spatial activity patterns in the AL become more different in response to nestmate and non-nestmate colony odor, indicating a change from spontaneous activity prior to stimulation to colony odor specific activity patterns upon stimulation.

In order to assess variability, we compared the trajectories representing individual odor trials (Fig. 5). Individual *NM* and *nNM* trajectories evolved into the same general direction upon stimulation and were not clearly segregated from each other, whereas the *control* trajectory evolved into a different direction (Fig. 5A&B). To quantify variability, we statistically tested i) EDs of similar odor pairs (NM-NM and nNM-nNM) in *pre* and *stim* conditions, and ii) EDs of the odor pairs NM-NM, nNM-nNM, and NM-nNM in the *stim* condition. EDs between odor pairs in the pre

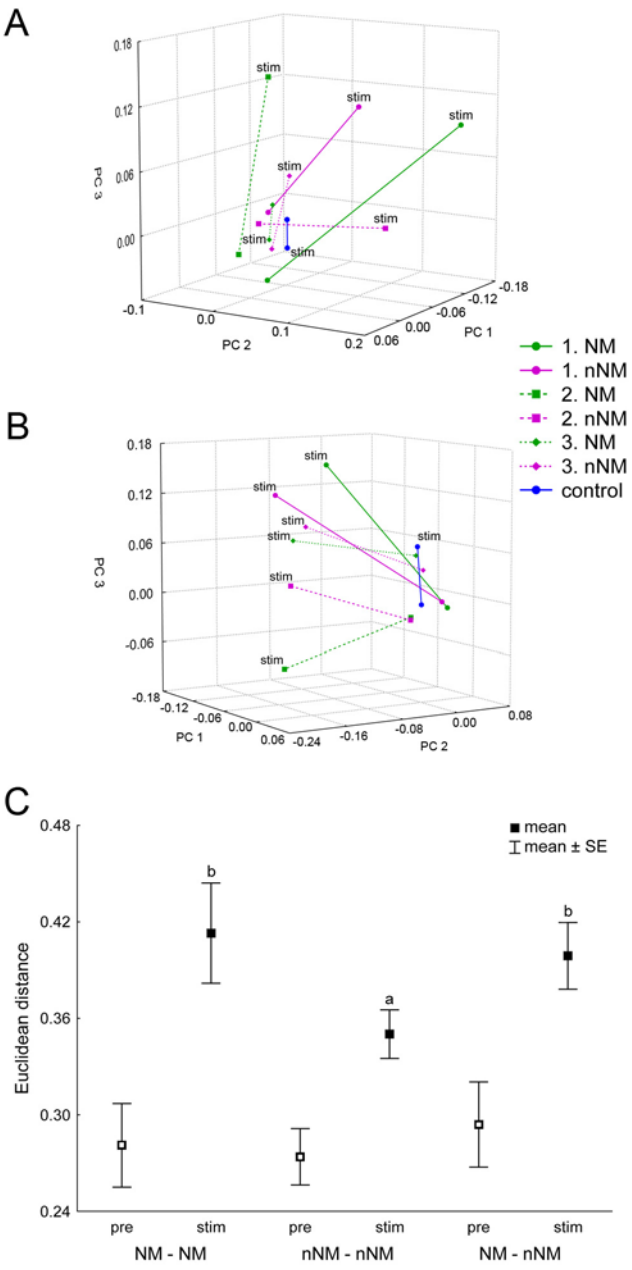

Figure 5: Principal component analysis: 3D-trajectories upon repeated colony odor stimulation. A&B: Exemplary 3-D trajectories representing the evolution of spatial activity patterns from pre-stimulus condition to stimulation (stim) of 2 animals (same specimens as in Fig. 4 A&B, respectively). Trajectories of repeated stimulation with nestmate (NM, green) and non-nestmate colony odor (nNM, magenta) and of one control stimulation (control, blue) are shown (first stimulation: solid lines with pointed ends; second stimulation: dashed lines with squared ends; third stimulation: dotted lines with diamonded ends). In contrast to the control stimulation trajectory, colony odor trajectories evolve generally into the same direction; however repeated nestmate and non-nestmate trajectories are variable and not segregated from each other. **C:** Mean Euclidean distances of eigenvectors between odor pairs of 9 animals before (pre) and during simulation (stim). Odor pairs are either consecutive repeated stimulations with the same colony odor (nestmate: NM-NM; non-nestmate: nNM-nNM) or consecutive stimulations with nestmate and non-nestmate colony odors (NM-nNM). This analysis allows for assessing the variability of spatial activity patterns in response to repeated stimulations with the same colony odor and compare it to the variability in spatial activity patterns in response to stimulation with a different colony odor. Euclidian distances before stimulation (pre, empty boxes) were significantly lower than during stimulation (stim, full boxes) for all odor pairs. During stimulation (stim, full boxes) Euclidean distances between nNM-nNM (a) were significantly lower than between NM-NM and between NM-nNM (b; see Tab. 3 for statistics). Thus, spatial activity patterns elicited by non-nestmate colony odor are less variable than activity patterns elicited by nestmate colony odor.

condition were significantly lower than in the *stim* condition (Tab. 3A). In the *stim* condition, EDs between nNM-nNM were significantly lower than between NM-NM and between NM-nNM. There was no significant difference in EDs between NM-NM and between NM-nNM (Tab. 3B). Thus, in a n-dimensional space, trajectories representing activity patterns elicited by nestmate

colony odor expand more fanned out than those representing non-nestmate colony odor; yet, the non-nestmate colony odor representations are not clustered distinctly outside the (larger) area of space occupied by nestmate colony odor representations. Thus, spatial activity patterns elicited by non-nestmate colony odor are less variable than activity patterns elicited by nestmate colony odor.

Previous behavioral studies have shown that modifying the sensory experience of ants by masking their antennae with non-nestmate colony odor results in a changed acceptance range after 15 h, where treated workers were no longer aggressive to the respective non-nestmates, while nestmates were still accepted and non-nestmates of other colonies are still rejected [107,108]. In a pilot experiment to investigate the effect of template reformation on the neuronal representation, antennae of 2 workers were masked with non-nestmate colony odor prior to calcium imaging experiments. To compare spatial activity patterns we plotted *odor* trajectories (Fig. 6). Pooled *nNM-masked* trajectories evolved in comparable directions as *NM* and *nNM* trajectories (Fig. 6A&B). Individual *nNM-masked* trajectories were variable and not distinctly segregated from *NM* or *nNM* trajectories (Fig. 6C&D).

