Chemical cues and how ants use them for recognizing colony members

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Summary

The most sophisticated form of altruism is found in eusocial insects, with workers usually sacrificing their own reproduction in order to support their mother (queen) in raising offspring (workers, virgin queens and males). Altruistic acts ultimately increase the workers’ indirect fitness, but only when directed towards relatives. Indeed, individuals from the same colony (nestmates) are usually all related and workers rigorously defend their colony against individuals from foreign conspecific colonies (non-nestmates). Ants are amazingly fast and accurate in discrimination of non-nestmates, however, in rare cases social parasites sneak into and live within a host colony exploiting its resources. Recognition of nestmates and discrimination from non-nestmates is based on mixtures of low-volatile components present on each individuals’ exoskeleton (cuticular hydrocarbons: CHCs). CHC profiles are species-specific and CHC profiles of individuals from neighboring conspecific colonies usually only differ in the quantitative ratios of CHCs. The individuals’ CHC profiles are further influenced by environmental factors and a frequent exchange of CHCs between nestmates results in a uniformation of CHC profiles of nestmates. However, inter-individual, task-specific differences in CHC profiles remain. The neuronal basis of nestmate recognition is unknown, but it is generally assumed that the nestmate recognition process is based on a process of label-template matching where the CHC profile of an encountered ant (label) is compared with a neural representation of the own colony odor (template). When label and template match, the encounter ant is recognized as nestmate and when they mismatch, the encounter ant is recognized as non-nestmate. An encounter of non-nestmates often results in aggression. Importantly, since the CHC profiles change over time due to environmental influences, the template needs to be updated.

In this thesis, I investigated different levels of nestmate recognition in ants. Specifically, I focused on which CHCs are produced, how CHC profiles are perceived, how the behavioral response is influenced by experience, and how CHC profiles are encoded and decoded in the brain.

In chapter 1, I investigate a host-social parasite system, where a social parasitic ant (*Megalomyrmex symmetochus*) parasitizes a colony of a fungus-growing ant (*Sericomyrmex amabilis*). I show that the CHC profiles of the two species are distinct and the parasite shares only a single hydrocarbon with its host. Furthermore, the parasite has fewer CHCs and in an overall lower quantity than its
host, suggesting that the parasite uses a ‘chemical insignificant’ strategy. My behavioral experiments provide evidence that colonies both with and without parasites discriminate between nestmates and conspecific non-nestmates and that parasites are not ‘chemically hidden’ but are in fact detected and attacked by host ants that are not familiar with parasites. Furthermore, I find that volatile chemicals emitted by the parasites likely induce submission responses in host ants. The results show that parasites do not need to match the label of their host, but use volatile chemicals in order to manipulate members of the host colony.

In chapter 2, I investigate in carpenter ants (*Camponotus floridanus*) how specific manipulations of CHC profiles of individuals originating from the same source colony influence their nestmate recognition. I provide evidence that ants can adjust to novel CHC profiles, while the previous CHC profiles are still accepted as nestmate. Furthermore, I find that not only an addition but also a lack of a hydrocarbon can lead to a discrimination as non-nestmate and I provide the first evidence that ants can form novel templates for nestmates.

In chapter 3, I investigate the influence of recent presence of nestmates (social context) on the propensity of aggression response towards non-nestmates in the territorial red wood ant, *Formica rufa*. When kept in a group of nestmates, workers showed a high probability of aggression against non-nestmates whereas the lack of social context reduces the workers’ probability of aggression against non-nestmates. Thus, social context is necessary to maintain or induce a physiological state in workers that allows aggression response against non-nestmates. Furthermore, my results suggest that the nestmate recognition system can be utilized at remote sites for an adaptive and flexible tuning of the response against competitors.

For chapter 4, I collected data on the encoding of colony odors in the first olfactory neuropil in the brain of ants (*C. floridanus*) using functional imaging at high-speed and standard frame recording of calcium signals, and I developed a method for simultaneously monitoring the behavioral response of a mounted ant while measuring calcium signals. The data set of this investigation is not complete and therefore this part has to be considered as pilot study for future experiments.

The four studies, compiled in my thesis revealed novel insights to several aspects of nestmate recognition in ants: Nestmate recognition depends on previous experience of individuals, and ants can store multiple templates for different nestmate labels and can discriminate them from similar non-nestmate labels. Furthermore, the behavioral response towards conspecific non-nestmates depends on recent social contact with nestmates. My data also shed light into host-parasite relationships and broadens our understanding on how parasites circumvent the nestmate recognition system of their host. Additionally, I provide a new, testable hypothesis on how nestmate recognition is implemented in the ant brain. Taken together, my studies provide solid bases for future experiments on the proximate mechanisms of nestmate recognition, learning and classification of labels and modulation of decision-making through changes of an individual’s internal state.
ZUSAMMENFASSUNG


In dieser Arbeit untersuche ich verschiedene Aspekte der Nestgenossen-Erkennung bei Ameisen. Speziell untersuche ich, welche Kohlenwasserstoffe produziert und wie die chemischen Profile wahrgenommen werden, wie die Verhaltensreaktion moduliert wird und wie chemischen Profile im Gehirn kodierte und dekodierte werden.


Forming social groups is not exclusive to us humans. Many animal species form social groups, where individuals from the same species live together and interact with each other in parts of or throughout their life. Some well-known examples are the herds of elephants (Katugaha, 1999), packs of wolves (Mech, 1999), and prides of lions (Packer et al., 1990). Within these social groups individuals often perform cooperative acts, e.g. cooperative hunting or cooperative defending. Individuals cooperating together have some apparent advantages compared to individuals acting alone. In African wild dogs, for example, it has been reported that cooperative hunting and an increase in group size enhances the rate of successful hunts, reduces the individual’s costs and enables the group to capture larger prey (Creel and Creel, 1995). Hence, all individuals in the group benefit from the cooperative acts and the costs are comparatively low.

However, in some cases, a single individual of a group has costs while the other individuals of the group benefit from the individual’s behavior. This phenomenon is called altruism. For example, Belding’s ground squirrels give multi-note trills in order to alert their group members to terrestrial predators. Those warn calls, however, increase the vulnerability to predators (Sherman, 1985). Darwin (1859) introduced the paradox of altruistic behavior. His initial theory of evolution is based on the idea that individuals’ ultimate goal is to survive and reproduce. An altruistic behavior that is beneficial for other individuals, but introduces some costs for the altruistic individual may hinder its reproduction. Still, altruistic behavior seems to be an evolutionary stable strategy. It was Hamilton who came up with the inclusive fitness concept (Hamilton, 1964): an individuals reproductive success (fitness) is measured as the proportion of the individual’s genes which are passed onto the next generations. Since relatives share partly the same genes, individuals can indirectly increase their own fitness by supporting their relatives in raising offspring (indirect fitness). Obviously, the ability to discriminate between kin and non-kin is of mayor importance for altruistic behavior and the selection due to indirect fitness is called kin selection (Hamilton, 1964). As for the Belding’s ground squirrels, they indeed live in groups of close relatives and when they help their group members to survive by warning them of predators, they may indirectly increase their own fitness (Sherman, 1977).
Eusociality in the animal kingdom

The most sophisticated form of altruism is found in groups of eusocial individuals. Eusociality is characterized by the following three features: cooperative brood care, overlapping generations of adults, and most importantly division of reproductive labor with reproductive and non-reproductive members. Eusociality has been described mainly in eusocial Hymenoptera (ants, bees and wasps (Wilson and Hölldobler, 2005)), but is also found in other insects, like termites (Bartz, 1979), (Thorne, 1997), aphids (Stern, 1994), gall thrips (Crespi, 1992), beetles (Kent and Simpson, 1992) as well as in crustacean, e.g. the sponge-dwelling snapping shrimp (Duffy et al., 2000) and in mammals, namely in naked mole rats and Damaraland mole rat (Burda et al., 2000).

The established paradigm is that eusociality has evolved through the combination of an increased number of offspring due to altruistic helpers and a close relatedness between the group members (Foster et al., 2006). In Hymenoptera, an unusual high relatedness arises from the haplo-diploidy, where males hatch from non-fertilized (haploid) eggs and females from fertilized (diploid) eggs. Due to this sex determination system, females are more related to their sisters than to their mother or their own offspring. As a result, helping their mother (queen) to raise reproductive sisters (new virgin queens) is more advantageous in contrast to producing own offspring. Importantly, only in colonies with one single-mated queen (monogynous, monandrous colonies), the relatedness between sisters is high and it has been proposed that high relatedness is rather a result than a cause for the evolution of eusociality (Wilson, 2008; Wilson and Hölldobler, 2005). However, there is evidence that mating with one male and colonies with only one queen is an ancestral trait, supporting that a high relatedness was critical for the evolution of eusociality (Hughes et al., 2008).

All ant species are eusocial and there is a high diversity between the life styles of ants (Hölldobler and Wilson, 1990). Irrespective of the species, ant colonies have to exclude strangers and individuals need to know to whom to direct altruistic behavior, and this requires an efficient kin recognition system. In eusocial insects, kin recognition is referred to as nestmate recognition, because usually all members of a colony (nestmates) are related.

Chemical basis of nestmate recognition in ants

Nestmate recognition in ants is based on mixtures of low-volatile components present on the body surface of each individual. Those components are long-chained cuticular hydrocarbons (CHCs), which have been suggested to have evolved primarily as a barrier against dessication (Lockey, 1988) and infection. Secondarily, CHCs have gained the function to inform about colony membership. The composition of CHCs is species-specific, with each species carrying a specific set of CHCs (Guillem et al., 2016; Martin et al., 2008). In a comparative study with
more than 70 different ant species, the chain lengths of the vast majority of CHCs ranged between 19 and 33 carbon atoms (Martin and Drijfhout, 2009a). The diversity of possible configurations for a hydrocarbon of a specific chain length – and hence its potential smell – is immense, since a single hydrocarbon can be either saturated (alkanes) or unsaturated with hydrogen due to double-bonds between carbon atoms (alkenes, dienes, trienes). Furthermore, a hydrocarbon can be either linear or branched (with one or several methyl branches). Linear and longer chained hydrocarbons generally have higher melting temperatures (Gibbs, 1995; Gibbs and Pomonis, 1995), which in turn improves the water proofing properties. While linear alkanes might be important for protection against desiccation, it was suggested that alkenes and dimethyl-branched alkanes might play an important role in nestmate recognition (Gibbs, 2002; Guerrieri et al., 2009; Martin et al., 2008). The hydrocarbons are thought to be produced in oenocytes (Moussian, 2013). These cells are often associated with fat bodies, which are the mayor source of fatty acids that could serve as the precursors for hydrocarbon synthesis (Provost et al., 2008). Hydrocarbons are released to the hemolymph and transported to the cuticle and the post-pharyngeal gland (PPG) (Soroker and Hefetz, 2000). The PPG acts as a reservoir of hydrocarbons, and from there hydrocarbons can be distributed on the cuticle via self-grooming.

Individuals from neighboring conspecific colonies usually carry the same CHCs, only the ratios of CHCs in the mixtures differ and are colony-specific. Behavioral experiments have shown that these quantitative differences of CHCs are sufficient for ants to discriminate between nestmates and non-nestmates (Lahav et al., 1999; Wagner et al., 2000). Besides the genes, also environmental factors (e.g. nesting material and diet) influence the CHC profiles of individuals from a colony (Jutsum et al., 1979; Vander Meer et al., 1989). CHCs are frequently exchanged between nestmates by allo-grooming and sharing of liquid food (trophallaxis), resulting in a uniformation of the CHC profiles between members of the same colony (Dahbi et al., 1999; Lenoir et al., 2001a). However, sufficient differences in the CHC profiles between members of a colony still exist (Kaib et al., 2000) and can be used by individuals to discriminate between nestmate workers of different tasks (Greene and Gordon, 2003). The sum of all CHC profiles of individuals from the same colony present at a time is called the colony odor. Colony composition, diet and nesting material for a particular colony may change over time, and, as a consequence, the individuals’ CHC profiles and hence the colony odor change as well (Lambardi et al., 2004; Vander Meer et al., 1989).

In general, individuals that are not recognized as nestmates, are rejected. Rejected ants are not allowed to receive food and are being attacked when entering the colony. Therefore, individuals of a colony know how their nestmates currently smell, generalize the perceived CHC profiles to a certain degree in order to not falsely reject nestmates, but also discriminate between the perceived CHC profiles in order to avoid falsely accepting non-nestmates.
Nestmate recognition mechanisms

Different mechanisms for recognizing nestmates and discriminating them from non-nestmates have been proposed. The individual recognition model assumes that individuals become familiar from previous encounters. Paper wasps use individual recognition and visually discriminate between different colony members based on their individual facial patterns (Baracchi et al., 2015, 2016). In ants, individual recognition based on visual cues is not known and individual recognition based on chemical cues is rare. Olfactory individual recognition has been demonstrated in the ant *Pachycondyla villosa* (d’Ettorre and Heinze, 2005) for which individual recognition is important for the development of a dominance hierarchy between the queens. However, ant colonies can become huge with several thousand individuals within one colony at a time. In this case, it is unlikely that an individual has been familiarized to and can recognize all her sisters in order to not falsely reject them. Crozier and Dix (1979) proposed a model for nestmate recognition in social insects which assumes that the components on the cuticle are transferred among all individuals from a colony by grooming and trophallaxis, resulting in a common colony signature. Only individuals with this common signature will be accepted as nestmates. However, as noted above, there are differences between individuals’ CHC profiles and different worker tasks from the same colony can be discriminated by their sisters (Greene and Gordon, 2003). The phenotype matching model by Holmes and Sherman (1983) (also termed label-template matching model) assumes that each individual carries both, phenotypic recognition cues (its own recognition label) and an internal template specifying a set of specific recognition cues that can be used to distinguish whether another individual belongs to the group or not (Lacy and Sherman, 1983; Sherman, 1977). The recognition cues could be learned from the individual itself by self-referent matching or from encounters with individuals of its group. When an ant then encounters another individual, it compares the perceived CHC profile (label) with its internal representation of the learned recognition cues (template). As long as a certain threshold of difference between label and template is not reached, the encounter ant is recognized as a nestmate. Several behavioral studies support that the acceptance threshold is not fixed but flexible, e.g. depending on parasite pressure (d’Ettorre et al., 2004; Fürst et al., 2012).

Acquisition of the template

Several studies demonstrated that the recognition of colony-specific cues is learned rather than innate (Lenoir et al., 1999). Now the question arises, when do ants learn the recognition cues, e.g. when do they acquire the template? Ants are holometabolous insects and undergo a complete metamorphosis including four different developmental stages. The queen lays fertilized eggs, and from each egg a larva hatches and grows until it is developing into a pupae. The final stage is the adult stage. A freshly eclosed adult is also called callow and can be easily
distinguished by human eye from older adults, because its cuticle is still soft and has a brighter color. Callow workers are not aggressive towards any other con- or heterospecific individuals (Fielde, 1903). Furthermore, they do not exhibit an own CHC profile yet (Lenoir et al., 1999), hence, when transferred to another foreign colony, callows also do not elicit aggression in their non-sister, but are fed and groomed by them (Fielde, 1903). This allows to study the acquisition of the template through cross-foster experiments. In such kind of experiments, individuals from different colonies of the same or even of different species are raised in one artificial colony without any aggression between individuals (Carlin and Hölldobler, 1983, 1986; Errard, 1994). In artificial mixed-species colonies, heterospecific individuals raised in the same colony accept each other, while genetic sisters raised in different colonies are aggressive (Carlin and Hölldobler, 1983). This demonstrates that ants may not be able to do true kin recognition, but rather a recognition based on colony membership. Those CHC profiles, which ants frequently encounter during a few days after their eclosion, are used for recognition of their colony members (Carlin and Hölldobler, 1983; Morel and Blum, 1988). Additionally, callows need social contact with nestmates in order to develop and/or acquire a colony-specific label (Morel et al., 1988). When callow ants become older, they are recognized by foreign ants as strangers and the callow ants themselves show discrimination behavior between nestmates and non-nestmates. Beside learning of recognition cues as a callow, it has been shown that the template can be updated and may be constantly updated during adult life (Guerrieri et al., 2009; Leonhardt et al., 2007). This is of great importance since, as noted above, the colony odor may change over time, when colony composition, diet and nest material change with time.