Table 3: Statistics on Euclidean distances between principal components of colony odor elicited neuronal responses (i.e. trajectories; see Fig. 5C).

A)

paired t-test comparing	DF	t-value	p-value
NM-NM (*pre*) vs. NM-NM (*stim*)	9	-5.92	0.0004
nNM-nNM (*pre*) vs. nNM-nNM (*stim*)	9	-6.30	0.0002
NM-nNM (*pre*) vs. NM-nNM (*stim*)	9	-5.20	0.0008

B)

paired t-test comparing	DF	t-value	p-value
NM-NM (*stim*) vs. nNM-nNM (*stim*)	9	3.02	0.016
NM-nNM (*stim*) vs. NM-NM (*stim*)	9	-1.00	0.346
NM-nNM (*stim*) vs. nNM-nNM (*stim*)	9	3.60	0.007

Degrees of freedom (DF), t-, and p-values are given for Bonferroni-Holm corrected paired t-tests on Euclidean distances between principal components of odor pairs **A**) prior to (*pre*) and during stimulation (*stim*) and **B**) during stimulation (*stim*). Odor pairs were: repeated stimulations with nestmate colony odor (NM-NM), repeated stimulations with non-nestmate colony odor (nNM-nNM), and stimulation with nestmate and non-nestmate colony odor (NM-nNM).

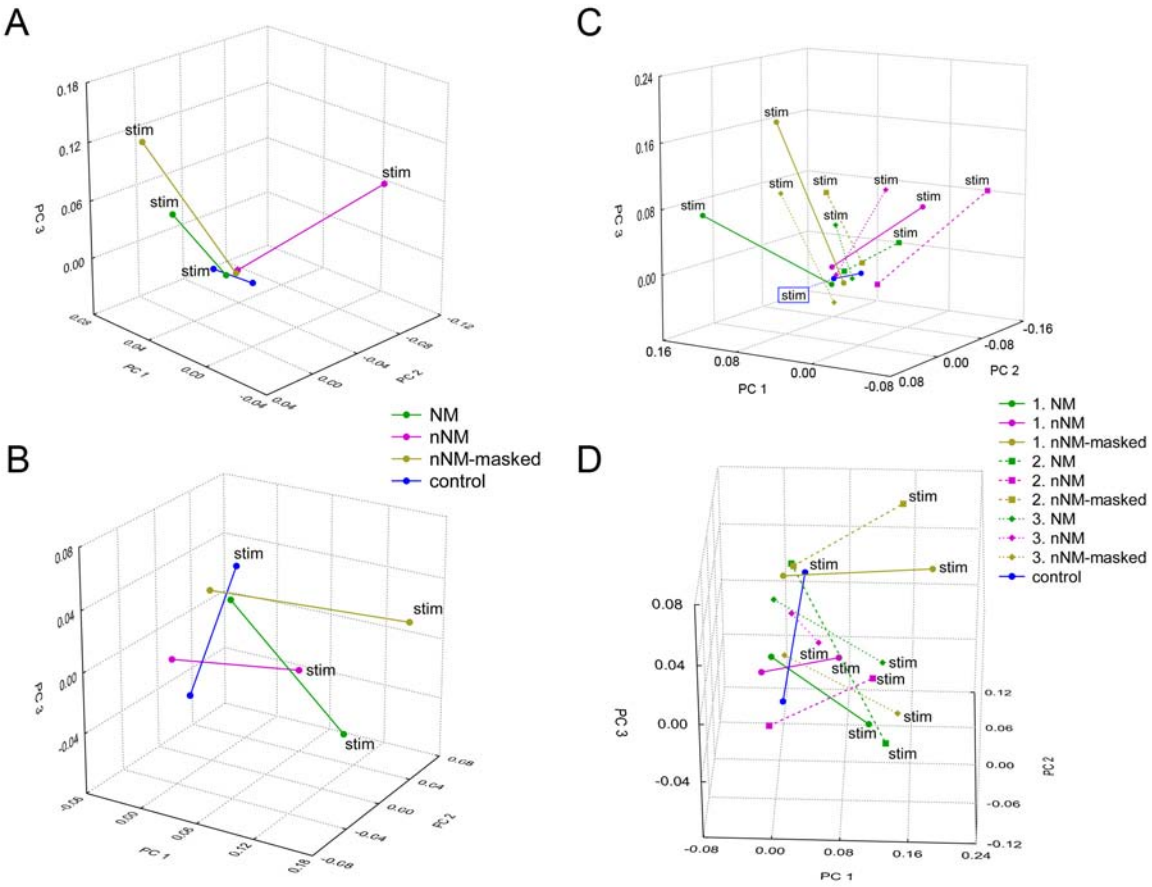

Figure 6: Principal component analysis: 3D-trajectories upon colony odor stimulation after artificially induced template reformation. Prior to calcium imaging, antennae of workers were masked with colony odor from non-nestmates. This has been shown to result in a reformation of the neuronal template: non-nestmates with the colony odor used for masking are not rejected anymore. **A-D:** Exemplary 3-D trajectories representing the evolution of spatial activity patterns from pre-stimulus condition to stimulation (stim; same specimens in A&C and B&D, respectively). In A&B trajectories upon repeated stimulations with colony odor of nestmates (NM, green), non-nestmate (nNM, magenta), and the non-nestmates used for masking (nNM-masked, ocher) were pooled. In addition, a trajectory describing the control stimulation is shown (control, blue). In C&D individual trajectories upon repeated stimulations are shown (first stimulation: solid lines with pointed ends; second stimulation: dashed lines with squared ends; third stimulation: dotted lines with diamonded ends). The nNM-masked trajectories evolve comparably to nestmate and non-nestmate trajectories. There are no marked differences that separate nNM-masked trajectories from nestmate or non-nestmate colony odor trajectories.

Discussion

In this study, we investigated the antennal lobe (AL) of ants and measured the neuronal representation of a social odor that is used to identify friends and foes. Multi-component colony odors of nestmates and non-nestmates were represented in overlapping spatial activity patterns. Overlapping representation was expected, since both colony odors consist of the same components at differing ratios. Although the activity patterns were not homogeneously

distributed, we did not find exclusive representations restricted to single AL compartments. Our results indicate that information about colony odors is processed in parallel, using the computational power of the whole AL network. Parallel processing might be advantageous, when the olfactory system has to reliably discriminate highly complex social odors. Activity patterns in response to repeated stimulation with the same colony odor were variable, yet variability was higher in response to nestmate than to non-nestmate colony-odor. We speculate that this finding may reflect plasticity of the AL network, which allows for adjustment of the neuronal template to a changing colony odor, i.e. template reformation.

The AL of *C. floridanus* consists of approximately 460 glomeruli, which are arranged in distinct neuroanatomical compartments [68,69]. Advanced two-photon microscopy allowed us to measure neuronal activity in various compartments by recording at three different focal planes in the AL (at 40 µm, 160 µm, and 200 µm below ventral AL surface). High spatial resolution in combination with high spontaneous activity allowed us to identify the majority of glomeruli at each focal plane. On average, we selected in each AL almost 200 regions of interest (ROIs) that correspond to glomeruli. Hence, recording at three different focal planes allowed us to monitor the neuronal activity of approximately 40 % of all AL glomeruli. We identified landmarks in the AL and this allowed us to allocate the glomeruli to either the ventral-rostral hemilobe of the AL (VR-hemilobe; l-APT innervated; glomeruli at 40 µm and lateral-rostrally located glomeruli at 160 µm) or the dorsal-caudal hemilobe (DC-hemilobe; m-APT innervated; medial-caudally located glomeruli at 160 µm and glomeruli at 200 µm). It is not possible to precisely allocate glomeruli to specific AL clusters; however based on their location within the AL and visual comparison to completely reconstructed ALs with allocated clusters [69,143], we conclude that glomeruli at 40 µm belong to the glomerular clusters T1-T3. At 160 µm, the brightly stained, medial-caudally located glomeruli are part of the T5-cluster, while the weakly stained, lateral-rostrally located glomeruli belong to the T4-cluster. In the most dorsal part of the AL, the T6-cluster is located, consisting of approximately 140 glomeruli [68,69,144]. Based on the high number of glomeruli and our 3D-reconstruction (Fig. 1 D), we are confident that we recorded from T6-glomeruli at the most dorsal focal plane, although glomeruli of other clusters might as well have been visible at this plane. The T6-cluster is innervated by *S. basiconica* [133], which are CHC-sensitive and which have been suggested to be important for colony recognition [90]. Neither did we find an exclusive representation of nestmate and/or non-nestmate colony odor in the T6-cluster, nor did we measure the strongest neuronal responses in the region where T6-glomeruli are located (at 200 µm). Since we measured neuronal responses to colony odors in clusters, which are not innervated by *S. basiconica* but by *S. trichodea curvata*, an exclusive role of a specific sensillum type for colony recognition can be ruled out. Furthermore, we did not find

any indications that the *S. basiconica* innervated T6-cluster might function as a center for colony recognition, as proposed previously [68].

Distributed activity across compartments rather suggests parallel processing of colony odor information across the dual olfactory pathway. On average, one fifth of all identified glomeruli in an animal responded to colony odors. The strongest responses were measured in the ventral part of the DC-hemilobe (at 160 µm). The inhomogeneous distribution of neuronal activity is based on the functional organization of the AL network and glomeruli with similar odor response profiles are not dispersed stochastically across the AL, but are often located closely adjacent to each other [145,146]. Colony odors of nestmates and non-nestmates are very similar, consisting of the same components with differing ratios [25]. On average, 27.3 % of all glomeruli activated by a colony odor in an animal responded to both nestmate and non-nestmate colony odor, and this was particularly true for those glomeruli responding most strongly to colony odors (at 160 µm).

Spatial activity patterns in response to repeated stimulation with nestmate and non-nestmate colony odor were variable in both hemilobes. What might have caused these variable neuronal representations in response to colony odors? Variable neuronal responses may reflect variability of the stimulus. Colony odors have a very low volatility and, hence, diffusion into headspace is low. For presentation of colony odors, we used a stimulation technique resembling the natural situation by simulating close-range colony odor detection from a nearby nestmate or non-nestmate. The multi-component odor stimulus arriving at the antenna of an ant does not only depend on the chemical composition of the colony odor, but may also vary depending on external physical factors like temperature, humidity as well as the distance and diffusion rate between colony odor source and receiver. Subtle differences in the arriving stimulus may result in variable neuronal responses. Nevertheless, ants are able to very accurately discriminate colony odors under such stimulus conditions [100].

In order to quantify variability, we compared the spatial activity patterns of odor pairs of repeated stimulations with the same colony odor (nestmate vs. nestmate and non-nestmate vs. non-nestmate) using a principal component analysis. We found that spatial activity patterns were less variable in response to repeated stimulation with non-nestmate than with nestmate colony odor. What might be the cause of different variability of nestmate and non-nestmate colony odor elicited activity patterns? The neuronal template needs to be plastic, as it has to be constantly adjusted to changing colony odors [48-51]. Learning has been shown to result in changes of the neuronal representation of odors in the AL, indicating plasticity of the AL network [86-89]. Representation of nestmate colony odor possibly is constantly adjusted, even between repeated stimulations. We propose that the variability in activity patterns reflects the

adjustment of the neuronal template, particularly apparent for nestmate colony odor representation. Variability might be a neuronal correlate of template reformation. In a pilot experiment to further investigate template reformation, we artificially induced a change in the neuronal template by modifying the sensory experience of ants. Earlier studies showed that masking an ant's antennae with a non-nestmate colony odor resulted in a modified acceptance range, where treated workers were no longer aggressive to non-nestmates of which colony odor for masking was obtained, while nestmates were still accepted [107,108]. Our current results show that such masking does not induce profound changes in the neuronal representation of colony odors. Hence, sensory adaptation is not the mechanism that allows colony recognition. Furthermore, this pilot experiment illustrates that it is possible to induce a change in the neuronal template and investigate the neuronal representation of colony odors during the artificially induced template reformation.