Factors influencing the behavioral response

It is important to note that even if recognition occurs (e.g., the recognition of a non-nestmates, when the perceived CHC label does not match the neural template), it does not necessarily result in behavioral discrimination (e.g., aggressive behavior towards the non-nestmate), when the ability or the ‘motivation’ to behaviorally respond is missing. The word ‘motivation’ is controversial and is used here in a non-anthropomorphic sense. For example, when an individual in a familiar environment is confronted with one and the same stimulus at two different time points, this may result in a behavioral responses that is different in both situation solely due to the motivational state of the animal.

Not all individuals behave the same, hence there is variability in aggression response of different workers from the same colony with consistent individual differences, where some individuals are always more aggressive than others (Newey et al., 2010; Van Wilgenburg et al., 2010). The factors influencing the aggression response are diverse. Prior winning experience, for example, increases the probability of responding with aggression towards non-nestmates (Van Wilgenburg et al., 2010).
It has been shown that different task groups have different aggressiveness, were partollers or foragers are usually aggressive against non-nestmates, while brood tenders are usually not aggressive (Nowbahari and Lenoir, 1989; Sturgis and Gordon, 2013). The group size of near-by nestmates also influences the individual’s propensity of aggressive response against hetero-specifics (Tanner, 2006). In the desert ant *Cataglyphis fortis*, individuals forage alone and the state of their path integrator has been shown to influence the individual’s aggressiveness (Knaden and Wehner, 2004). Furthermore, familiarity with the encounter may influence the aggression response. For some species, a dear-enemy effect has been described, where the aggression towards non-nestmates from familiar (neighboring) colonies was lower than towards non-nestmates from unfamiliar (distant) colonies (Heinze et al., 1996; Langen et al., 2000). However, in other species the opposite, a nasty-neighbor effect, has been described (Frizzi et al., 2015; Knaden and Wehner, 2003).

**Neural template in the brain**

Little is known about the neural mechanisms of nestmate recognition and about where the template is stored in the brain. As other insects, ants detect general odorants and also CHCs with their antennae. Odorants are detected by olfactory receptor neurons (ORNs), housed in sensilla on the antenna (Schneider and Boeckh, 1962). ORN axons project to the first olfactory center of the brain: the antennal lobe (AL). ORNs which express the same olfactory receptors are sorted by converging into the same functional units in the AL. These units are called glomeruli which are connected via a dense network of inhibitory and excitatory interneurons (Hansson and Anton, 2000). From the AL, the olfactory information is further transmitted to higher brain centers, like the mushroom bodies (MB) and the lateral horn (LH), via project neurons (PNs). The MB is important for learning and memory (Giurfa, 2007; Heisenberg, 2003) and the LH is considered as an important structure for evaluating the biological significance of odors (Sandoz, 2011). Upon stimulation with general odorants, the connectivity in the AL results in a spatio-temporal activity pattern, carrying information about odorant identity. Comparing the glomerular activity patterns with odor discrimination abilities revealed that odors perceived as being similar induce similar spatial activity patterns in the AL (Guerrieri et al., 2005). Also both nestmate and non-nestmate CHC profiles evoke neuronal activity in the AL (Brandstaetter and Kleineidam, 2011). Spatial activity patterns elicited by nestmate and non-nestmate CHC profiles are overlapping and based on the patterns, the colony identity of the CHC profile cannot be predicted (Brandstaetter and Kleineidam, 2011). Similar spatial activity patterns in the AL were assumed since nestmate and con-specific non-nestmate CHC profiles consist of the same CHCs, hence, activating the same glomeruli. However, the temporal onset of glomerular activation should be different due to different ratios of the CHCs. An interesting finding of the study by Brandstaetter and Kleineidam (2011) was, that repeated stimulations with nestmate CHC profiles elicited more variable activity
patterns than repeated stimulations with non-nestmate CHC profiles - which is puzzling at the first glance. As a possible explanation for this phenomenon, the authors suggested that ants need to update their template of their own colony odor and therefore, plasticity is needed. The plasticity may be seen as highly variable activity patterns when nestmate CHC profiles are presented.

In a nutshell, the nestmate recognition process can be divided into the following parts: i) the expression involving producing and presenting of a CHC profile on the cuticle of each individual, ii) the reception involving the detection of the CHC profile by ORNs, iii) the sensory processing of the olfactory information in the AL network and in higher brain areas, iv) the perception involving the presence of a percept, attributing the olfactory information with a specific meaning (classification), and v) the decision of a behavioral response based on the perceived odor and the ‘motivation’ to respond.

**Scope of this thesis**

In this thesis, I investigated different aspects of the nestmate recognition process (expression, processing, perception and behavioral response). For each topic, I selected a specific ant species that is well suited to investigate the question. In a particular host–social parasite system, I investigated which recognition cues are produced by the parasite ants to integrate into host ant colonies (chapter 1). In another species, I investigated, whether the acceptance range is broadened by widening the present template or by forming several templates (chapter 2). Furthermore, I investigated how the current social context affects the propensity of individuals to respond aggressively (chapter 3). Finally, I investigated the neural encoding of nestmate and non-nestmate CHC profiles in the AL (chapter 4).

*Chapter 1 - Host colony integration: Megalomyrmex guest ant parasites maintain peace with their host ants through weaponry*

Although, ants are very precise and fast in nestmate recognition, there are some species which are able to enter (infiltrate) and live within (integrate into) ant colonies without being recognized as non-nestmates. Such species, parasitizing colonies of social insects, are called social parasites. Social parasites can use one or a combination of several chemical strategies to live in nests of their host ant colonies. This chapter is a study on a specific host-social parasite system (fungus-growing ant *Sericomyrmex amabilis* colonies parasitized by *Megalomyrmex symmetochus*) and deals with the question: Which chemical strategy does the social parasite ants use to integrate into colonies of the fungus-growing ant species? We compared the CHC profiles of the host and the social parasite and conducted behavioral experiments.
Chapter 2 - Learning distinct chemical labels of nestmates in ants
It was previously considered that ants are not able to perceive, if a component is missing in a label. This chapter investigates in the species *Camponotus floridanus*, whether CHC profiles are perceived as an entity or rather as a sum of its components and whether the acceptance range is broadened by template widening or by developing several templates. To this end, we chemically manipulated the CHC profiles of ants and conducted behavioral experiments.

Chapter 3 - Social interactions promote adaptive colony defense in ants
Whether an ant is aggressive or not when encountering a non-nestmate is influenced by many factors, e.g. the worker’s task or distance to the nest. In this chapter, we investigate in *Formica rufa*, how the current social context influences the worker’s propensity of aggression. We manipulated the recent social context of individuals (allowing or prohibiting contact with nestmates) and conducted behavioral experiment.

Chapter 4 - Investigating the neural encoding of colony odors with calcium imaging experiments
A previous calcium imaging study found that repeated stimulations with the same nestmate or non-nestmate CHC profile induce variable glomerular activity patterns and suggested that nestmate and non-nestmate CHC profiles are not encoded in distinct spatial patterns of glomerular activity in the antennal lobe. In this chapter, we asked whether a CHC profile could be encoded in the temporal pattern of glomerular activity and whether the high variability in encoding of repeated stimulations is a specific feature of the own colony odor. We used calcium imaging in the AL of the carpenter ant *Camponotus floridanus* during repeated stimulation with nestmate and non-nestmate CHC profiles and developed a behavioral monitor (electro-physiological recordings of the mandible muscles) in order to investigate simultaneously the encoding of nestmate and non-nestmate CHC profiles and the individuals’ behavior towards a presented CHC profile.
Abstract

Social parasites exploit resources of other social species, to the detriment of their host. In order to enter and integrate in a host colony, social parasites must avoid being detected as a non-nestmate. The parasites, therefore, use one or a combination of chemical strategies: 1) producing recognition cues that match host’s (mimicry), 2) acquiring recognition cues from the hosts or its nest (camouflage), 3) not producing recognition cues (insignificance), and/or 4) using substances for confusing, suppressing or appeasing the host (weaponry).

In this study, we investigate the integration strategy of *Megalomyrmex symmetochus* ants into colonies of the fungus-growing ant *Sericomyrmex amabilis*. We compared the chemical odour profiles of parasitized and non-parasitized *S. amabilis* colonies with the profiles of the parasites. Additionally, we conducted behavioural assays, where we introduced a single ant, being either a nestmate, a conspecific non-nestmate, or a parasite into an arena with five *S. amabilis* workers and scored the behaviour of the latter ants. The chemical analysis revealed that the social parasites have distinct odour profiles and share only one hydrocarbon with its host, have a low overall abundance of cuticular hydrocarbons, and have high concentrations of venom-derived alkaloids. In behavioural experiments, we found that workers of non-parasitized colonies fight against parasite intruders, whereas workers of parasitized colonies treat introduced parasites (from their own and from another parasitized colony) similar to their conspecific nestmates. All workers (parasitized or not) show more submissive behaviour towards parasitized workers and parasites than towards non-parasitized workers. The chemical analysis of odour profiles suggests that the parasites use a chemical insignificance strategy.
Furthermore, the chemical and behavioural data suggest that the parasites use weaponry to maintain an amiable association with their host ants. We discuss the biological significance of the lack of aggression in *S. amabilis* workers from parasitized colonies.

**Keywords**

Host-parasite system, cuticular hydrocarbon, nestmate recognition, alkaloid, tolerance, resistance, submission, aggression, *Sericomyrmex amabilis, Megalomyrmex symmetochus*

**Introduction**

Sophisticated mechanisms have evolved to protect social insect colonies (e.g., ants, some bees and wasps) from invasion (Breed and Bennett, 1987; Gamboa et al., 1986; Hölldobler and Wilson, 1990). Therefore, a successful exploitation of social insect colonies requires strategic evasion of organized defence tactics. The recognition of colony members is a fundamental component of being social, and thus allows amiable social groupings and loyalty between colony members. The nestmate recognition system of social insects is predominantly chemically-based and allows individuals to discriminate between members from their own colony (nestmates) and members from a different colony (non-nestmates). In ants, nestmate recognition is based on long-chained species-specific cuticular hydrocarbons (CHCs) present on the exoskeleton (Brandstaetter et al., 2008; Lahav et al., 1999; Martin and Drijfhout, 2009a). CHCs are thought to have evolved primarily as protection against desiccation (Lockey, 1988), and gained secondarily a function for identification of colony membership, where neighbouring colonies of the same species have the same CHCs, which only differ in quantity (d’Ettorre and Lenoir, 2010). The odour profile of a colony is not only genetically determined, but also influenced by environmental factors, like nest material and diet (Jutsum et al., 1979; Liang and Silverman, 2000), and thus varies over time (Vander Meer et al., 1989). It is generally assumed that an ant compares the perceived odour profile (label) of an encountered ant with an internal neuronal representation (template) of its own colony odour, a process called label-template matching (van Zweden and d’Ettorre, 2010). When label and template are similar, the encountered ant will be recognized as a nestmate, if mismatched, the encountered ant is recognized as a non-nestmate and may be attacked (Vander Meer and Morel, 1998). Since the colony odour changes over time, the neuronal template needs to be updated as well (Vander Meer et al., 1989). Social parasites, defined as a social organism that exploits another social organism, avoid host attack by circumventing detection or through host domination (Buschinger, 2009).
Social parasites overcome host detection by using one or more chemical strategies: mimicry, camouflage, insignificance, and/or weaponry (Akino, 2008; Lenoir et al., 2001b). Some parasites can produce nestmate recognition cues that match the host’s chemical profile (chemical mimicry) while others can acquire them by exposure to the nest environment or host individuals (chemical camouflage). An impressive example for these two strategies is the caterpillar of the butterfly Maculina rebeli that parasitizes Myrmica ant colonies (Akino, 2008; Nash et al., 2008). The caterpillars produce host recognition cues and are carried by ant workers into the nest. Later they acquire additional hydrocarbons within the nest, making their CHC profile nearly identical to their host’s (Akino et al., 1999). Similarly, social parasite wasp CHC profiles change to match colony-specific host odour following infiltration and during host colony integration (Sledge et al., 2001). Other parasite species lack an abundant CHC profile, when recognition cues are absent the parasites appear to be chemically ‘invisible’ to the host (i.e., chemical insignificance). This strategy is used during host colony infiltration by the social parasitic ant Acromyrmex insinator. The parasites avoid host colony aggression by producing fewer hydrocarbons relative to their host and bearing increased n-alkane levels (Nehring et al., 2015). Besides circumventing detection, parasites can also produce chemicals to attack or confuse the host, disrupting nestmate recognition and host defence behaviour (chemical weaponry). This strategy is used in the slave-making ant species Polyergus rufescens. The usurping queen uses secretions from its Dufour’s gland as an appeasement allomone (Mori et al., 2000) or repellent (d’Ettorre et al., 2000).

The expected evolutionary response of hosts towards social parasites can be either in the form of resistance or tolerance, both adaptive solutions to parasite exploitation. Parasite resistance may involve direct host aggression towards the parasite, preventing a successful attack. It may also involve a hierarchical sequence of resistance behaviours that occur over time (Kilner and Langmore, 2011). In other circumstances, hosts use a ‘tolerance’ strategy to minimize detrimental fitness impacts of parasites, in other words, it is better to consent than to risk death (Svensson and Råberg, 2010). Tolerance behaviour would be expected in host species that lack an effective defence (e.g., toxic poison, strong mandibles) or species that do not recognize the parasite as a threat but instead as a harmless nestmate.

The social parasitic ant species Megalomyrmex symmetochus (Formicidae: Solenopsidini) (Wheeler 1925) parasitizes the fungus-growing ant Sericomymrex amabilis (Formicidae: Attini: Attina) (Wheeler 1925) by living within the nest and consuming the brood and fungus garden of the host (Adams et al., 2013; Bruner et al., 2014; Wheeler, 1925). The interactions between the host and parasites are typically amiable. However, aggression has been observed in field and laboratory colonies when the two species are producing sexuals (Boudinot et al., 2013). In addition, the parasite workers chew the wings from the host female sexuals (i.e., gynes), prohibiting these individuals from dispersing. The parasite workers are armed with
Chemical integration into a host colony

toxic alkaloid venom that can kill the host and the hosts’ enemies (Adams et al., 2013). In contrast, S. amabilis workers do not appear to have a toxic venom, but are capable of biting off legs and antennae from their opponents (Adams et al., 2013; Boudinot et al., 2013). Their reaction to threat is often crypsis, during which ants tuck their antennae and head under and play dead, similar to other attine host species (Adams et al., 2015).
The infiltration strategy (i.e., initiation of the association) and integration strategy (i.e., maintenance of the association) of M. symmetochus parasites into colonies of S. amabilis are currently unknown. In this study, we investigate the integration strategy of M. symmetochus parasites using chemical analysis and a behavioural approach. If host colonies and parasites have similar CHC profiles (i.e., mimicry or camouflage) then we predict that workers from a parasitized colony would not react to the parasites but workers from a non-parasitized colony would react aggressively, just as they would to a conspecific non-nestmate. If the parasites’ CHCs are very low in abundance then we predict that the host as well as non-parasitized ants would behave as if they did not detect the parasites (i.e., chemical insignificance). If the CHC profile of the parasite is not similar to their host’s and causes a behavioural reaction by S. amabilis workers from parasitized and non-parasitized colonies, it would suggest the a weaponry strategy. We found no evidence for mimicry or camouflage. In contrast, our chemical analysis suggests that the parasites use a chemical insignificance strategy. Furthermore, the chemical and behavioural data support the hypothesis that the parasites use weaponry to maintain an amiable association with their host ant species S. amabilis.