Due to their high variability, spatial activity patterns do not provide sufficient information to discriminate nestmate from non-nestmate colony odor. How are colony odors classified by the nervous system as being nestmate or non-nestmate specific? Additional parameters of neuronal activity are most probably necessary to allow discrimination and recent studies emphasize the importance of precise timing of neuronal activity for discrimination of chemically similar odors and odor blends [74-79]. In this study, we found less variable spatial activity patterns in response to non-nestmate compared to nestmate colony odor and this might facilitate the detection and classification of foes at the next level of the olfactory pathway by increasing the chance of precisely timed coincidental activity, e.g. in Kenyon cells of the mushroom bodies. Based on behavioral data, colony recognition was recently suggested to be effectively mediated by *non-nestmate recognition* [21,119]. Commonly used behavioral assays are designed to identify rejection of foes by an aggressive response. However, they do not allow for determining why individuals are accepted, be it because they are recognized as nestmates or classified as friends due to learning and memory of several colony odors. As a result of the biased experimental design, interpretations drawn from aggression-based behavioral experiments tend to underrate the high discriminatory power exhibited by ants, allowing them to discriminate between members of different castes and life stages within their colony [43,45-47]. We show that ants are not anosmic to nestmate colony odor. Information about both, nestmates and non-nestmates, is passed on to higher brain centers, as we measured the AL output. For a neuronal process as complex as colony recognition, it may be advantageous to use information about nestmates and non-nestmates side by side in order to allow reliable recognition.

Functional segregation within single compartments and parallel processing distributed across compartments are two different processing mechanism and examples for both have been

described in the insect olfactory system [53]. Depending on the requirements on odor detection or odor discrimination, selective pressure may ultimately favor either one or the other mechanism. Complex blends, like e.g. colony odors, demand a high discriminatory power of the nervous system. In this case, distributed activity and parallel processing may be advantageous, as the computational power of the whole AL network can be used to solve the discriminatory task. On the other hand, a functional segregation with exclusive processing in specialized centers may be favored in case high sensitivity is needed for detecting even minute quantities of single components, e.g. of a sex pheromone. However, these two principles are not irrevocably imperative: Trail pheromone is used by many different ant species, and often sensitivity is remarkably high [16]. However, whereas major workers of leaf-cutting ants exhibit functional segregation using a trail pheromone specific macroglomerulus for detection [67,106,133-135], carpenter ants do not feature such a specialized compartment and the releaser component of their trail pheromone is represented in the AL in distributed patterns of activity [69,104].

In conclusion, the organization of the AL is shaped to balance the requirements of discriminatory power and sensitivity, and eventually opposing selective pressures result in a complex olfactory system adapted to the behavioral repertoire of a species. As the highly developed olfactory system of ants is easily accessible for a range of neurophysiological techniques, this system is ideally suited to unlock the principles underlying the processing of complex odors.

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Authors' contributions

A.S.B. and C.J.K. designed the experimental procedure. A.S.B. collected calcium imaging data and did the data analysis. A.S.B. and C.J.K. wrote the manuscript.

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IX. General Discussion

Survival of social insect colonies depends on accurate identification of friends and foes. Understanding the neuronal basis of colony recognition requires an integrative approach, in which behavioral assays that describe the animals' behavioral repertoire, are combined with neurophysiological experiments that allow directly investigating neuronal correlates of the processes underlying colony recognition. Behavior, which is relevant in the context of colony recognition, has been described in great detail [16,21,22,81]. However, to date, no data are available about the neuronal processes in the brain during colony recognition. In this thesis, I investigated for the first time the neuronal processes in the central nervous system underlying discrimination of friends and foes in ants.

IX.1 Stimulus delivery for colony odors

IX.1.1 Colony odor detection is possible at short range

Colony odors are very low-volatile [25]. Due to the enormous sensitivity of the olfactory system of ants, several authors have speculated that colony recognition at close range might be possible [24,26,81,147,148], however, this has been never tested experimentally. In a behavioral assay, I investigated, whether ants can detect and discriminate colony odor over short distances when contact is not permitted (Chapter 2). My results show that ants are able to discriminate multi-component colony odors presented on dummies without tactile interaction over a distance of at least 1 cm. Contact chemo-sensilla are not necessary and airborne cues received via olfactory sensilla are sufficient for colony recognition. As the postpharyngeal gland (PPG) extracts used for this experiment contain remarkably few short-chain components (tested via gas chromatography) the same long-chain hydrocarbons are probably used for discrimination on close range as on contact. My results are supported by other studies, which emphasize the high sensitivity of the olfactory system of ants. Ants use low-volatile CHCs to assess the reproductive status of colony members [37,97,117,149] and it has been shown that fertility signals can be detected over short distances via olfactory sensilla [36,150].

I used a C23 alkene (cis-9-tricosene) to show that a long-chain hydrocarbon, which does not belong to *C. floridanus'* colony odor, interfered with colony recognition if added to nestmate colony odor and could, thus, be detected without contact. Presenting cis-9-tricosene alone resulted in even more aggressive responses and the strong effect on the behavior of workers seems to superpose the response to nestmate colony odor. At least some workers, however, perceived the nestmate colony odor, as fewer workers responded aggressively towards the mixture of nestmate colony odor and cis-9-tricosene than towards cis-9-tricosene alone. In the

natural environment, cis-9-tricosene may act as a cue to detect other insects, e.g. heterospecific ants [151]. The possibility to recognize non-nestmates without tactile interaction considerably increases an ant's chances of survival when encountering rivals by allowing early behavioral reaction to avoid or prepare for aggressive interaction.

IX.1.2 Effective stimulus delivery for low-volatile odors

The low volatility of colony odors considerably complicates stimulus delivery in neurophysiological experiments, which are extremely motion-sensitive. Hence, an important task at the beginning of my experimental work was to develop a stimulation technique, which can be used for contact-free stimulation with very low-volatile colony odors. I tested whether the dummies used for presentation of colony odors in Chapter 2 could be used for stimulus delivery in neurophysiological experiments (dummy-delivered stimulation; Chapter 3). Electroantennography revealed that dummies can be used for stimulation in a relative simple neurophysiological approach. Dummies loaded with highly volatile alarm pheromone undecane and low-volatile trail pheromone nerolic acid elicited strong sensory responses in ORNs of the antenna. To evaluate whether dummy-delivered stimulation is especially advantageous for stimulation with low-volatile odors, I used calcium imaging, which is more sensitive than electroantennography, and compared neuronal responses in the AL using either dummy-delivered stimulation or conventional stimulation via an air-stream (air-delivered stimulation) [67,69,128,129,131,146,152-154]. Dummies were loaded with highly volatile undecane, low-volatile nerolic acid, and the very low-volatile behaviorally active cis-9-triciosene. Whereas undecane elicited strong neuronal responses with both air- and dummy-delivered stimulation, detection level for nerolic acid was only reached when dummy-delivered stimulation was used. Hence, this novel stimulation technique is better suited for stimulation with low-volatile odors than conventional air-delivered stimulation. I propose that for low-volatile odors the threshold concentration at the receptor organ is reached at a lower loading quantity when dummies are used compared to conventional air-delivered stimulation, where an air-stream is directed over odor-loaded filter paper. Furthermore, dummy-delivered stimulation better resembles the natural situation of odor dispersal, e.g. from a food source or a foraging trail, than air-delivered stimulation. Hence, dummies do not only provide a technical improvement for studying olfaction of low-volatile odors but are additionally well suited to simulate natural conditions in neurophysiological experiments.

Neuronal activity in response to air-delivered cis-9-tricosene was negligible in all cases, while dummy-delivered stimulation sometimes worked very well, eliciting strong neuronal responses, and sometimes not at all. Stimulation efficiency with odors of very low-volatility, thus, was not yet optimal and needed to be further increased to allow reliable stimulation with colony odors.

To this end, dummies were moderately heated, in order to increase colony odor concentration in headspace. A behaviorally assay conducted in parallel to the neurophysiological experiments assured that the odor quality of colony odor stimuli was not changed by the moderate increase in temperature and workers readily discriminated between temperature-controlled dummies loaded with nestmate or non-nestmate colony odors (data not shown). Hence, moderately heated dummies provided a very effective stimulus delivery, which was used not only in this study on colony recognition, but which is already employed successfully in two other projects (not part of this thesis): i) long-chained CHCs are used to signal reproductive status in ants [97,116,117] and the newly developed stimulation technique allowed for investigating the neuronal representation of *C. floridanus'* fertility signal in the AL; ii) honey bees use several low-volatile CHCs to facilitate recruitment during waggle dancing [155] and by increasing dummy-temperature while recording the neuronal responses in the AL, it is investigated how honey bees may use different thorax-temperatures during waggle dancing for signal modulation [156].

IX.2 Neuronal correlates of colony recognition

The newly developed, effective stimulation technique allowed me to directly investigate the neuronal representation of colony odors in the nervous system, in order to investigate the neuronal processes underlying colony recognition in ants (Chapter 4 and 5). Here, the key findings of my neurophysiological experiments are recapitulated and discussed.

IX.2.1 Ants perceive their own colony odor

According to the sensory filter hypothesis, sensory adaptation of CHC-sensitive *sensilla basiconica* results in specific anosmia to nestmate colony odor, and as a consequence, only non-nestmate specific information is transferred to the brain [90]. As *S. basiconica* innervate the T6-cluster of the AL [133], this neuronal compartment has been suggested to function as a center for colony recognition [68]. Contradictory to the sensory filter hypothesis, I measured neuronal activity in response to both, nestmate and non-nestmate colony odors, in the peripheral (ORN level) and the central nervous system (AL level) and this clearly shows that ants are not anosmic to their own colony odor. Since I measured neuronal responses to colony odors in AL clusters, which are not innervated by *S. basiconica* but by *S. trichodea curvata*, an exclusive role of a specific sensillum type for colony recognition can be ruled out. Neither did I find an exclusive representation of nestmate and/or non-nestmate colony odor in the T6-cluster, nor did I measure the strongest neuronal responses in the region of the AL where T6-glomeruli are located. Hence, I did not find any indication that the *S. basiconica* innervated T6-cluster has a prominent role for colony recognition. My results show that colony odor information is not exclusively processed in functionally segregated centers of the ant AL.

IX.2.2 Colony odor information is processed in parallel

Colony odors of nestmates and non-nestmates were represented in distributed spatial activity patterns, yet the neuronal activity was not distributed homogeneously across the AL. The inhomogeneous distribution is based on the functional organization of the AL network and glomeruli with similar odor response profiles are not dispersed stochastically across the AL, but are often located closely adjacent to each other [145,146]. Distributed activity across AL hemilobes indicates parallel processing of colony odor information across the dual olfactory pathway of Hymenoptera.

Parallel processing across neuroanatomical compartments and functional segregation of odor information processing within single compartments are two different processing mechanism and examples for both have been described in the insect olfactory system [53]. Depending on the requirements on odor detection or odor discrimination, selective pressure may ultimately favor either one or the other mechanism. Complex blends, like e.g. colony odors, demand for a high discriminatory power of the nervous system. In this case, distributed activity and parallel processing may be advantageous, as the computational power of the whole AL network can be used to solve the task of discrimination. On the other hand, a functional segregation with exclusive processing in specialized centers may be favored in case high sensitivity is needed for detecting even minute quantities of single components, e.g. of a sex pheromone [65,130-132]. However, these two principles are not irrevocably imperative: Trail pheromone is used by many different ant species, and often sensitivity is remarkably high [16]. However, whereas major workers of leaf-cutting ants feature a functional segregation with a macroglomerulus in the AL that allows high-sensitive trail pheromone detection [67,106,134,135], carpenter ants do not have such a specialized compartment and the releaser component of their trail pheromone is represented in the AL in distributed patterns of activity [69,104]. In conclusion, my results support the idea that the organization of the AL is shaped to balance the requirements of discriminatory power and sensitivity, and that eventually opposing selective pressures result in a complex olfactory system adapted to the behavioral repertoire of a species.

IX.2.3 How are friends and foes classified?