Material and Methods

Study Animals

Sericomyrmex amabilis colonies or sub-colonies (referred to as colonies hereafter) were collected along Plantation Road in Gamboa (9.16 N, 79.74 W) and on Barro Colorado Island (9.16 N, 79.84 W), Republic of Panama between 2011 and 2013. Queen-right and queen-less laboratory colonies can be kept alive for years under humid laboratory conditions as long as they forage to feed their garden (Adams personal observation). In our experiments, 16 laboratory host colonies contained Megalomyrmex symmetochus social parasites (referred to as parasitized colonies) and 19 S. amabilis colonies were without parasites (non-parasitized colonies).

Creating CHC extracts

We randomly collected workers from parasitized and non-parasitized colonies (host and parasites, if present) from their nest boxes into filter paper-lined Petri dishes, allowed them to acclimate, then froze them over night at -80°C (colonies and species were kept in separate dishes). For each extract, we selected three workers
and put them in a 2 ml vial with ca. 100 µl of pentane (cuticular wash). The vials were gently agitated for one minute using a vortex machine with a slow rotating speed. After waiting 10 minutes and gently shaking the vial again, the solution was moved to a 200 µl insert, and evaporated. The samples were frozen until chemically analysed. In total, we created 19 host ant extract samples from four parasitized colonies, 36 extracts of eight non-parasitized colonies, and 24 extracts of parasites from five different parasitized colonies.

Chemical analysis of CHC extracts

Before the gas chromatography-mass spectrometry (GC-MS) analysis, 10 µl of hexane was added to each vial (C22 standard was added to a subset of the samples). Then using an autosampler, 3 µl of extract were injected (split-less) into a GC (Agilent 7890) coupled to MS (Agilent 5975C). The components were separated on a non-polar column (ZB-5HT, 30 m x 0.32 mm, film thickness 0.25 µm). The carrier gas was helium with a constant flow of 1 ml/min. Chromatograms were recorded with Agilent Chemstation using the following temperature program: 1) constant temperature of 100°C for 1 min, 2) increasing temperature at 30°C/min to 250°C, 3) increasing temperature at 4°C/min to 360°C, and 4) constant temperature at 360°C for 2 min. Chromatograms were analysed using Enhanced ChemStation (MSD ChemStation Agilent Technologies, 2005). Peaks were aligned manually to ensure identical compounds were compared between runs. Peak areas of individual cuticular hydrocarbons were quantified with respect to the total CHC area within single chromatograms. CHCs were identified based on diagnostic ions and mass spectra were compared with entries of the NIST11 library.

Calculation of cuticular surface

In order to compare the total CHC abundance between the host and the parasite species (insignificance hypothesis), we used only the samples with C22 standard (non-parasitized host samples: n=15, parasitized host samples: n=16, and parasite samples: n=11). We calculated the sum CHC area for each chromatogram and normalized the area to the standard peak area. To take into account potential differences in body surface area between the two species (since individuals with a small total body surface area may automatically have a lower total abundance of CHCs), the body surface area was estimated for parasite and host species individuals (for detailed information on calculation of the surface area, see Supplementary Material Materials on page 27). We used a similar approach as described in (Kroiss et al., 2009). We measured length, width and height of the three body parts: head, thorax (mesosoma) and gaster of 10 individuals per species with a stereo microscope equipped with an eyepiece reticle. From these measures, we approximated the surface area of each individual’s body parts using corresponding geometric shapes. We calculated the total surface area, by adding the surface areas of the three body parts. We compared the surface areas of the two species using a
two-sample t-test. We then calculated the total CHC peak area per chromatogram corrected with respect to body size of the corresponding species.

**Principle component analysis for CHC profiles**

We also compared the similarity of the CHC profiles between the host and the parasite (mimicry and camouflage hypothesis) using a principal component analysis (PCA), based on all chromatograms. We used the peak areas of the CHCs normalized to the total CHC peak area within each chromatogram as input. Furthermore, we compared the CHC profiles between parasitized and non-parasitized host colonies with another PCA. For quantification of the differences, we calculated the average pairwise Bray-Curtis distance based on the relative abundance of CHCs (normalized to the overall CHC area of each chromatogram) between all samples. Bray-Curtis distances were calculated with the `bcdist` function in R (3.4.0) and range from zero to one. A value of zero represents a complete matching between two data points, while a value of one represents a complete separation in the n-dimensional space.

**Behavioural Assay**

For one trial, five *S. amabilis* workers (referred to as ‘focal ants’ hereafter) were selected from either a parasitized or non-parasitized colony and directly put into a small Petri dish (35 mm in diameter) with a piece of fungus garden (ca. 100 mm³). This setting, resembling the nest environment, should provide a social context for the focal ants ensuring realistic colony defence responses (Kleineidam et al., 2017). We allowed the focal ants to acclimate for at least 5 min. Then, another ant was introduced into the dish and is referred to as the ‘stimulus ant’ hereafter. The stimulus ant was either a *S. amabilis* nestmate, a conspecific *S. amabilis* non-nestmate, or a heterospecific *M. symmetochus* parasite. New focal ants and *S. amabilis* stimulus ants were used in each trial, but it was necessary to use some parasite stimulus ants multiple times (due to a low number of parasite ants). The behaviour of all five focal ants in response to the stimulus ant was video recorded for 5 min and later scored blindly (the person was unaware of colony origins for focal and stimulus ants). Solomon Coder (Version: beta 15.11.19 by András Péter) was used to record the behaviour of the focal ants following an interaction. An interaction was identified when a stimulus and a focal ant were within an antennal length distance from each other or when they touched. The behaviours were scored as being from one of several behavioural categories (Table 1.1). The behaviours ‘short antennation’, ‘prolonged antennation’, ‘opening mandibles’, ‘carrying’ and ‘biting/pulling’ were used for the statistical analysis of aggression. The behaviours ‘turning’ and ‘head tucking’ were used for the statistical analysis of submission. Sometimes, a focal ant did not show an obvious reaction or a change in behaviour after being contacted by a stimulus ant or in some cases, head and mandibles of a
1.3 Material and Methods

Table 1.1 Ethogram of behavioural responses from focal ants towards stimulus ants used in statistical analyses.

<table>
<thead>
<tr>
<th>Behavioural Response</th>
<th>Description</th>
<th>Analysis Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short Antennation</td>
<td>Focal ant’s antennae are moving towards or touching the stimulus ant and lasts less than three seconds.</td>
<td>Aggression</td>
</tr>
<tr>
<td>Prolonged Antennation</td>
<td>Focal ant antennation lasts for more than three seconds.</td>
<td>Aggression</td>
</tr>
<tr>
<td>Opening Mandibles</td>
<td>Focal ant opens her mandibles in response to the stimulus.</td>
<td>Aggression</td>
</tr>
<tr>
<td>Carrying</td>
<td>Focal ants displace a stimulus ant via dragging or lifting with their mandibles for more than two seconds.</td>
<td>Aggression</td>
</tr>
<tr>
<td>Biting/Pulling</td>
<td>Focal ant bites at or on the stimulus ant, including pulling at one of her extremities.</td>
<td>Aggression</td>
</tr>
<tr>
<td>Turning</td>
<td>Focal ant changes direction (&gt;45 degrees) after close contact with stimulus ant.</td>
<td>Submission</td>
</tr>
<tr>
<td>Head Tucking</td>
<td>Focal ant retracts her antennae into her scrobes and tucks her head down for at least two seconds.</td>
<td>Submission</td>
</tr>
</tbody>
</table>

focal ant were not visible, and therefore no discernible behaviour could be noted. Both cases were classified as ‘unknown’.

We performed the trials with nine different pairings of focal and stimulus ants. The different pairings were necessary to disentangle, whether the type of stimulus ant accounted for differences in behaviour of focal ants, or whether the type of focal ant (being from a parasitized or non-parasitized colony) also explained some of the differences. In two pairings, we tested focal ants from parasitized and non-parasitized colonies against their conspecific nestmates as stimulus ants (pairings #1 and #2, respectively). In four pairings, we tested the behaviour of focal ants from parasitized and non-parasitized colonies against conspecific non-nestmates from parasitized and non-parasitized colonies (pairings #3, #4, #5, and #6). In the remaining three pairings, we tested the behaviour of focal ants against parasites. Focal ants from parasitized colonies were tested against their own parasites (pairings #7) and also against parasites from a foreign colony (pairing #8), while focal ants from non-parasitized colonies were tested only against foreign parasites (pairing #9), as by definition they do not have parasites in their colony. In our experiments, we used queen-right and queen-less colonies for both parasitized and non-parasitized colonies. If the queen-status affects nestmate recognition in the corresponding individuals (focal and stimulus ants), it would influence our experiments by increasing the variance in the data within all pairings.
Analysis of behavioural data

_Sericomyrmex amabilis_ ants respond to various perturbations with a crypsis defence strategy (submission). Submission is therefore not unique to ant-to-ant interactions; however, it is a recognized defence behaviour in many attine ant species (Adams et al., 2015; Wheeler, 1925). Since submission behaviour is not typically linked to nestmate recognition, we investigated the submissive behaviours ‘head tucking’ and ‘turning’ independently from the other behavioural responses (e.g., ‘short antennation’, ‘prolonged antennation’, ‘opening mandibles’, ‘carrying’ and ‘biting/pulling’).

The behavioural data were analysed with respect to the differences in aggression behaviour between the nine different pairings. We ran a multinomial logistic regression model (a model with a categorical outcome variable) on the aggression data. This kind of analysis allowed us to account for differences in the number of encounters per trial by investigating the proportion of a single behaviour within each trial. Furthermore, it enabled us to account for repetitions of trials from the same colony (non-independent data). For each trial, we counted the total number of occurrences for each of the behavioural responses ‘short antennation’, ‘prolonged antennation’, ‘opening mandibles’, ‘carrying’ and ‘biting/pulling’. We used these counts as the multinomial response variable. In the model, we included an explanatory variable for the specific pairing (#1–#9). The colony origin of focal ants and stimulus ants were added as random effects. The parameters of the multinomial logistic regression model were estimated in a Bayesian framework using Markov chain Monte Carlo simulations. We ran OpenBUGS from within R using the 'R2OpenBUGS' package (Sturtz et al., 2005), and generated random initial values, then ran three Markov chains for 100 000 iterations. The burn-in was set to 10 000 and the chain was thinned by 10 (i.e., sampled every 10th iteration) to reduce autocorrelation. We assessed convergence graphically and by the R-hat values (i.e., MCMC convergence statistic), which were always close to one (Brooks and Gelman, 1998). From the Markov chains, we extracted the means using them as estimates and the 2.5% and 97.5% quantiles to describe the 95% credible intervals (Gelman et al., 2004). In contrast to the null hypothesis testing in frequentist methods (via p-values), Bayesian statistics allow the assessment of significance through probabilities of meaningful hypotheses (measure of certainty). We tested if there are differences in the proportions of behavioural responses (e.g., ‘short antennation’, ‘prolonged antennation’, ‘opening mandibles’, ‘carrying’ and ‘biting/pulling’) within a trial between two pairings. Therefore, we compared the proportion of a specific behaviour by calculating the percentage of simulated values from the posterior distribution that are larger (or smaller) in one pairing compared to another pairing. We considered a difference between the proportions of a specific behaviour between the two pairings to be significantly different if the calculated percentage was larger than 0.98 (certainty of 98%).
We also investigated the differences of submission responses between the nine different pairings. We used a binomial generalized linear mixed model (GLMM) with submission (‘head tucking’ and ‘turning’) as the binary response variable. Again, this kind of analysis allowed us to account for repetitions of trials from the same colony (non-independent data). Whenever submission (‘head tucking’ or ‘turning’) occurred within one trial, the trial was counted as submissive. If no submission occurred, the trial was counted as non-submissive. Additionally, we included the specific pairing (#1–#9) as explanatory variables in the model. We added origin of the focal ants as well as origin of the stimulus ant as the two random effects. Furthermore, we used a Bayesian framework in order to reveal certainty measures for the parameter values. In contrast to other methods, Bayesian methods are the only exact way to draw inferences from GLMMs (Bolker et al., 2009). We used an improper prior distribution (flat prior) and directly simulated 5000 values from the posterior distribution of the model parameters by using the 'sim' function of the R package 'arm' (Gelman and Hill, 2007). For each pairing, we used the fitted values as estimates and the 2.5% and 97.5% quantiles from the simulated model parameters as the upper and lower limits of the 95% credible intervals. We tested for differences in submission probabilities between two pairings by comparing the probabilities of submissive behaviour. We calculated the proportion of simulated values from the posterior distribution that were larger (or smaller) in one pairing compared to another pairing. We considered a difference between the probability of submission between two pairings to be significantly different if the calculated proportion was larger than 0.98 (certainty of 98%). The statistical analysis was done using R 3.4.0 (R Core Team, 2017).

Results

Chemical analysis

To determine odour profiles, we analysed the chemical content of cuticular washes. The GC-MS analysis showed that the CHC profiles differed between the host and parasite ants (Figure 1.1). Together, the *M. symmetochus* parasites and *S. amabilis* workers from parasitized and non-parasitized colonies revealed 33 different CHC compounds (Figure 1.1, Table 1.2). Both *S. amabilis* workers from parasitized and non-parasitized colonies had 24 hydrocarbons between C29 to C37, sharing the same CHCs (Table 1.2). In contrast, the CHC profile of *M. symmetochus* parasites ranged from C33 to C39 with ten distinct compounds. Among those compounds were possibly several rarely occurring methyl-branched dienes (branched C35:2, C37:2, and C39:2). The reason why we are not certain is, that peaks 20, 21, 27, 28, 32, and 33 had profiles of the mass spectra up to m/z 100 that looked very much the same as alkadienes compared to corresponding mass spectra in the NIST11 library. However, we also found indications that they were possibly branched alkadienes because there were a few ions at the higher mass end (m/z 200–420).
that were more elevated, but this could also be due to higher ion abundances. We found only a single hydrocarbon (the alkene C35:1, Figure 1.1, Table 1.2) that was shared between the host and parasites. The parasite workers had a smaller amount of CHCs on their cuticle than the host species (Figure 1.1a). To control for the differences of body size, we calculated the body surface area of host and parasite workers. The parasites had on average an 18.8% smaller body surface area than the host workers (two-sample t-test: t = 4.2911, df = 17.965, P < 0.001). However, when we compared the total CHC area normalized to the C22 peak and corrected for the difference in body surface area, we still found significant differences between host and parasite workers. The total CHC area was significantly smaller in parasites than in the hosts (two sample t-Test: t = -6.23, df = 15.72, P < 0.001). The PCA showed that host CHC profiles (from parasitized and non-parasitized colonies) were separated from the parasite profiles, both forming distinct clusters (Figure 1.1a, S1 Figure on page 29). The chemical profiles between parasitized or non-parasitized host and parasites were very different having a mean Bray-Curtis distance of 0.97 or 0.98, respectively. In contrast, host CHC profiles (from parasitized and non-parasitized colonies) were similar and overlapping with a mean Bray-Curtis distance of 0.26 between parasitized and non-parasitized host samples. For comparison, the mean Bray-Curtis distance between single parasite samples and single *S. amabilis* (parasitized and non-parasitized) samples was 0.11 and 0.25 respectively. Samples from the same *S. amabilis* host colonies were generally more similar to each other than they were to other colonies (S1 Figure on page 29, S2 Figure on page 30).

In addition to the CHCs, we found large amounts of volatile compounds in all 24 parasite samples (retention times of 8-10 min in Figure 1.1). These included a mixture of the two isomers (5Z,8E)- and (5E,8E)-3-butyl-5-hexylpyrrolizidine alkaloids (Adams et al., 2013) ranging from 16 to 43 µg per ant (T. Jones, personal communication). These isomers are dispensed from the parasite’s specialized sting as an aerosol or contact venom (Adams et al., 2013).

**Behavioural analysis**

In total, we conducted 348 trials. From all behaviours observed, 65% were used for the aggression analysis and 7% were used for the submission analysis. The missing 28% corresponds to the ‘unknown’ behaviour category, where either no obvious reaction from the focal ants towards the introduced stimulus ants occurred (no reaction: < 8%) or the behaviour could not be evaluated because it was out of view (unclear: ca. 20%).

**Aggression analysis**

The focal ants encountered the stimulus ant frequently during the 5 min trials, and the proportion of behaviours depended on the kind of pairing (Figure 1.2). In the aggression analysis, the highest proportion of behaviours were ‘short antennation’,

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Table 1.2 Mean relative abundances (±SD) of cuticular hydrocarbons in the chemical profiles of the host *S. amabilis* and its social parasite *M. symmetochus*. The shared hydrocarbon across all three categories is indicated in bold. Compounds that were found in <0.5% on average or absent from >90% of the samples were omitted (x). Unknown branching site is indicated in with '?'. Peak # = number of the peaks in Fig. 1.1. RT = retention time. N = sample size.

<table>
<thead>
<tr>
<th>Peak Nr.</th>
<th>RT</th>
<th>Compound Name</th>
<th>S. amabilis Non-parasitized Colonies</th>
<th>S. amabilis Parasitized Colonies</th>
<th><em>M. symmetochus</em> Parasite</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19.2</td>
<td>C29</td>
<td>1.96 ± 0.44</td>
<td>0.97 ± 0.44</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>20.5</td>
<td>C30</td>
<td>1.27 ± 0.47</td>
<td>0.95 ± 0.42</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>20.9</td>
<td>14,16MeC30</td>
<td>1.05 ± 0.4</td>
<td>0.7 ± 0.23</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>21.9</td>
<td>C31</td>
<td>13.73 ± 5.72</td>
<td>12.99 ± 4.33</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>22.3</td>
<td>13,15MeC31</td>
<td>11.42 ± 3.02</td>
<td>11.41 ± 2.56</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>22.7</td>
<td>11,19DMeC31; 15,17DMeC31; 15,16DMeC31</td>
<td>5.3 ± 0.94</td>
<td>3.85 ± 1.24</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>22.9</td>
<td>3MeC31</td>
<td>3.41 ± 2.13</td>
<td>4.9 ± 2.45</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>23.3</td>
<td>C32</td>
<td>4.61 ± 1.01</td>
<td>5.08 ± 1.51</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>23.6</td>
<td>12,14,16,18MeC32</td>
<td>3.95 ± 0.84</td>
<td>4.52 ± 1.03</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>4MeC32</td>
<td>2.69 ± 0.49</td>
<td>2.72 ± 0.59</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>24.6</td>
<td>C33</td>
<td>16.22 ± 6.54</td>
<td>12.98 ± 4.97</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>25</td>
<td>11,13MeC33</td>
<td>-</td>
<td>1.73 ± 0.25</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>25.1</td>
<td>13,15MeC33</td>
<td>16.13 ± 3.17</td>
<td>15.08 ± 2.83</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>25.2</td>
<td>Unknown</td>
<td>3.34 ± 2.42</td>
<td>4.8 ± 4.19</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>25.4</td>
<td>13,19DMeC33; 15,21DMeC33; 17,21DMeC33; 11,21DMeC33</td>
<td>9.26 ± 1.86</td>
<td>7.89 ± 2.86</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>25.6</td>
<td>3MeC31</td>
<td>5.25 ± 2.82</td>
<td>4.91 ± 1.85</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>26</td>
<td>C34</td>
<td>2.92 ± 1.85</td>
<td>1.91 ± 1.04</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>26.3</td>
<td>12,14,16MeC34</td>
<td>1.83 ± 0.66</td>
<td>1.68 ± 0.22</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>26.7</td>
<td>12,22DMeC34</td>
<td>1.68 ± 0.55</td>
<td>1.36 ± 0.32</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>26.7</td>
<td>possibly a branched C35:2</td>
<td>-</td>
<td>-</td>
<td>45.24 ± 49.88</td>
</tr>
<tr>
<td>21</td>
<td>26.9</td>
<td>possibly a branched C35:2</td>
<td>-</td>
<td>-</td>
<td>3.55 ± 0.49</td>
</tr>
<tr>
<td>22</td>
<td>27.1</td>
<td>C35:1</td>
<td>2.37 ± 0.91</td>
<td>3.32 ± 1.46</td>
<td>18.18 ± 25.79</td>
</tr>
<tr>
<td>23</td>
<td>27.2</td>
<td>C35</td>
<td>2.17 ± 0.94</td>
<td>1.68 ± 0.87</td>
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<tr>
<td>24</td>
<td>27.6</td>
<td>13,15,17MeC35</td>
<td>2.08 ± 0.66</td>
<td>2.88 ± 0.78</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>28</td>
<td>3MeC35</td>
<td>3.29 ± 1.7</td>
<td>3.16 ± 1.53</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>28.2</td>
<td>7,13DMeC35</td>
<td>0.66 ± 0.53</td>
<td>1.04 ± 0.53</td>
<td>-</td>
</tr>
<tr>
<td>27</td>
<td>29.3</td>
<td>possibly a branched C37:2</td>
<td>-</td>
<td>-</td>
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<tr>
<td>28</td>
<td>29.5</td>
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<td>-</td>
<td>-</td>
<td>22.86 ± 3.68</td>
</tr>
<tr>
<td>29</td>
<td>30.7</td>
<td>C37:1</td>
<td>-</td>
<td>-</td>
<td>12.55 ± 7.4</td>
</tr>
<tr>
<td>30</td>
<td>30.6</td>
<td>13,15MeC37</td>
<td>1.06 ± 0.59</td>
<td>1.31 ± 0.33</td>
<td>-</td>
</tr>
<tr>
<td>31</td>
<td>31.6</td>
<td>possibly a C39:2</td>
<td>-</td>
<td>-</td>
<td>7.57 ± 1.61</td>
</tr>
<tr>
<td>32</td>
<td>31.9</td>
<td>possibly a branched C39:2</td>
<td>-</td>
<td>-</td>
<td>8.84 ± 1.35</td>
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<tr>
<td>33</td>
<td>32</td>
<td>possibly a branched C39:2</td>
<td>-</td>
<td>-</td>
<td>7.29 ± 1.12</td>
</tr>
</tbody>
</table>
Fig. 1.1 Odour profile differences between hosts and parasites. a) Example chromatograms of *S. amabilis* and *M. symmetochus* odour profiles. The upper trace is a sample of *S. amabilis* workers from a parasitized colony and the lower trace is a sample of *M. symmetochus* parasite workers. Peaks with retention times between 8-10 minutes are parasite venom alkaloids. The peak marked with an asterisk refers to the C22 standard (11.5 min). The relative abundance of the two profiles were adjusted to the C22 standard to make them comparable. Numbers at the peaks refer to the peak numbers in Table 1.2. Peak 22 (arrow) is the alkene hydrocarbon that is shared between host and parasite. b) Comparison of CHC profiles between host and parasites using a principal component analysis (PCA). We used the peak areas of the CHCs normalized to the total CHC peak area within each chromatogram as input. Visualized are the first two principle components explaining 68.1% of the total variance. Parasite profiles (red) are separated mainly along the first principal component (PC1), non-parasitized and parasitized *S. amabilis* host profiles are separated mainly along the second principal component (PC2). ‘NP host’ indicates that *S. amabilis* samples originate from a non-parasitized colony (green), ‘P host’ means *S. amabilis* samples originate from a parasitized colony (blue) and ‘Parasite’ means samples are parasites (red).
1.4 Results

‘mandible opening’ and ‘biting/pulling’. In the following description of the results, we focus only on these behaviours and compare them between the different pairings. ‘Short antennation’ was the most frequent behaviour observed between nestmates (pairing #1 and #2). The mean proportion of ‘short antennation’ for focal ants from parasitized and non-parasitized colonies was 0.77 and 0.78 respectively (Supplementary Table S1). In contrast, ‘opening mandibles’ and ‘biting/pulling’ were rarely or never seen in focal ants towards nestmate stimulus ants in pairings #1 and #2 (‘opening mandibles’: mean proportion is 0.06 and 0.13, respectively; ‘biting/pulling’: mean proportion of 0.0 for both). Focal ants encountering a conspecific non-nestmate stimulus ant (pairing #3–#6) showed ‘biting/pulling’ and ‘mandible opening’ behaviours significantly more often than in nestmate encounters (>98.9% in all comparisons between #1 or #2 and #3–#6). When the focal ants originated from a parasitized colony and the stimulus ant was a parasite from the same colony (pairing #7), the proportions of the behaviours ‘short antennation’, ‘opening mandibles’, and ‘biting/pulling’ were similar as towards their own (conspecific) nestmates (pairing #1). However, when the stimulus ant was a foreign parasite (pairing #8), ‘opening mandibles’ was shown significantly more often (certainty of >99.9%) compared to their (conspecific) nestmates (pairing #1) and compared to their own parasites (pairing #7 certainty of >99.9%). Focal ants from non-parasitized colonies encountering a parasite (pairing #9), show more ‘opening mandibles’ (certainty 97.2%) and ‘biting/pulling’ behaviours (certainty >99.9%) within a trial, than focal ants from a parasitized colony towards foreign parasites (pairing #8). The proportions of ‘opening mandibles’ and ‘biting/pulling’ behaviour were similar to the proportions in encounters with conspecific non-nestmates (pairing #5 and #6).

Submission analysis

Focal ants from parasitized and non-parasitized colonies are significantly more submissive when tested against non-nestmates from a parasitized colony (pairings #3 and #5, respectively) than when tested against a non-nestmate from a non-parasitized colony (pairings #4 and #6, respectively) (certainty for focal ants from parasitized colonies: 99.6%; certainty for focal ants from non-parasitized colonies: 99.4%; Fig. 3). Focal ants from parasitized colonies were less submissive towards parasites (own and foreign, pairings #7 and #8, respectively) than focal ants from non-parasitized colonies (pairing #9) (certainty of 95.6% and 99.9%, respectively). Note that focal ants from non-parasitized colonies approaching a parasite frequently showed ‘head tucking’ right after they showed ‘opening mandibles’ (S5 Supplemental Video on page 32).
Fig. 1.2 Proportions of single behaviours shown by *S. amabilis* focal ants within a trial. Aggressive behaviours of focal ants were much higher towards conspecific non-nestmates (pairings #3–#6) than towards nestmates (pairs #1 and #2), regardless if the focal ants were from parasitized or non-parasitized nests. Furthermore, non-parasitized focal ants showed more ‘biting/pulling’ and ‘carrying’ towards parasites than did the parasitized focal ants. Bars represent the means of the model parameters from the posterior probabilities and lines represent the 95% credible intervals for each single behaviour based on the Markov chain Monte Carlo simulations. ‘P’ indicates that stimulus or focal ants originate from a parasitized colony and ‘NP’ means they originate from a non-parasitized colony. Sample sizes are indicated at the top of the figure and specific pairings are numbered directly below. All means and credible intervals are given in S3 Table on page 31.
1.4 Results

Fig. 1.3 The probability of submission behaviour in *S. amabilis* focal ants within a trial. The probability that focal ants showed submission during a trial was higher when the stimulus ant was a parasite (pairings #7–#9) or a conspecific non-nestmate from a parasitized colony (pairings #3 and #5) than when the stimulus ant was a conspecific from a non-parasitized colony (pairings #4 and #6) or a nestmate (pairings #1 and #2). Dots represent the mean of the model parameters from the posterior probabilities and lines depict the 95% credible intervals for submissive behaviour based on the binomial generalized linear model (parameter estimates and standard errors are given in S4 Table on page 32). ‘P’ indicates that stimulus or focal ants originate from a parasitized colony and ‘NP’ means they originate from a non-parasitized colony. Sample sizes are indicated at the top of the figure and specific pairings are numbered directly below.
Discussion

In this study, we investigated the integration strategy of the social parasite *M. symmetochus*. We compared CHC profiles of parasite and host species, and tested the behaviour of parasitized and non-parasitized colonies against an introduced stimulus ant. We found very different CHC profiles for both ant species, ruling out mimicry and camouflage. The chemical analysis revealed low amounts of CHCs suggesting that the parasites use an insignificance strategy. Both the chemical profiles of parasites and the behavioural data support the hypothesis that the parasites use weaponry to maintain an amiable association with their host ants.

Support for the chemical insignificance strategy

One of the predictions for the chemical insignificance strategy is a low abundance of CHCs and/or unusually long-chained CHCs compared to the host colony, as it has been shown in other social parasites (Lambardi et al., 2007). We found support for this strategy as the CHCs of the parasite *M. symmetochus* were less abundant and the CHCs were generally longer-chained than the host CHCs. The muted CHC profiles of the parasites could reflect an evolutionary adaptation making *M. symmetochus* workers stealthy inside the host nest. Alternatively, the insignificant CHC profile could also be a by-product of the parasites’ life in the host nest, as the parasite workers do not leave the humid nest environment and, therefore, they lack the need for CHC protection against desiccation. In addition, in the CHC profiles of the parasites, we potentially found a high proportion of methyl-branched alkadienes, these compounds are found in other ant species in interspecific, amicable associations (Menzel and Schmitt, 2012). However, further work is needed to unequivocally determine whether methyl-branched alkadienes are also present in our host-social parasite system.

Another prediction for the chemical insignificance strategy is that workers from parasitized and non-parasitized colonies behave as if they do not detect the parasites. Our results are contradictory to this prediction, since we found that workers of non-parasitized colonies respond with similar aggression towards parasites and conspecific non-nestmates. This recognition may be because the workers of non-parasitized colonies can detect either, (1) the parasite’s CHCs, (2) traces of host-derived CHCs on the cuticle of the parasites (not detectable with our GC-MS analysis), or (3) something else in the parasite’s odour profile. Scenario 1 and 2 are possible, because focal workers from parasitized colonies showed significantly more often ‘opening mandibles’ towards foreign parasites than towards their own parasites. However, focal ants from parasitized colonies do not escalate to high-level aggression (‘biting/pulling’). Scenario 3 is most likely because the chemical analyses demonstrate that parasite odour profiles not only consist of CHCs, but also volatile alkaloids. The aggressive responses (‘biting/pulling’) in *S. amabilis*
workers from non-parasitized colonies towards parasites were likely due to the parasite venom alkaloids rather than to the parasite CHC profile.

**Workers from parasitized colonies tolerate parasites**

Previous studies showed that ants and honeybees can maintain several neuronal templates for nestmate odours simultaneously (Breed et al., 2004; Errard, 1994; Greene and Gordon, 2003). Similarly, in our experiments, workers from parasitized colonies may have developed a parallel neuronal template for the parasites’ odour and this could explain their tolerance towards their parasites. We exclude the possibility that workers from parasitized colonies are unable to recognize nestmates because we show that *S. amabilis* workers (irrespective of being from a parasitized or non-parasitized colony) were aggressive towards conspecific non-nestmates and not aggressive towards their sisters. Social parasites may affect the nestmate recognition abilities of the host as has been shown in social wasps (Lorenzi, 2003). However, we did not find evidence of this in our host-parasite system. Interestingly, the long-term association of coexistence between *S. amabilis* and *M. symmetochus* (>7 years) can lead to host rebellions where the host ants turn against the parasites and kill them (Boudinot et al., 2013; Adams personal observation). This supports the idea that *S. amabilis* workers from parasitized colonies can recognize parasites, but accept them as nestmates. However, when their acceptance threshold shifts due to factors that are still unknown for our host-social parasite system, they can turn aggressive towards the parasites, as has been shown in other species (d’Ettorre et al., 2004). In the laboratory, host rebellion can result in the death of the parasite colony but in the field, with the looming threat of other parasites, it may simply be a ‘culling’ of the parasite worker forces (Boudinot et al., 2013), since hosting *M. symmetochus* parasites can have context-dependent advantages (i.e., host protection against more lethal parasites) (Adams et al., 2013). Workers from non-parasitized colonies are unlikely to have encountered the parasite odour profile (both, the CHCs and the alkaloids) before, and therefore recognize the parasites as non-nestmates and react with aggression. The alkaloids originating from the venom gland contain the volatile isomers (5Z,8E)- and (5E,8E)-3-butyl-5-hexylpyrrolizidine in a 59:41 ratio (Adams et al., 2013). The large amount of volatile alkaloids (16–43 µg/ant) suggests that the parasite *M. symmetochus* uses chemical weaponry.