How does the nervous system classify colony odors as nestmate or non-nestmate specific? Since odors activate specific subsets of ORNs, this results in an odor specific glomerular activation patterns in the AL (spatial activity patterns) [103]. Earlier studies revealed that odors, which elicit similar spatial activity patterns in the AL, are perceived similarly, i.e. a similar odor quality is attributed [64,75]. This correlation led to the suggestion that the brain readily uses activity patterns in the AL to assess odor quality. However, my experiments showed consistently that spatial activity patterns in response to colony odors are variable and not sufficiently distinct to

allow discrimination between nestmates and non-nestmates (Chapter 4 and 5). Nevertheless, behavioral experiments show that the nervous system is perfectly well able to classify nestmate and non-nestmate colony odors [93-96,100,107], despite the variable neuronal representation of colony odors described here. My results challenge the notion that spatial activity patterns in the AL are generally sufficient to code for odor quality. Other neuronal parameters are most likely necessary for attribution of odor quality to complex, multi-component odors. Several studies emphasize the importance of precise timing of neuronal activity for discrimination of chemically similar odors and odor blends [74-79]. Odor information is integrated and processed in the AL network by interactions between glomeruli via local interneurons and this results in distinct temporal firing patterns of projection neurons of the AL [103,111]. Specific colony odors may then elicit synchronous activity in ensembles of projection neurons leading to patterns of coincidence in postsynaptic neurons at the next levels of the olfactory pathway, i.e. the mushroom bodies or the lateral horn. Thus, temporal activity patterns of AL projection neurons may suffice to code for nestmate or non-nestmate specificity. Interestingly, calcium imaging with advanced two-photon microscopy revealed less variable spatial activity patterns in response to non-nestmate compared to nestmate colony odor (Chapter 5). Lower variability of activity patterns in response to non-nestmate colony odor might facilitate the detection and classification of foes at the next level of the olfactory pathway by increasing the chance of precisely timed coincidental activity, e.g. in Kenyon cells of the mushroom bodies.

Based on behavioral data, it has been recently suggested that colony recognition is rather mediated by *non-nestmate recognition* than by *nestmate recognition* [21,119]. Commonly used behavioral assays are designed to identify rejection of foes by an aggressive response. However, they do not allow for determining why individuals are accepted, be it because they are recognized as nestmates or classified as friends due to learning and memory of several colony odors. As a result of the biased design, interpretations drawn from aggression-based behavioral experiments tend to underrate the high discriminatory power exhibited by ants, allowing them to discriminate between members of different castes and life stages within their colony [43,45-47]. In this thesis, I show that information about both, nestmates and non-nestmates, is passed on to higher brain centers, as I measured the AL output. For a neuronal process as complex as colony recognition, it may be advantageous to use information about nestmates and non-nestmates side by side in order to allow reliable recognition.

So far, it remains elusive in which part of the nervous system colony odors are classified as nestmate or non-nestmate specific. My experiments show that information about colony odors is available at higher integration centers of the ant brain; hence, recognition may be achieved at this level of the olfactory system. On the other hand, my results indicate that other parameters

of neuronal activity than spatial activity patterns are most probably important for colony recognition; synchronous activity of AL projection neurons is possibly sufficient for classification of colony odors. Electrophysiological approaches may allow elucidating the role of precise timing for the discrimination of colony odors. Eventually, different mechanisms at multiple levels of the olfactory system might be used in combination with each other, in order to assure fast and accurate recognition.

IX.2.4 What causes the high variability of spatial activity patterns?

Spatial activity patterns in response to colony odors are variable. Variability between animals is expected, as colony odors change over time [25,35-47]. Surprisingly, however, variability was high even upon repeated stimulation with the same colony odor. What might have caused this variability?

IX.2.4.1 A highly complex stimulus

Variable neuronal responses to repeated stimulation with the same colony odor may reflect variability of the colony odor stimulus under natural conditions. Colony odors have a very low volatility and, hence, diffusion into headspace is low. For presentation of colony odors, I used a stimulation technique resembling the natural situation by simulating close-range colony odor detection from a nearby nestmate or non-nestmate. The multi-component odor stimulus arriving at the antenna of an ant does not only depend on the chemical composition of the colony odor, but may also vary depending on external physical factors like temperature, humidity as well as the distance and diffusion rate between colony odor source and receiver. Airborne colony odors may vary in their ratios, but are still recognized by workers in behavioral assays (see Chapter 2). I conclude that the variable activity patterns I measured in response to repeated stimulation with the same colony odor reflects the natural variability of the multi-component colony odor stimulus. A recent study in moth showed that the ratios of odor components can vary to some degree without reducing its behavioral effect [110]. Under natural conditions, multi-component odors constitute varying and fluctuating stimuli, and most probably animals are generally faced with the problem that these elicit variable neuronal responses which have to be classified correctly by the nervous system to allow accurate odor recognition.

IX.2.4.2 Plasticity of the olfactory system

Using calcium imaging with a two-photon microscope, I found that spatial activity patterns were even more variable in response to repeated stimulation with nestmate than with non-nestmate colony odor (Chapter 5). What might be the cause of different variability of nestmate and non-nestmate colony odor elicited activity patterns? Colony odors are not stable and as a consequence, the neuronal template needs to be plastic, as it has to be constantly adjusted to

changing colony odors [48-51]. Learning has been shown to result in changes of the neuronal representation of odors in the AL, indicating plasticity of the AL network [86-89]. Representation of nestmate colony odor possibly is constantly adjusted, even between repeated stimulations. I propose that the variability in activity patterns reflects the adjustment of the neuronal template, particularly apparent for nestmate colony odor representation. The measured variability might be a neuronal correlate of template reformation. In order to understand the neuronal processes underlying template reformation, integrative approaches may prove to be highly useful; in the following paragraph I exemplify such an integrate approach, combining behavioral manipulation with neurophysiological measurements.

IX.2.5 Template reformation

Template reformation can be induced artificially by manipulating the sensory experience of workers and this can be used to investigate the dynamics of the reformation process (Chapter 1). Masking of the antennae of workers with non-nestmate colony odor resulted in a modified acceptance range, where treated workers were no more aggressive to non-nestmates with the colony odor used for masking, while nestmates were still accepted. Masking with nestmate colony odor did not induce a change in behavior and this shows that the treatment itself did not influence the ants' general discriminatory ability. Social interaction was not needed to induce a change in the neuronal template and this result has been confirmed by other studies [108,119]. The reformation process was very slow, taking more than 2 h, and sensory adaptation at receptor neuron level cannot explain these findings. Several different adaptation mechanisms were described for insect ORNs [157-159]. However, all of them are acting on a shorter time scale as the behavioral change described in Chapter 1 (> 2 hours) and, presumably, even on a shorter time frame than the faster template reformation process (25 min) found in honeybees [160-163]. The results of this behavioral experiment, hence, provide further evidence that the sensory filter hypothesis cannot sufficiently explain colony recognition.