**Evidence for chemical weaponry strategy in *M. symmetochus***

In general, submissive behaviours in fungus-growing ants are not observed between nestmates. Considering the parasite occasionally threatens or even kills host workers (Adams et al., 2013), it is important that the host is behaviourally adapted to avoid an escalation that results in death. Accordingly, in our study, submission
Chemical integration into a host colony

by *S. amabilis* focal ants from parasitized colonies was more prevalent towards parasites than towards conspecific nestmates. In focal ants, ‘opening mandibles’ often preceded ‘head tucking’ behaviour as they approached the parasite. This observation and the fact that the alkaloids are volatile suggest they are found in the air surrounding the parasites (Adams et al., 2013). Focal ants may perceive the alkaloids in increasing concentrations as they approach the parasite, eliciting a shift in behavioural response from aggression to submission. Furthermore, the high probability of submission in trials with ‘alkaloid-exposed’ stimulus ants (i.e., *S. amabilis* workers from parasitized colonies or the parasites) also suggests that the parasite-derived alkaloids are not only found on the cuticle of parasites, in the fungus garden (Adams et al., 2013), but also on the cuticle of the host ants. Further work is needed to unequivocally identify that the venom alkaloids facilitates *M. symmetochus* host colony integration.

In this study, we focused on colony integration rather than infiltration and found tolerance for social parasites by host workers, especially those individuals that had been exposed to social parasites. From these results, we hypothesize that virgin *S. amabilis queens* originating from colonies that had social parasites might have incorporated the parasite chemical profile (CHCs and alkaloids) into their neuronal template for nestmate recognition. It is possible, that these queens could be more likely to accept parasite queens during nest founding than host queens from a non-parasitized colony. Future work on alkaloid dispensing behaviour in the nest environment and the host’s propensity for accepting a parasite queen will shed light on invasion tactics and success.

**Acknowledgement**

We thank two anonymous referees and the editor for their helpful comments on the manuscript. We are grateful for permit and facilities support from the Smithsonian Tropical Research Institute (STRI). Autoridad Nacional del Ambiente y el Mar (ANAM) provided permission to collect and export Panamanian ants. Jon Shik, Anders Illum, Joanito Liberti helped with colony collections in conjunction with the graduate course, Tropical Behavioural Ecology and Evolution (TBEE) hosted by the Centre for Social Evolution, University of Copenhagen and STRI. We thank the TBEE students and co-instructors Koos Boomsma and Jon Shik for discussion and Fränzi Korner-Nievergelt and Tobias Roth for valuable help in the statistical analysis of the behavioural data. We thank Matthew Boot for measuring the body sizes of individual ants. We also thank David Nash for helpful discussion while we designed the behavioural experiments and for comments on the final statistical analysis. RMMA thanks Patrizia d’Ettorre, David Nash and Koos Boomsma, University of Copenhagen for fruitful conversations and encouragement. Additional financial support was received from The Ohio State University for RMMA and AD and by the International Max Planck Research School for Organismal Biology and the University of Konstanz for SN.
Supplemental Materials

Approximation of the body surface areas

With a micrometer fitted to a Wild Heerbrug compound microscope using a 25x objective lens, we measured the length, width and height of the head, thorax (mesosoma) and gaster of the host *Sericomyrmex amabilis* and the parasite *Megalomyrmex symmetochus*. We assumed the appendages (legs and antennae) are similar between the two species and are negligible in surface area. The micrometer measurements were recorded on a per mark basis and multiplied by a conversion factor of 0.0185 to attain values in millimeters. Ten specimens of each species were measured and standardized taxonomic measurements were performed following established protocols (i.e., Head length 1, Head width, Weber’s length, Pronotal width, Gaster length, Gaster width) (Ješovnik and Schultz, 2017). Additional height measurements were also required for the head, thorax and gaster to determine the surface area. Head height was measured in profile view as the line perpendicular to the major axis of the head directly behind the compound eye. Throax or mesosomal height was measured in profile view as the line perpendicular to the major axis of the mesosomal line created by the Weber’s length and originating from the base of the prometanotal suture. Gaster height was measured as the line perpendicular to the major axis created by gaster length in profile view at the point of maximum distance. We determined a geometrical shape that best describes the different body surface types for each species (*M. symmetochus*: head – mix of prolate spheroid and cylinder, thorax – half-cylinder, gaster – prolate spheroid; *S. amabilis*: head – cylinder, thorax – rectangular prism, gaster – prolate spheroid). The formula used for calculations are as follows:

\[ A = 2\pi a \left( a + \frac{c^2}{\sqrt{c^2 - a^2}} \cdot \arcsin \left( \frac{\sqrt{c^2 - a^2}}{c} \right) \right) \]  \hspace{1cm} (1.1)

where \( a \) is length and \( c \) is (height + width)/4

\[ A = \pi \cdot r \cdot (r + H) + r \cdot H \]  \hspace{1cm} (1.2)

where \( r \) is (width + height)/4 and \( H \) is length
Cylinder:

\[ A = 2\pi \cdot r \cdot (r + H) \]  

where \( r \) is \((\text{width} + \text{height})/4\) and \( H \) is length

Rectangular prism:

\[ A = 2ab + 2ac + 2bc \]  

where \( a \) is length, \( b \) is width and \( c \) is height

Mix of prolate spheroid and cylinder:

\[ A = (A_1 + A_2)/2 \]  

where \( A_1 \) is the area of a prolate spheroid (see equation 1.1) and \( A_2 \) is the area of a cylinder (see equation 1.3)
S1 Figure: Comparison of CHC profiles between parasitized and non-parasitized host workers using a principal component analysis (PCA). We used the peak areas of the CHCs normalized to the total CHC peak area within each chromatogram as input. Visualized are the first two principal components explaining 61.2% of the total variance. Ellipses illustrate 95% confidence area.
S2 Figure: Cluster tree of samples from host and parasite workers. For quantification of the differences between host and parasite workers, we calculated the average pairwise Bray-Curtis distance based on the relative abundance of CHCs (normalized to the overall CHC area of each chromatogram) between all samples. Bray-Curtis distances were calculated with the `bcdist` function in R (v 3.4.0) and range from zero to one. A value of zero would represent a complete matching between two data points, while a value of one would represent a complete separation in the n-dimensional space. The figure shows a cluster analysis tree based on Bray-Curtis distances between individual samples from parasites (red) and *S. amabilis* workers from parasitized and non-parasitized colonies (blue and green, respectively).
### S3 Table: Results from the multinomial logistic regression model on the aggression data.

Shown are the means, which were used as the estimates (and 95% credible intervals in parentheses) for the five distinct behaviours and nine different pairings. P – ant from a parasitized colony, NP – ant from a non-parasitized colony, NM – nestmate, NNM – non-nestmate.

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Pairing #1</th>
<th>Pairing #2</th>
<th>Pairing #3</th>
<th>Pairing #4</th>
<th>Pairing #5</th>
<th>Pairing #6</th>
<th>Pairing #7</th>
<th>Pairing #8</th>
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<td>NP</td>
<td>P</td>
<td>P</td>
<td>NP</td>
<td>NP</td>
<td>P</td>
<td>P</td>
<td>NP</td>
</tr>
<tr>
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<td>NM</td>
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<td>parasite</td>
<td>parasite</td>
<td>parasite</td>
</tr>
<tr>
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<td>foreign</td>
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<td>own</td>
<td>foreign</td>
<td>foreign</td>
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<td>0.78 (0.69 - 0.85)</td>
<td>0.44 (0.3 - 0.58)</td>
<td>0.45 (0.27 - 0.61)</td>
<td>0.35 (0.25 - 0.46)</td>
<td>0.48 (0.35 - 0.6)</td>
<td>0.92 (0.85 - 0.97)</td>
<td>0.69 (0.54 - 0.81)</td>
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<td>Prolonged antennation</td>
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<td>0.09 (0.05 - 0.15)</td>
<td>0.11 (0.05 - 0.2)</td>
<td>0.19 (0.08 - 0.34)</td>
<td>0.1 (0.06 - 0.17)</td>
<td>0.16 (0.09 - 0.27)</td>
<td>0.02 (0 - 0.05)</td>
<td>0.05 (0.02 - 0.09)</td>
<td>0.05 (0.03 - 0.08)</td>
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<td>Mandible opening</td>
<td>0.06 (0.02 - 0.13)</td>
<td>0.13 (0.06 - 0.22)</td>
<td>0.35 (0.19 - 0.54)</td>
<td>0.2 (0.08 - 0.37)</td>
<td>0.4 (0.25 - 0.56)</td>
<td>0.26 (0.13 - 0.42)</td>
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<tr>
<td>Carrying</td>
<td>0 (0 - 0.01)</td>
<td>0 (0 - 0)</td>
<td>0.01 (0 - 0.05)</td>
<td>0.01 (0 - 0.05)</td>
<td>0.02 (0.01 - 0.06)</td>
<td>0.02 (0.0 - 0.05)</td>
<td>0 (0 - 0.02)</td>
<td>0 (0 - 0.01)</td>
<td>0 (0 - 0.01)</td>
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<tr>
<td>Biting/pulling</td>
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<td>0 (0 - 0.01)</td>
<td>0.09 (0.02 - 0.25)</td>
<td>0.15 (0.02 - 0.45)</td>
<td>0.12 (0.04 - 0.27)</td>
<td>0.08 (0.01 - 0.24)</td>
<td>0 (0 - 0.02)</td>
<td>0 (0 - 0.01)</td>
<td>0.19 (0.07 - 0.39)</td>
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</tbody>
</table>
S4 Table: Parameter estimates and se from the binomial GLMM with submission as response variable. Reference is pairing #1 (parasitized towards their own conspecific nestmates).

Fixed effects:

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<th>Estimate</th>
<th>se</th>
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<td>Pairing #9</td>
<td>3.905</td>
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</table>

Screen shot of S5 Supplemental Video: Submission behaviour in *Sericomyrmex amabilis* host workers. The video shows five focal workers (*S. amabilis* from a non-parasitized colony) and one parasite (at the lower part of the petri dish). One of the focal workers first opens her mandibles towards the parasite and then shows a submissive response without having touched the parasite with its antennae.
Chapter 2

Learning distinct chemical labels of nestmates in ants

Stefanie Neupert, Manuel Hornung, Jocelyn G. Millar, Christoph J. Kleineidam

Abstract

Colony coherence is essential for eusocial insects, since it supports the inclusive fitness of colony members. Ants quickly and reliably recognize who belongs to the colony (nestmates) and who is an outsider (non-nestmates) based on chemical recognition cues (cuticular hydrocarbons: CHCs) which as a whole constitute a chemical label. The process of nestmate recognition often is described as a matching of neural template with the label. In this study, we tested the prevailing view that ants use commonalities in the colony odor that are present in the CHC profile of all individuals of a colony or whether different CHC profiles are learned independently. We created and manipulated sub-colonies by adding one or two different hydrocarbons that were not present in the original colony odor of our Camponotus floridanus colony and later tested workers of the sub-colonies in one-on-one encounters for aggressive responses. We found that workers adjust their nestmate recognition by learning novel, manipulated CHC profiles, but still accept workers with the previous CHC profile. Workers from a sub-colony with two additional components showed aggression against workers with only one of the two components added to their CHC profile. Thus, additional components as well as the lack of a component can render a label as ‘non-nestmate’. Our results suggest that ants have multiple-templates to recognize nestmates carrying distinct labels. This finding is in contrast to what previously has been proposed, e.g. a widening of the acceptance range of one template.

We conclude that nestmate recognition in ants is a partitioned (multiple-template) process of the olfactory system that allows discrimination and categorization of nestmates by differences in their CHC profiles. Our findings have strong implica-
tions for our understanding of the underlying mechanisms of colony coherence and task allocation, because they illustrate the importance of individual experience and task associated differences in the CHC profiles that can be instructive for the organization of insect societies.

Keywords

pattern recognition, olfaction, label-template matching, cuticular hydrocarbons, synthetic odor processing, social insects, *Camponotus floridanus*

**Introduction**

In eusocial insects, the ability to discriminate between members from one’s own colony (nestmates) and members from a foreign colony (non-nestmates) is of fundamental importance for colony coherence, and ultimately benefits colony fitness (Hölldobler and Wilson, 1990). Discrimination between nestmates and non-nestmates prevents the colony’s resources (e.g. food storage, brood) from being exploited by competing conspecific and heterospecific colonies, predators, and parasites. In ants, the discrimination between nestmates and non-nestmates relies on olfaction and is based on mixtures of low-volatile chemical components on the ants’ exoskeleton (cuticular hydrocarbons: CHCs) (Lahav et al., 1999). The primary functions of CHCs are to prevent desiccation (Lockey, 1988) and protect against infections. Secondarily, CHCs were exploited as recognition cues for colony membership. The composition of CHCs is species-specific (Martin and Drijfhout, 2009b) and genetically determined, but individuals’ CHC profiles are additionally shaped by diet and nest material (Jutsum et al., 1979; Vander Meer et al., 1989). Frequent exchange of CHCs between nestmates through trophallaxis and allogrooming (Lenoir et al., 2001c) results in a uniformed, colony-specific chemical signature (Crozier and Dix, 1979). This signature commonly is called the colony odor and consists of all CHC profiles carried by individuals of one colony. The colony odors of neighboring, conspecific colonies generally differ only in the quantitative ratios of CHCs. Because diet, nest material, and colony composition may change, the colony odor is not constant but varies over time (Vander Meer et al., 1989). Importantly, CHC profiles among individual workers of a colony are not as uniform as previously assumed. Workers have task-specific CHC profiles because task performance, e.g., nest building, brood tending, or foraging outside the nest influence the CHC profiles (Kaib et al., 2000; Wagner et al., 1998). Furthermore, the mixing of CHCs between individuals of a colony is not complete because workers within a task-group encounter and interact with each other more frequently than between task-groups (Mersch et al., 2013; Pamminger et al., 2014; Sendova-Franks and Franks, 1995; Tschinkel and Hanley, 2017). Based on these systematic differences in CHC profiles, workers are able to discriminate between nestmates performing different tasks (Bonavita-Cougourdan et al., 1993; Greene
and Gordon, 2003). Although CHC profiles vary within a colony, aggression among nestmates is rare and nestmates are discriminated from conspecific non-nestmates rapidly and with high reliability - even though conspecific non-nestmates can have CHC profiles which are qualitatively identical and only differ in component ratios (Stroeymeyt et al., 2010). It is not known how workers discriminate between nestmates and non-nestmates based on the ratios of CHCs. To understand the neuronal and behavioral mechanisms of nestmate recognition, we need to examine all parts of the recognition system from CHC profiles to behavioral reactions. This involves analyzing how information contained in the CHC profile is detected and processed by the nervous system, how ‘nestmate’ and ‘non-nestmate’ is encoded in the brain, how this influences decision making, and eventually results in an observable behavioral outcome during encounters.

Different models and possible strategies to discriminate between nestmates and non-nestmates have been proposed. Based on the phenotype matching model (Holmes and Sherman, 1983), each individual carries both recognition cues of its own CHC profile (a label) and a neural representation (a template) of its own colony odor (Lacy and Sherman, 1983). In theory, the label from an encountered individual is compared with the ‘own’ template, denoted as a label-template matching process. In its most simple form, individuals are hypothesized to be anosmic to their own colony odor and so colony-specific labels are not perceived, and all labels causing a salient perception are considered as belonging to non-nestmates. Although this idea has been proposed prominently (Ozaki et al., 2005), it fails to explain how workers can discriminate between nestmates of different task-groups (Bonavita-Cougourdan et al., 1993; Greene and Gordon, 2003) and subsequent studies have shown that workers are not anosmic to their own colony odor (Brandstaetter and Kleineidam, 2011; Brandstaetter et al., 2011; Sharma et al., 2015).

The most widely accepted model for nestmate recognition considers the frequently experienced labels of nestmates during early adulthood as being instructive for the formation of a template (Carlin and Hölldobler, 1983; Errard, 1994; Morel and Blum, 1988). This learning process allows individuals to recognize nestmates and to discriminate between ‘we’ vs. ‘others’. Because the colony odor can change over time, individuals need to adjust their template accordingly. Manipulation of the colony odor leads to acceptance of both the current colony odor as well as the previous colony odor, and for a single template this would require a widening of the acceptance range (Bos, 2014; Bos and d’Ettorre, 2012; Guerrieri et al., 2009; Leonhardt et al., 2007). Because diverse labels can be employed for recognizing nestmates (Errard, 1994; Fielde, 1903), it is puzzling how workers can still achieve high reliability in nestmate recognition by using a single template. The specificity range of this neural template has to be broad enough to cover the variety of different labels that are present within the colony, and at the same time exclude labels of non-nestmates (Reeve, 1989).

As an alternative to a single, unifying-template, we hypothesize that workers use
Learning distinct chemical labels of nestmates in ants

**Fig. 2.1 Schematic acceptance ranges** as ovals (dashed lines) for the one-template and as circles (solid lines) for the multiple-template nestmate recognition system (0: no additional components, A: one additional, AB: two additional components added to the CHC profile)

multiple templates, each with its own specificity range, for recognizing nestmates. In this case, chemically distinct labels are recognized with different templates, which possibly are attributed with further information about the label carrier. Labels identified from distinct templates may be perceptually kept separately or are generalized as all belonging to ‘nestmates’. Such a recognition system with multiple templates is comparable to associative learning, analogous to honeybees learning the association between flowers and floral odorants, where flowers provide a species-specific chemical pattern and many different types of floral odorant profiles can be learned (Bitterman et al., 1983; Gerber et al., 1996). The difference between these two nestmate recognition hypotheses is that in the one-template nestmate recognition system, commonalities in the CHC profiles of the colony odor are used for nestmate recognition, whereas in the multiple-template nestmate recognition system chemically distinct CHC profiles within a colony are recognized independently of each other and categorized as belonging to nestmates.

To investigate these two possible mechanisms of nestmate recognition, we systematically manipulated the CHC profiles of workers originating from the same colony by adding none, one or two hydrocarbon(s) and tested if they still accept each other as nestmates. Based on a one-template nestmate recognition system, we predict that workers which incorporated two additional hydrocarbons into their CHC profile also accept individuals that have only one hydrocarbon added (Figure 2.1). In contrast, if workers with two additional hydrocarbons do not accept individuals with only one of the hydrocarbons added, the idea of a multiple-template nestmate recognition system is supported.

**Materials and Methods**

For all experiments, we used adult workers of the species *Camponotus floridanus* (Buckley, 1866) from a mature colony that was collected at Sugarloaf Shores in Florida by A. Endler in July 2003. Collection of founding queens for laboratory colonies conformed to the laws of the United States of America effective at the time of collection. Colonies of this species have one mated queen (monogynous).
which mates with only one male (monoandrous) (Gadau et al., 1996) resulting in high genetic homogeneity within a colony compared to polygynous/polyandrous species. In the laboratory, the queen-right colony was kept in an artificial plaster nest at a constant temperature of 25°C and about 40-50% humidity with a 12:12h L/D photo period. The colony was fed with honey water and dead locusts twice a week and was provided with water ad libitum. The experiments and protocols performed comply with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health published by National Academic Press (1996) and the current laws of the Federal Republic of Germany (“Tierschutzgesetz”).

### Manipulating colony profiles of sub-colonies

From the mature colony (source colony), we created four different sub-colonies maintained in separate plaster nests. Each sub-colony received a different treatment by adding either no, one, or two synthetic hydrocarbons to the colony odors of the sub-colonies. For manipulation, we used the following hydrocarbons, which were not present in the original CHC profile of C. floridanus: the linear alkane triacontane (C30, Sigma-Aldrich) and hereafter referred to as hydrocarbon A, was added to two sub-colonies (sub-colony A and sub-colony AB). The branched alkane 5-methylheptacosane (5-MeC27), synthesized for this study and hereafter referred to as hydrocarbon B, was added together with A to sub-colony AB. The branched alkane 11-methylheptacosane (11-MeC27), synthesized for this study, hereafter referred to as hydrocarbon C, was added to sub-colony C. One sub-colony was sham-treated as the control (sub-colony 0).

We used a two-step approach to manipulate the CHC profiles. In the first step, we induced an immediate change of the CHC profiles of all workers before transferring them to the respective sub-colonies. In the second step, we counteracted the diminishing of introduced hydrocarbons from the workers’ cuticles by providing hydrocarbon-coated granules at the nest entrance and in the food.

For the first manipulation step, we coated the inner wall of an Erlenmeyer flask (25 ml) with the corresponding hydrocarbon(s) by adding 3 mg of hydrocarbon A (sub-colony A) or 3 mg of hydrocarbon A and 3 mg of hydrocarbon B (sub-colony AB) or 6 mg of hydrocarbon C (sub-colony C), and 6 ml of pentane to the flask. We added a relatively large amount of hydrocarbon C to sub-colony C in order to induce a strong change in the CHC profiles of one sub-colony which should be recognized with a high probability as unfamiliar and belonging to non-nestmates. The flask was gently shaken and briefly heated to 60°C on a hot plate to evaporate the solvent, and then cooled to room temperature. We randomly selected 5 workers from the source colony, cooled them on ice and transferred them to the coated flask. The workers were gently swirled in the flask for 10-15 sec, and then transferred to a new plaster nest. This was repeated until 100-150 workers had been treated. Workers for sub-colony 0 received the same treatment, except no hydrocarbon was added to the pentane (sham-treatment).
For the second manipulation step, we coated 600 mg of granules (superabsorbent polymers, poly-acrylic acid sodium salt, Stockosorb® 500) with 6 ml of pentane and 3 mg of hydrocarbon A (sub-colony A) or 3 mg of hydrocarbon A and 3 mg of hydrocarbon B (sub-colony B), or 6 mg of hydrocarbon C (sub-colony C) in a beaker. The beaker was swirled and then put on a hot plate at 60°C to evaporate the pentane. The coated granules then were transferred to a glass tube and small glass vials. We connected the glass tube (diameter: 17 mm; length: 100 mm) to the nest (approx. 100 mm x 80 mm), which served as a long entrance area. Workers entering and leaving the nest passively took up some hydrocarbons from contact with the granules. We added honey water or water to the glass vials and provided them to the sub-colonies in order to facilitate hydrocarbon uptake while feeding. After manipulation, sub-colony 0, A, and AB contained approximately 150 adult workers each, and colony C had approximately 100 workers. We provided brood (eggs, larvae, and pupae) to each of the sub-colonies to maintain their natural social structure. Workers in the sub-colonies that were tested later in behavioral experiments were all familiar with the previous, original CHC profile and in the manipulated sub-colonies workers also became familiar with a novel CHC profile. Thus, we created workers with different experience, being familiar with one or two different CHC profiles and unfamiliar with three or two CHC profiles, for sham-treated and manipulated sub-colonies, respectively.

**Behavioral assay**

Starting one day after manipulations, we performed one-on-one encounters in small Petri dishes between two workers, either from the same or from different sub-colonies. Because social isolation of workers reduces the propensity for aggression against non-nestmates (Kleineidam et al., 2017), we first transferred one of the workers (stimulus worker) into the Petri dish, and after one hour, we started the experiment by adding the second worker (focal worker) to the dish. We scored the behavior of the focal worker during the first interaction with the stimulus worker as either being ‘aggressive’ when the focal worker showed one of the behaviors widely opened mandibles, snapping, body raising or gaster flexing, all of which are typical aggressive behaviors for this species (Brandstaetter et al., 2008). Focal workers which exhibited none of these behaviors were classified as being ‘non-aggressive’. We inferred nestmate recognition and discrimination based on the focal workers’ behavioral responses. In cases, where focal workers did not respond with aggression, we categorized this as recognition and possible acceptance as nestmates. In cases where workers from a sub-colony show low probability of aggression against stimulus workers from different sub-colonies, either their CHC profiles are not discriminated, or they are discriminated but without a behavioral readout by the observer. Thus, the workers’ nestmate recognition, as categorized by the observer, either is a generalization with the assumption that workers can discriminate a diversity of CHC profiles or the categorization is based on non-discriminable CHC profiles.
Scoring of the behavioral recordings was conducted blind, with the observer not knowing the stimulus worker type. Immediately after the first interaction, focal and stimulus workers were separated either for examination of CHC profiles (described below), or the focal worker was transferred back to its sub-colony. On the four consecutive days after establishing the sub-colonies and manipulating their colony odors, a total of 360 one-on-one encounters were conducted in 6 sessions with (almost) balanced types of encounters. For each of the 12 possible encounter types, 25-31 repetitions were conducted; the variation in numbers resulted from the observer-blind experimental design. Because some of the focal workers were transferred back to their sub-colonies after an experiment, it is possible that some focal workers were tested more than once against the same sub-colony. Based on a simulation (5000 times) with the number of focal workers that were not used for the analysis of their CHC profiles, we calculated that no more than 7.5% of all tested focal workers were selected more than once for an encounter against a stimulus worker from the same sub-colony as before.

Analysis of behavioral data

First, we tested whether the time since establishing the sub-colony significantly influenced the probability of aggression in focal workers. We ran a generalized linear model (GLM) with the binary response variable aggression (‘aggression’/’no aggression’) and added time (experimental sessions 1 to 6) as the only explanatory variable. Because the slope estimate was not significant (p > 0.05), we did not include time in the final model (model2).

Second, we tested for differences in the probability of aggression in focal workers against stimulus workers from the different sub-colonies, using a GLM with the binary response variable aggression (‘aggression’/’no aggression’), and as explanatory variables the origin of stimulus worker, origin of focal worker, and an interaction of both variables (model2).

In order to draw inferences about differences in behavior between focal workers from the different sub-colonies, we used a Bayesian framework. We calculated 20,000 values that are random draws from the posterior distribution of the model2 estimates. We compared the probability of a worker being aggressive towards stimulus workers from their own and from different sub-colonies. We calculated the proportions of simulated values from the posterior distribution that were larger (or smaller) for focal workers encountering stimulus workers from their own sub-colony compared to focal workers encountering stimulus workers from another sub-colony (measure of certainty). We used the 2.5% and the 97.5% quantiles as the lower and upper limits of the 95% credible interval. Statistical analyses were done using R (v3.4.3) (R Core Team 2017), including the package arm with the sim function to draw random samples from the posterior distribution of model parameters (Gelman & Hill 2007). Level of significance for the Bayesian statistics was set to >99% of certainty.
CHC extractions

To verify that our manipulation of CHC profiles was successful, we collected workers for CHC extractions after aggression tests (always pairs of focal and stimulus workers) and immobilized the workers on ice. Each worker was held in a 1 ml glass vial and completely covered with pentane for one hour. The vial was shaken gently and then the pentane was transferred to a clean vial with a 200 µl glass insert. The pentane was allowed to evaporate at room temperature and sealed vials then were stored in a freezer at -20°C until analysis.

Analysis of CHC profiles

The vials were defrosted to room temperature, 1-3 drops of pentane were added, and the vials were agitated for 1 min with a vortexer. We then concentrated the sample under a constant stream of air to approximately 20 µl. For coupled gas chromatography mass-spectrometry analysis (GC-MS), we manually injected 1 µl of the samples into a GC (Trace GC Ultra coupled to a DSO II mass spectrometer, Thermo Scientific). The CHCs were analyzed on a fused silica column (Optima-5-MS - 0.25µm, 30m x 0.25mm, Macherey-Nagel GmbH & Co. KG) with helium as carrier gas (1.2ml/min). Chromatograms were recorded with Xcalibur software 1.4 SR1 (Thermo Scientific). The GC oven was programmed as follows: 1) 70°C for 1 min, 2) increase at 30°C/min to 200°C, 3) increase at 3°C/min to 290°C, 4) hold at 290°C for 5 min. We analyzed the chemical similarity of workers from different sub-colonies by principal component analysis (PCA) based on the normalized peak areas, including the 10 most prominent peaks from the GC-MS analysis plus the signal at the retention times where the three components (A, B, C) were detected when present.

CHC identification

We ran a standard solution of linear hydrocarbons ranging from 21 to 40 carbons, using the same method as for the CHC profiles. We calculated the Kovats index for each peak in the profile. The CHC compounds were identified based on their Kovats indices and their mass spectra, which were compared to entries of known components in the Wiley and NIST libraries.

Synthesis of methyl-branched hydrocarbons

5-Methylheptacosane
Butyllithium (2.2 M in hexane) was added dropwise to a slurry of docosyltriphenylphosphonium bromide (3.26 g, 5 mmol) in 100 ml dry tetrahydrofuran (THF) in an oven-dried flask under argon until the mixture retained a pink tinge, followed by addition of a further 2.5 ml of butyllithium solution (5.5 mmol). The resulting clear cherry-red solution was stirred 30 min, followed by addition of 2-hexanone
(0.50 g, 5 mmol) in 5 ml THF with a syringe pump over 30 min. The resulting mixture was stirred overnight, then quenched by addition of saturated aqueous NH4Cl. The mixture was then diluted with water and extracted with hexane. The hexane layer was washed with water and brine, dried over anhydrous Na2SO4, concentrated, and purified by vacuum flash chromatography on silica gel, eluting with hexane. The resulting semicrystalline product was taken up in 25 ml hexane in a 50 ml flask, 5% Pd on carbon catalyst was added (250 mg), and the flask was flushed with hydrogen, sealed, and stirred under hydrogen for 3 h, when GC analysis showed that all the starting material had been consumed. The mixture was filtered through a celite plug, rinsing well with hexane, and after concentration, the resulting solid was recrystallized from 30 ml acetone overnight at 4°C. Filtration yielded the product as white crystals (0.60 g).

EI mass spectrum (70 eV) (m/z, abundance): 394 (M+, trace), 379 (7), 365 (7), 351 (3), 337 (54), 336 (31), 309 (7), 308 (10), 295 (1), 281 (2), 267 (2), 253 (3), 239 (3), 225 (3), 211 (3), 197 (4), 183 (5), 169 (5), 155 (7), 141 (8), 127 (10), 113 (13), 99 (19), 85 (100), 84 (73), 71 (56), 57 (81), 43 (71).

11-Methylheptacosane

11-Methylheptacosane was made in similar fashion, starting with hexadecyltriphenylphosphonium bromide and 2-dodecanone, with the exception that the final product was recrystallized first from isoctane and then again from acetone at 4°C, yielding white crystals.