The finding that sensory adaptation does not play an important role for colony recognition is also supported by the pilot experiment, in which I investigated the neuronal representation of colony odors in the AL, after artificially inducing template reformation (Chapter 5). The results of this experiment show that masking of the antennae does not induce profound changes in the neuronal representation of colony odors, again ruling out sensory adaptation to colony odor. This pilot experiment illustrates that it is possible to induce a change in the neuronal template and investigate the neuronal representation of colony odors during the artificially induced template reformation. Monitoring neuronal activity, while the template changes, may prove to be helpful in elucidating the learning mechanism underlying template reformation.

Template reformation fundamentally differs from classical olfactory learning, which has been described in honey bees in great detail [83]. During associative learning, a conditioned stimulus (CS; e.g. an odor) is presented directly before a positive, unconditioned stimulus (US; e.g. sugar). Thus, the US is predicted by the CS, and this can induce learning, after which the CS alone is sufficient to induce the behavioral response originally induced by the US. In this case, the US acts as a reinforcer for the CS. In the case of artificially induced template reformation, colony odor used for modifying the sensory experience of ants does not qualify as a CS, because it has no predictive value for an US, which could act as a positive reinforcer. Under natural conditions, allo-grooming and trophallaxis within a colony may act as a positive reinforcer to allow learning of a changing colony odor, i.e. template reformation. However, as shown in Chapter 1, social interaction is not necessary for template reformation. A speculative explanation may be that the lack of negative feedback (i.e. being aggressed while experiencing the unknown colony odor) possibly positively reinforces the colony odor used for masking of the antennae. Investigating to which extent and in which aspects template reformation relates to classical odor learning is a promising approach for future experiments and will help to understand how the recognition system continuously manages to keep track of who is a friend and who is a foe.

IX.3 Significance of the work

In this thesis, I present an integrative approach to investigate the neuronal basis of colony recognition in ants. I developed a novel stimulation technique, which provides a useful tool for olfaction research and allows studying the neuronal processing of very low-volatile odors, used for colony recognition (this study) and fertility signaling in ants or as a recruitment signal in honey bees. My research efforts provide the necessary means to study the colony recognition system directly, using neurophysiological methods. This is of high significance for the research field of insect olfaction as colony recognition in social insects is an excellent model system to study the coding of odor quality and long-term memory mechanisms underlying recognition of complex, multi-component odors. I present the first scientific findings from a new and promising research field, where neuroscience helps to answer the basic questions of insect sociobiology. My results invalidate the sensory filter hypothesis of colony recognition and challenge our current notion of how odor quality is coded by the nervous system. Furthermore, I provide first neurobiological information on how a discrimination task as complex as identifying friends and foes is solved by the tiny brains of social insects.

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Einer noch zum Schluss: Warum gehen Ameisen nicht in die Kirche?

XI. Curriculum vitae

Andreas Simon Brandstaetter

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Education

May 2007 – Dec 2010	**University of Wuerzburg, Germany** Department of Behavioral Physiology & Sociobiology PhD candidate (Dr. rer. nat. cand.)
Oct 2001 – Mar 2007	**University of Wuerzburg, Germany** Diploma with distinction in Biology Main subject: Neurobiology Minor subjects: Behavioral Physiology and Sociobiology Cell and Developmental Biology
Jan 2005 – Jun 2005	**University of Umea, Sweden** Courses: Functional Genomics Academic Writing, English Swedish as a Foreign Language
Aug 2000 – Jun 2001	**Clinic of the University of Regensburg, Germany** Department of Medical Microbiology & Hygiene Civilian service
Sep 1991 – Jun 2000	**Secondary School, Regental-Gymnasium Nittenau, Germany** University-entrance diploma (grade: 1.8)

Research Experience

May 2007 – Dec 2010	**University of Wuerzburg, Germany** Department of Behavioral Physiology & Sociobiology PhD thesis: Neuronal correlates of nestmate recognition in the carpenter ant Supervision: Dr. habil. Christoph J. Kleineidam Prof. Wolfgang Roessler Prof. Juergen Liebig (Arizona State University, Tempe, AZ, USA)
Mar 2010 & May 2010	**University of Konstanz, Germany** Department of Biology Research stay: Two-photon microscopy & calcium imaging of colony odors in *Camponotus floridanus* Supervision: Dr. habil. Christoph J. Kleineidam
Oct 2009 – Dec 2009	**Arizona State University, Tempe, AZ, USA** School of Life Sciences Research stay: Neuronal representation of fertility signals in the carpenter ant Supervision: Prof. Juergen Liebig Prof. Brian H. Smith
Jun 2006 – Mar 2007	**University of Wuerzburg, Germany** Department of Behavioral Physiology & Sociobiology Diploma thesis: Perception of nestmate recognition cues in ants Supervision: Prof. Wolfgang Roessler Dr. Christoph J. Kleineidam Second referee: Prof. Martin Heisenberg (Department of Genetics and Neurobiology, University of Wuerzburg, Germany)

Teaching Experience

2006 – 2009	**University of Wuerzburg, Germany** Lectured and supervised practical courses for bachelor students in Neurophysiology
2006 – 2009	**University of Wuerzburg, Germany** Lectured and supervised practical courses and literature seminars for master students in Neurobiology and Behavioral Physiology

Scientific Initiatives

May 2008 – Jul 2009	Organized the 20th Neuro-DoWo 2009 (neurobiology PhD student workshop) in Wuerzburg
May 2008 – Jun 2008	Organized the 1st Fellow Retreat 2008 of the Graduate School of Life Sciences Wuerzburg (GSLS)

Awards & Honors

May 2007 – Oct 2010	Scholarship of the Graduate School of Life Sciences (GSLS), University of Wuerzburg funded by a grant of the German Excellence Initiative
Sep 2009	"Price for the best poster of the neurobiology section" on occasion of the 102nd Annual Meeting of the German Zoological Society (Deutsche Zoologische Gesellschaft) 2009, Regensburg, Germany
Aug 2007	Heiligenberg Student Travel Award of the International Society for Neuroethology
Jan 2005 – Jul 2005	Grant of the ERASMUS student exchange program of the European Union

Memberships

Jul 2009 – present	International Union for the Study of Social Insects (IUSSI)
Jun 2009 – present	J.B. Johnston Club (Comparative, Evolutionary, and Behavioral Neuroscience)
Apr 2007 – present	International Society for Neuroethology

Languages

Mother tongue:	German (fluent)
Second languages:	English (fluent) Swedish (basic knowledge)

Würzburg, ……..