EI mass spectrum (70 eV) (m/z, abundance): 394 (M+, trace), 379 (5), 365 (3), 351 (2), 337 (2), 323 (2), 309 (1), 295 (1), 281 (1), 267 (1), 253 (22), 252 (26), 239 (1), 225 (3), 224 (7), 211 (2), 197 (3), 183 (4), 169 (21), 168 (45), 155 (5), 141 (7), 140 (7), 127 (11), 113 (18), 99 (27), 85 (65), 71 (80), 57 (100), 43 (53).

Results

Discrimination of different CHC profiles

We tested workers of different sub-colonies in one-on-one encounters and used aggressive responses as a measure of the workers’ categorization of the encounter worker as either ‘nestmate’ or ‘non-nestmate’. Our manipulations allowed us to address the question as to whether aggression is related either to chemical similarity, or to the worker’s experience with the different CHC profiles (familiar and unfamiliar CHC profiles). The CHC profile of the sub-colony where both the linear and the methylated hydrocarbons were added (sub-colony AB), is more similar to the CHC profile of the sub-colony where only the methylated hydrocarbon was added (sub-colony A) than from that of the sham-treated sub-colony (sub-colony 0).

In almost all one-on-one encounters (353 of 360) between focal workers and stimulus workers, we were able to classify the behavior of the focal worker as aggressive or non-aggressive. In 7 encounters, we were unable to classify the focal worker’s
behavior because one of the two workers escaped the arena by climbing the wall, thus avoiding an encounter.

We rarely found aggression in encounters between focal and stimulus workers from matching (same) sub-colonies (10.7-16.7%, Figure 2.2), indicating that coherence within the sub-colonies is maintained. The majority of focal workers from the manipulated sub-colony A also did not show aggression to stimulus workers from the sham-treated sub-colony (sub-colony 0; Figure 2.2 blue; 17.2%), which shows that the original colony odor is still accepted. Focal workers from sub-colony AB more often (level of certainty: 93%) responded with aggression towards stimulus workers from sub-colony 0 (26.7%) than towards stimulus workers from their own sub-colony (10.7%), indicating that about 3/4 of the workers of sub-colony 0 are still accepted.

The percentage of focal workers from sub-colony 0 that showed aggression towards stimulus workers treated with A was also low (22.6%), and we found little support that it differs from workers’ responses towards stimulus workers from their own sub-colony (level of certainty only 71.6%). It seems that the presence of component A (linear alkane C30) in stimulus workers does not signify non-nestmate status to focal workers from sub-colony 0. By contrast, focal workers from sub-colony 0 and sub-colony A displayed aggression towards stimulus workers from sub-colony AB (42% and 34% increase of aggression, respectively; levels of certainty > 99% in both cases). Thus, adding the two components A and B (C30 and 5-MeC27) to the CHC profile of stimulus workers induces aggressive responses in focal workers from sub-colonies in which none (sub-colony 0) or only one of the two components (C30 in sub-colony A) was used for manipulation of the colony odor.

Furthermore, focal workers from sub-colony AB significantly more often responded with aggression towards stimulus workers from sub-colony A (38.7%) than towards stimulus workers from their own sub-colony (10.7%). In this test scenario, the lack of component B in the CHC profile of the stimulus workers induces more often aggressive behavior in focal workers of sub-colony AB compared to encounters with their current nestmates (level of certainty > 99% ). With a certainty of 83.8%, workers of sub-colony AB are more often aggressive towards workers from sub-colony A than towards workers from sub-colony 0. Thus, both the presence of an additional component and the lack of a specific component can alter the CHC profile of a nestmate to that of a non-nestmate.

In addition, we tested focal workers from all three sub-colonies (0, A, AB) against stimulus workers from another sub-colony that was treated with a methylated hydrocarbon at high concentration (11-MeC27, sub-colony C), to quantify aggression against an excessively manipulated CHC profile that should easily be recognized as unfamiliar and belonging to non-nestmates. All focal workers, irrespective of which sub-colony they were selected from, showed significant aggression towards stimulus workers from sub-colony C (levels of certainty >99%, Figure 2.2 green).
2.4 Results

Fig. 2.2 Aggression of focal workers towards stimulus workers from different sub-colonies. Focal workers from all sub-colonies rarely showed aggression towards stimulus workers from their own sub-colony (matching colors), or from the sham-treated sub-colony 0 (blue squares). In contrast, a significant number of focal workers from all sub-colonies showed aggression towards stimulus workers from sub-colony C (green diamonds). Focal workers from sub-colony 0 significantly more often showed aggression towards stimulus workers from sub-colony AB (red triangles) but not so towards stimulus workers from sub-colony A (orange circles). Focal workers of sub-colony A showed aggression significantly more often towards stimulus workers from sub-colony AB (red triangles) than towards stimulus workers from their own sub-colony. Focal workers of sub-colony AB showed aggression significantly more often towards stimulus workers from sub-colony A (orange circle) than towards stimulus workers from their own sub-colony. Symbols are fitted values from the binomial GLM (model2) and vertical lines represent the 95% credible intervals based on Bayesian statistics. Between 25 and 31 workers from each group were tested. Asterisks indicate levels of certainty above 99% for a difference between matching (from same sub-colony) and mismatching (from different sub-colonies) pairs.
Table 2.1 Relative amounts (mean ± SD) of the 10 most abundant CHCs plus the additional hydrocarbons in extracts obtained from individual workers (normalized to total peak area of most abundant peaks without additional hydrocarbons). Peak indices as marked in Figure 2.3.

<table>
<thead>
<tr>
<th>Peak</th>
<th>sub-colony 0 n=11</th>
<th>sub-colony A n=12</th>
<th>sub-colony AB n=8</th>
<th>sub-colony C n=7</th>
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<tr>
<td>A</td>
<td>0.2 ± 0.4</td>
<td>8.9 ± 6.9</td>
<td>4.8 ± 5.1</td>
<td>0.8 ± 1.3</td>
</tr>
<tr>
<td>B</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>11.7 ± 6.9</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>C</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>174.4 ± 115.5</td>
</tr>
<tr>
<td>1</td>
<td>6.5 ± 0.8</td>
<td>8.1 ± 0.5</td>
<td>7.8 ± 0.8</td>
<td>7.4 ± 0.8</td>
</tr>
<tr>
<td>2</td>
<td>5.3 ± 1.3</td>
<td>5.8 ± 1.1</td>
<td>5.8 ± 0.9</td>
<td>6.4 ± 1.1</td>
</tr>
<tr>
<td>3</td>
<td>14.1 ± 0.7</td>
<td>14.6 ± 0.6</td>
<td>14.7 ± 0.6</td>
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<tr>
<td>4</td>
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</tr>
<tr>
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<tr>
<td>8</td>
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<td>14.5 ± 0.9</td>
</tr>
<tr>
<td>9</td>
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</tr>
<tr>
<td>10</td>
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Manipulation of CHC profiles

Following the behavioral experiments, we collected workers and analyzed their CHC profiles in order to verify that the synthetic hydrocarbons had been transferred to their cuticle by our manipulation. GC-MS analyses of cuticular washes from a total of 38 individual workers revealed that our chemical manipulation of the colony odor was successful. CHC extracts of individuals contained the same prominent CHCs as the sham-treated sub-colony (sub-colony 0) plus one or two additional hydrocarbons depending on the manipulation (Table 1). The additional hydrocarbons A and B were detected as distinct peaks in the chromatograms at retention times where no components of the original CHC profile of *C. floridanus* appear (Figure 2.3). Based on the GC-MS data, we cannot rule out that we potentially collected synthetic hydrocarbons that were taken up by the ants but not presented as part of the CHCs on the cuticle.

We visualized the similarities of CHC profiles between the sub-colonies by a principal component analysis (PCA). CHC profiles of workers were spread along PC1, whereas workers from sub-colonies A and AB were separated from workers of sub-colony C along PC2 (Figure 2.4). Chemically, the most homogeneous group were the workers from sub-colony 0, and on average the workers from sub-colony A were chemically more similar than workers from sub-colony AB, compared to workers from sub-colony 0.
2.4 Results

Fig. 2.3 Manipulation of CHC profiles of the different sub-colonies. Example chromatograms of single workers from sub-colonies 0, A, AB, and C. Components are indicated by letters and numbers: A: C30, B: Me-5C27, C: Me-11C27, 1: 3,7-di-methyl- and 3,9-di-methylnonacosane, 2: 4-methyltriacontane, 3: 4,10-di-methyl- and 4,8-di-methyltriacontane, 4: 9-methylhentriacontane and unknown, 5: 5,9-di-methylhentriacontane, 6: 8-methyl- and 12-methylotriacontane, 7: 3,7,11-tri-methylhentriacontane, 8 + 9 + 10: unknown. Note, in these examples the two components A and B are integrated in different amounts into the CHC profile.
Learning distinct chemical labels of nestmates in ants

Fig. 2.4 Principal component analysis (PCA) of CHC profiles obtained from single workers. Workers differ systematically, depending on treatment and are aligned on different axes. PCA reveals that the amounts of components integrated into the CHC profile within particular sub-colonies are variable. Ellipses represent 95% confidence areas. Blue: 11 workers of sub-colony 0, yellow: 12 workers of sub-colony A, red: 8 workers of sub-colony AB, green: 7 workers of sub-colony C. Grey arrows indicate loadings for the same 13 hydrocarbons as in Table 2.1.

Discussion

We investigated whether workers use commonalities found in CHC profiles to recognize nestmates, or whether nestmate recognition is based on the generalization of distinct CHC profiles that can be discriminated by workers. We successfully manipulated the colony odor of different sub-colonies by adding one or two different hydrocarbons that were not present in the original CHC profiles in *C. floridanus*, at levels similar (for component A and B, not for component C) to the amounts of the original hydrocarbons. This allowed us to test the effects of adding novel components to the profile, and also, to test the effect of the lack of one component of an otherwise very similar CHC profile. We found that workers can adjust their nestmate recognition to novel, manipulated CHC profiles, but still accept workers as nestmates that carry the previous CHC profile. Furthermore, we show that additional components as well as the lack of a component in the CHC profile can be discriminated and can render a label as belonging to non-nestmates. Workers having both the linear and the methyl-branched hydrocarbon in their novel CHC profile perceive workers having only the linear hydrocarbon, but not the methylated hydrocarbon, as being non-nestmates. Thus, our results support the hypothesis that workers can recognize nestmates with different CHC profiles in a multiple-template recognition process and categorize them as all belonging to nestmates. Whether they discriminate between the different nestmate CHC profiles remains to be tested.
Discrimination of CHC profiles

For our manipulation of the CHC profiles, we used two methyl-branched and one straight-chain hydrocarbon, knowing that hydrocarbons containing methyl groups may be more important as recognition cues compared to unbranched alkanes (Dani et al. (2001); Guerrieri et al. (2009); Martin and Drijfhout (2009b), but see Akino et al. (2004); Greene and Gordon (2007)). Indeed, we found that adding a linear alkane (C30) to the CHC profile did not significantly affect nestmate recognition in workers that did not have this component in their own colony odor. We hypothesize that components of the CHC profile can be insignificant or important recognition cues, based on whether their presence has predictive power for discrimination extending the concept of key recognition cues in CHC profiles (Dani et al., 2001; Guerrieri et al., 2009; Martin and Drijfhout, 2009b; van Zweden et al., 2014).

Our observations that after manipulation of the colony odor, the novel as well as the previous CHC profile is accepted for nestmate recognition led to the assumption that the underlying mechanism is a widening of the acceptance range of the template for nestmate labels (Guerrieri et al., 2009; Leonhardt et al., 2007; Meskali et al., 1995). In one setting, aggression against workers with the previous CHC profile was comparatively high (workers from sub-colony AB towards workers of sub-colony 0). We do not know the reason for this, but we consider the variation in mean aggression probability across the different groups tested against their previous CHC profile as a result of small sample size. The results presented here cannot be explained in the framework of diverse labels that are recognized with a unifying-template. A unifying-template would be based on commonalities found in the majority of all nestmate CHC profiles and their corresponding neural representation, which led to the concept of ‘inclusion theory’ (Bos and d’Ettorre, 2012; Bos et al., 2013; Guerrieri et al., 2009). However, in one of our test scenarios (workers from sub-colony AB encountering workers from sub-colony A), the inclusion criterion with respect to the chemical composition of the CHC profiles was fulfilled. We showed that workers were still able to discriminate based on CHC profiles that contain all except one of the components, compared to their own (manipulated) colony odor. Thus, with respect to the labels, i.e., composition of CHC profiles, the workers’ discrimination cannot be explained by the inclusion theory, because additional components were not more important than missing components for nestmate recognition, as had previously been suggested (Guerrieri et al., 2009).

Neural mechanisms for label-template matching and template formation

Ants learn about their colony odor during early adult life (Carlin and Hölldobler, 1983; Errard, 1994) and of novel labels later in life (Guerrieri et al., 2009), as is the case in our experiments. Because they match labels with a neural template, we propose that this takes place in the mushroom body (MB) - a brain area which is important for learning and memory (Giurfa, 2007; Heisenberg, 2003).
The components of a label are detected by broadly tuned olfactory receptor neurons (Sharma et al., 2015) which project to the antennal lobe (AL) where olfactory receptor neurons that express the same olfactory receptors coalesce in the same functional units (Couto et al. (2005); Zube and Roessler (2008); Figure 2.5a). Here, the label-specific activity patterns are reformatted and conveyed via projection neurons (PNs) to higher brain centers, such as the MB (Brandstaetter and Kleineidam, 2011; Deisig et al., 2010). At the calyces of the MB, PNs synapse onto Kenyon cells (KCs) which transform dense and overlapping activity patterns into sparse and much less overlapping activity patterns (Perez-Orive et al., 2002; Szyszka et al., 2005). In seeking for the neural substrate of templates, we adopt the current knowledge on evaluation and learning processes in the MB of the fruit fly Drosophila (Aso et al., 2014; Cohn et al., 2015; Hige et al., 2015a;b) and of the honey bee (Strube-Bloss et al., 2011; 2016) in order to propose the following mechanism for label-template matching:

Many KCs (~2000 in Drosophila and ~170 000 in honey bees: Aso et al. (2014); Witthöft (1967)) synapse onto a few output neurons (MBON; 34 in Drosophila and ~400 in honey bees: Rybak and Menzel (1993); Tanaka et al. (2008)) in different compartments of the MB. One compartment of the MB is positive and another, corresponding one is negative, each of both contains an extrinsic MBON. We postulate that a label will be associated with positive or negative valence when the strength of MBON activity from different compartments becomes unbalanced (Figure 2.5b). Unbalanced activity across MBONs results from compartment-specific differences in synaptic transmission from KCs to MBONs, and we assume that synaptic transmission is reduced in the compartment providing negative valence (Hige et al., 2015a). A lower synaptic transmission is restricted to those synapses of KCs that are activated by nestmate label. Based on this framework, the neural template would be implemented as weaker synaptic strength between KC and MBON in the negative compartment compared to the positive compartment (Figure 2.5c). Plasticity in KC synapses can be induced by coincident activity of compartment-specific dopaminergic neurons (DANs), which predominantly leads to synaptic depression (honey bee: Grünewald (1999); Mauelshagen (1993), Drosophila (Hige et al., 2015a), but can additionally lead to recruitment of MBON activity (see Okada et al. (2007); Strube-Bloss et al. (2011; 2016)).