Date Signature

XII. List of publications

Andreas Simon Brandstaetter

Peer-reviewed articles

Brandstaetter A.S. and Kleineidam C.J. (2011) Distributed representation of social odors indicates parallel processing in the antennal lobe of ants. Journal of Neurophysiology 106(5): 2437-2449, doi: 10.1152/jn.01106.2010

Brandstaetter A.S., Rössler W., and Kleineidam C.J. (2011) Friends and foes from an ant brain's point of view – neuronal correlates of colony odors in a social insect. PLoS One (6):e21383, doi: 10.1371/journal.pone.0021383

Brandstaetter A.S., Rössler W., and Kleineidam C.J. (2010) Dummies versus air puffs: efficient stimulus delivery for low-volatile odors. Chemical Senses 35(4): 323-333, doi: 10.1093/chemse/bjq022

Brandstaetter A. S., Endler A., and Kleineidam C. J. (2008) Nestmate recognition in ants is possible without tactile interaction. Naturwissenschaften 95: 601–608, doi: 10.1007/s00114-008-0360-5

Leonhardt S. D., Brandstaetter A. S., and Kleineidam C. J. (2007) Reformation process of the neuronal template for nestmate recognition cues in the carpenter ant (*Camponotus floridanus*). Journal of Comparative Physiology A 193: 993-1000, doi: 10.1007/s00359-007-0252-8

Oral presentations

Brandstaetter A.S., Rössler W. and Kleineidam C.J. (2010) Friends and foes from an ant brain's point of view - functional imaging of colony odors in *Camponotus floridanus*. 16th Congress of the International Union for the Study of Social Insects (IUSSI), Copenhagen, Denmark, p319

Brandstaetter A.S., Rössler W., and Kleineidam C.J. (2010) Neuronal correlates of colony recognition cues in ants. 9th International Congress of Neuroethology, Salamanca, Spain

Brandstaetter A.S. (2010) How to know your foe – a functional imaging approach to colony recognition in ants. L.E.G.S. – Laboratoire Evolution, Génomes et Spéciation, CNRS, Gif-sur-Yvette, France

Brandstaetter A.S. (2010) Neuronal representation of low volatile odors in social insects. Meeting of the SFB554 "Mechanisms and Evolution of Arthropod Behavior: Brain – Individual – Social Group", Bronnbach, Germany

Brandstaetter A.S., Leonhardt S.D., Rössler W. and Kleineidam C.J. (2008) A neurophysiological approach to nestmate recognition in the Carpenter ant, *Camponotus floridanus*. 4th European Meeting of the International Union for the Study of Social Insects (IUSSI), La Roche-en-Ardenne, Belgium, p69

Brandstaetter A.S., Rössler W. and Kleineidam C.J. (2008) A neurophysiological approach to nestmate recognition in the Carpenter ant, *Camponotus floridanus*. 1st Fellow Retreat of the Graduate School of Life Sciences Würzburg, Zeilitzheim, Germany , p11

Brandstaetter A.S. and Kleineidam C.J. (2008) Nestmate recognition in the ant *Camponotus floridanus*: insights from behavior and neurophysiology. 19th Neuro-DoWo (neurobiology PhD student workshop), Saarbrücken, Germany, p36

Poster abstracts

Brandstaetter A.S., Rössler W., and Kleineidam C.J. (2009) Chemical pattern recognition in a social insect and its neuronal basis. 39th Annual Meeting of the Society for Neuroscience, Chicago, IL, USA, 68.16/U37

Brandstaetter A.S., Karl C., Vogt K., Rössler W, and Kleineidam C.J. (2009) Friend or foe? Nestmate recognition in the Florida carpenter ant. 102nd Annual Meeting of the German Zoological Society (Deutsche Zoologische Gesellschaft), Regensburg, Germany, P NB.4, p149

Rittmeyer M., Brandstaetter A.S., Kleineidam C.J. (2009) Functional imaging of a volatile recruitment signal in honey bees. 102nd Annual Meeting of the German Zoological Society (Deutsche Zoologische Gesellschaft), Regensburg, Germany, P NB.13, p154

Kleineidam C.J., Rittmeyer M., Brandstaetter A.S. (2009) Pattern recognition of social odors in ants and bees. 31st International Ethological Conference, Rennes, France, L38, p296

Brandstaetter A.S., Rössler W., and Kleineidam C.J. (2009) Neuronal correlates of pattern recognition in a social insect. 8th Göttingen Meeting of the German Neuroscience Society, Göttingen, Germany, TS19-2A

Brandstaetter A.S., Rössler W., and Kleineidam C.J. (2008) Dummies versus air puffs: most efficient stimulus delivery depends on odor volatility. XV. International Symposium on Olfaction and Taste / Annual Meeting of the Association for Chemoreception Sciences, San Francisco, CA, USA, #P336, p147

Brandstaetter A.S., Leonhardt S.D. and Kleineidam C.J. (2007) Nestmate recognition in the ant *Camponotus floridanus*: towards neuronal correlates of behavior. Kleinsthirnkonferenz "Insect brain and control of behavior", Tutzing, Germany

Brandstaetter A.S., Leonhardt S.D., and Kleineidam C.J. (2007) Nestmate recognition in the ant *Camponotus floridanus*: towards neuronal correlates of behavior. 8th Congress of the International Society for Neuroethology, Vancouver, Canada, PO363, p222

Brandstätter A.S. and Kleineidam C.J. (2007) Short range detection and neural representation of nestmate recognition cues in the ant *Camponotus floridanus*. 7th Göttingen Meeting of the German Neuroscience Society, Göttingen, Germany, TS8-11B, p117

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