Template formation through associative learning requires the activation of the DAN pathway by a reward (e.g. social interaction or food) and possibly also by recurrent activation of DANs from MBONs (Zhao et al., 2018). Experiencing a novel label that eventually becomes valid for nestmates would lead to synaptic depression at all synapses of KCs that are activated by this novel label in the compartment providing negative valence. Synaptic depression due to distinct nestmate labels could occur on the same MBON and we assume that a high specificity to different labels is realized by the large number of KCs and their sparse activity patterns for different labels. Specifically, we assume that there are KCs which show the response property of responding to one stimulus (e.g. label A), but not to a
similar stimulus where only one additional component is added (e.g. label AB) (Figure 2.5b). Note that in Drosophila such KCs (responding to one component but not in combination with a second component) have not been described yet (Campbell et al., 2013). However, studies in Drosophila and honey bees suggest that such synthetic processing of information about components occurs in the AL (Deisig et al., 2010; Joerges et al., 1997; Silbering and Galizia, 2007; Silbering et al., 2008). Moreover, associative odor learning alters the activity patterns in functional units of the AL which enhances the difference between mixtures and their components (Daly et al., 2004; Faber et al., 1999; Fernandez et al., 2009; Rath et al., 2011). For our study, this implies modification of AL activity patterns for the label with one additional component (label A), thereby separating it from the activity pattern for the label with the same and another component added (label AB). Our result that the label with one additional component is 'strange' only for those workers that learned the label with two additional components as being valid, could also be explained by synthetic processing.

This framework for a template-formation mechanism could also explain the ability of workers to discriminate between several task-associated label from the same colony (Bonavita-Cougourdan et al., 1993; Greene and Gordon, 2003), if one assumes that different task-related labels would be encoded by different MBONs.

**Significance for colony organization**

Our proposed mechanism of multiple-template for nestmate recognition changes the paradigm on what information about colony members is acquired and how it is used. Under a previous hypothesis (the one-template system), membership initially is recognized and subsequently additional information such as cues about recent task performance is processed. Instead, our data suggests that recognition cues of colony members can be perceived and classified as belonging to distinct entities, and different labels can be attributed independently with more than one attribute of e.g. 'nestmate & forager' or 'nestmate & nurse'. Such a partitioned nestmate recognition system is highly selective for different labels while remaining flexible enough to accommodate developing differences due to e.g. task engagement, seasonality or, diet. Because the proposed associations in the multiple-template system takes place along parallel pathways in the MB, it requires less time for processing compared to a sequential categorization with additional information such as task engagement in a one-template system. Workers can recognize nestmates very quickly (Stroeymeyt et al., 2010), and conversely, fast reactions against non-nestmates are probably selected for because a delayed response increases the risk of being injured, and the first to react will probably have an advantage in any ensuing conflict. Because individual experience shapes the templates, we expect that template formation is an individual process and colony members probably have different templates, even for the same label. Inter-individual differences in nestmate recognition have been discussed as one potential mechanism for a reliable defensive response of the collective (Esponda and Gordon, 2015).
Fig. 2.5 Simplified circuit model proposed for template formation and label-template matching. (a) Recognition cues (RC) from a stimulus ant are received by broadly tuned olfactory receptor neurons (ORN) on the antennae (AN) of the evaluating ant. The odor-induced activity is reformatted along the olfactory pathway through the antennal lobe (AL) and the projection neurons (PN), resulting in label-specific activation patterns of Kenyon cells (KC) in the mushroom body (MB). In this example, label AB induced activity in KCs is indicated as thick red lines. During learning of a novel label (e.g. label AB), a reward results in activation of dopaminergic neurons (DAN) in one of the two compartments. Coincident activity of DAN and KC in this compartment results in synaptic depression between the KC and the MBON. Based on the relative output strength from different MB compartments, a positive, neutral or negative valence is provided, a decision is made and the focal ant eventually responds with a behavioral action towards the stimulus ant. In this example, label AB activates a specific set of KCs, during a learning process. The synapses from KC that are also active when the label 0 is presented are already depressed (from previous learning events), whereas synapses of label AB-specific KCs undergo depression by coincident activity in DAN (dashed circle). (b) Examples of synaptic transmission between KC and MBON at the two different compartments after learning label 0 and label AB as nestmate. All three labels (label 0, A and AB) activate different sets and the same number of KCs (colored thick lines indicate activity; grey thin lines indicated no activity). Due to weaker synapses between KC and MBON in the negative compartment, label 0 and label AB have an overall positive valence, while label A has a neutral valence. Functional connectivity of DAN and MBON in the MB is derived from studies on Drosophila (Aso et al. 2014). KC color code: black, unspecific KC for colony odors; blue, label 0 specific KC; orange, label A specific KC, red, label AB specific KC. LN: local interneurons. (c) Based on our proposed model, the template is implemented as reduced synaptic strength between nestmate label-specific KCs and MBON in the negative MB compartment compared to the positive compartment.
further consequence of the individual reinforcement during template formation and maintenance is that frequent interactions within a task-group can adjust templates accordingly. For example, workers involved in brood care interact more with nurses, and thereby may tune their corresponding template for ‘nestmate & nurse’ more than workers which are engaged in a different task somewhere else. This may result in experience-dependent improvement for detection of additional cues within the label that characterize the task-group. In this scenario, nurse workers know more about nurses, foraging workers more about foragers, guards probably more about foragers, and so on. Indeed, workers show spatial fidelity within the nest (Mersch et al., 2013; Tschinkel and Hanley, 2017) and although task allocation is flexible, workers tend to reengage in the same task over longer periods of time (Beshers and Fewell, 2001; Jeanson and Weidenmüller, 2014). A partitioned nestmate recognition system with multiple highly specific templates can contribute to task allocation when distinct sensory experience affects future decisions on task engagements (Pamminger et al., 2014; Sendova-Franks and Franks, 1995).

The concept of label-template matching

Describing the process of nestmate recognition in terms of label-template matching originally was intended to provide a conceptual framework for studies on kin recognition. The assumption that only one neural template is necessary and implemented probably was stimulated by the findings of systematic differences in CHC profiles among and low variability within colonies. Later, it turned out that CHC profiles are dynamic within a colony over time, as well as there being differences among colony members according to their functional roles, which challenges the one-template hypothesis. However, the question as to whether workers may employ multiple templates has never been addressed systematically before. In addition, the complexity of the CHC profiles and the rapid discrimination ability of workers resulted in research focusing on possible data reduction mechanisms from detection to perception, such as the inclusion theory, rather than acknowledging the high efficiency and discriminatory abilities of the highly evolved olfactory system of workers in assessing and categorizing complex CHC blends. We suggest that using the term label-template matching is still justified for highlighting the chemical uniformation within colonies, given that all colony members can recognize their own colony odor. However, when addressing the underlying mechanisms of nestmate recognition, this simplification is more misleading than informative, because it does not adequately describe a partitioned nestmate recognition system that allows fine-tuned categorization of chemically distinct nestmates.

Glossary

CHC profile - Cuticular hydrocarbons that are present on the insect cuticle. Commonly used solvents to extract these hydrocarbons include nonpolar solvents such as pentane, hexane, dichloromethane, and isooctane.
Recognition cues - Components of the CHC profile that can be detected and are used for nestmate recognition.

Label - The sum of recognition cues in a CHC profile.

Colony odor - The sum of all chemical components that are present on the cuticle of colony members. It includes nestmate recognition cues and other components not used for nestmate recognition. Note: this commonly used definition refers to the chemical environment and not to the perception and evaluation of chemical components.

Template - A neural representation of a nestmate label.

Reception - Detection of chemical components (odorants) by olfactory receptor neurons which encode odorants as changes in timing/rate of action potentials.

Activity pattern - Parallel pathways in the olfactory system that relay information about the stimulus to functional units, such as glomeruli in the antennal lobe or Kenyon cells in the mushroom body. The activity across these functional units (assembly code) is stimulus specific and can be recorded as activity patterns over time.

Representation - An activity pattern that is attributed with a value (learned or innate) and thereby becomes meaningful. The representation of chemical stimuli (odorants) in the brain allows the perception of odors.

Generalization - Chemical stimuli that can be discriminated are evaluated or categorized as belonging to a common entity.

Author contributions

CJK, MH and SN conceived and designed the experiments. MH performed the experiments, collected the data on workers’ behavior and the extracts for GC-MS analysis. SN conducted the formal analysis of the behavioral data. SN and MH analyzed the GC-MS data. JGM synthesized the components 5-MeC27 and 11-MeC27 for the study. SN prepared the first draft of the manuscript. CJK revised it critically for important intellectual content. Final version of the manuscript was approved by all authors.

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Social insects vigorously defend their nests against con- and heterospecific competitors. Collective defense is also seen at highly profitable food sources. Aggressive responses are elicited or promoted by several means of communication, e.g. alarm pheromones and other chemical markings. In this study, we demonstrate that the social environment and interactions among colony members (nestmates) modulates the propensity to engage in aggressive behavior and therefore plays an important role in allocating workers to a defense task. We kept *Formica rufa* workers in groups or isolated for different time spans and then tested their aggressiveness in one-on-one encounters with other ants. In groups of more than 20 workers that are freely interacting, individuals are aggressive in one-on-one encounters with non-nestmates, whereas aggressiveness of isolated workers decreases with increasing isolation time. We conclude that ants foraging collectively and interacting frequently, e.g. along foraging trails and at profitable food sources, remain in a social context and thereby maintain high aggressiveness against potential competitors. Our results suggest that the nestmate recognition system can be utilized at remote sites for an adaptive and flexible tuning of the response against competitors.

Keywords

Nestmate recognition, collective dynamics, task allocation, biogenic amines, socio-centric
Social interactions promote adaptive resource defense in ants

Introduction

Securing resources often requires active defense against competing con- and heterospecifics. The most valuable resource of social insects is their nest, housing the reproductive individuals (queens), the developing and immobile brood, and food stores. Nests provide shelter from harsh environment and the opportunity for microclimatic control of the nest interior (Horstmann, 1983; Kleineidam et al., 2001; Kleineidam and Roces, 2000), but inevitably lead to a clustered occurrence of brood and food other species may prey on (Alloway, 1979; Hölldobler and Lumsden, 1980). Ants, bees and wasps vigorously defend their nests, and at the nest entrance workers that are engaged in patrolling and attacking potential intruders can be identified based on their behavior (e.g. guard bees (Seeley, 1996)) or in several ant species even morphologically as soldiers with larger body size and distinct morphological traits (Kuebler et al., 2010; Wilson, 1953; 1980).

In response to a threat, guarding and defending workers may use alarm pheromones to recruit nestmates (Blum, 1996; Hölldobler and Wilson, 1990; Lofqvist, 1976). In this scenario, perceiving the alarm pheromone influences the internal physiological state of the receiver (aggression context), making it more likely for the receiver to respond with aggression towards unfamiliar objects and intruders (Hölldobler and Wilson, 1990). Such context-specific behavioral responses are common, either decreasing a response threshold or increasing a propensity for responding to a task-associated stimulus (Weidenmüller, 2004). Besides the well described pheromonal communication, social interactions among workers and the nest environment have an impact on the individuals’ internal state, changing it to a social context (Hölldobler and Wilson, 1990; LeBoeuf and Grozinger, 2014).

While there are frequent interactions and signals among nestmates within the nest that may modulate a worker’s context for an adequate behavioral response, comparable situations may occur at food sources (Buczkowski and Silverman, 2005). Mass recruitment along pheromone trails and markings at highly profitable food sources result in aggregations and defense of these sites against conspecifics (Boulay et al., 2007; Skinner, 1980) and heterospecifics (Tanner, 2008; Tanner and Adler, 2009). Since an individual ant itself might not be able to assess profitability of a resource, the aggressive defense of these remote ‘jackpot sites’ is surprising (Sakata and Katayama, 2001).

We hypothesize that frequent worker-worker interactions, e.g. mutual antennation and trophalaxis change the propensity of workers to respond aggressively against foreign workers. The nestmate recognition system that primarily functions for achieving colony coherence may play an important role in establishing a social context within the individual (d’Ettorre and Lenoir, 2010; Greene and Gordon, 2003; Greene et al., 2013; van Zweden and d’Ettorre, 2010; Vander Meer and Morel, 1998).

Ants can discriminate between nestmates and conspecific non-nestmates, using the odors found on the body surface (Brandstaetter et al., 2008; Lahav et al., 1999).
These odors consist of many species-specific low-volatile cuticular hydrocarbons (i.e. CHCs), which further become colony specific by environmental influences, e.g. diet and nest material (Jutsum et al., 1979; Leonhardt et al., 2007; Vander Meer et al., 1989). An almost uniform, colony specific odor is achieved by frequent exchange of CHCs between nestmates, mostly through trophallaxis and allogrooming (Boulay et al., 2004; Lenoir et al., 2001c).

Recognition of nestmates and discrimination from non-nestmates is achieved by phenotype matching of the CHC-profiles (Crozier and Dix, 1979). The concept behind is that ants compare the perceived CHC profile (one colony odor: label) with a neuronal representation (template) of their own colony odor, a process often termed label-template matching. Any mismatch between a label and template may be recognized as a non-nestmate (Vander Meer and Morel, 1998).

It has to be noted that we are far from understanding the nature of the neuronal template and how label-template matching is achieved in the brain (Brandstaetter and Kleineidam, 2011; Brandstaetter et al., 2011). Recent studies indicate that incomplete CHC profiles are sufficient for an acceptance and classification of nestmates (inclusion theory) (Bos et al., 2013; Guerrieri et al., 2009), and that perceptual differences among individuals may contribute to the collective response of defense (Esponda and Gordon, 2015; Newey et al., 2010). Colony coherence and the almost complete absence of aggression against colony members suggest a binary classification of nestmates vs non-nestmates. However, even within a colony, groups of nestmates can be recognized based on task-related changes of their CHC-profile by other ants, modifying their behavior and eventually leading to specific task allocation (Davidson et al., 2016; Gordon, 1996). Thus, nestmate recognition cues may influence the internal state of an individual (social context) and may also provide specific information about a recently performed tasks of an encountered worker.

In this study, we addressed the question whether the ant’s social context affects aggressiveness, and we tested *Formica rufa* (Linneaus, 1761) workers for aggressive responses against non-nestmates in one-on-one encounters. In our reductionist approach, we excluded any territorial markings or the presence of food. The experimental groups of workers differed only in the amount of social interactions prior to the encounter with nestmates or non-nestmates in a test arena.

**Materials and Methods**

**Animals**

For all experiments we used the territorially aggressive ant species *Formica rufa*. This species belongs to the IUCN Red List of Threatened Species (Category: near threatened. Social Insects Specialist Group. 1996. *Formica rufa*. Downloaded on 11 July 2017). Permit for collection was issued by the “Untere Naturschutzbehörde, Regierungspräsidium Freiburg, BW, Germany; Befreiung nach § 45/7 BNatSchG”.

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- Crozier and Dix, 1979
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- Brandstaetter et al., 2011
- Bos et al., 2013
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- IUCN Red List of Threatened Species
- Social Insects Specialist Group. 1996
- Linneaus, 1761
- § 45/7 BNatSchG
Social interactions promote adaptive resource defense in ants

Two colonies were collected in May 2012 near Markelfingen, BW, Germany (lat. 47°43'53.32"N, long. 9°1'51.42"E and lat. 47°43'53.17"N, long. 9°1'50.35"E, respectively). Both colonies were originally polygynous, and after establishing the colonies in the laboratory in artificial plaster nests with about 5000 workers each, colony 1 had two queens and colony 2 had one queen. The colonies were kept at a constant temperature of 25°C and 50%-60% humidity (12:12 h photoperiod), fed with honey water and frozen cockroaches twice a week and were provided with water ad libitum. We decided on studying this Red-List species in order to obtain information that may aid protection measures, and also because we think that the underlying mechanism for resource defense is widespread across the majority of ant species.

Behavioral assay

Workers were collected from a colony by branching them off from an exposed area located close to the nest. They climbed voluntarily onto a provided toothpick on which they were transferred into a plastic tube for cooling on ice until immobilized. This procedure prevented workers from releasing formic acid during the following procedure in which workers were placed either alone or together in groups of about 30 nestmates in a manipulation-arena with a Fluon coated wall and filter paper on the ground (diameter: 60 mm, height: 40 mm; Figure 3.1). Our manipulations were i) workers kept in isolation (isolated) and ii) workers kept in a social environment (social). After a separation time of 20 up to 286 min, workers from these manipulation-arenas were transferred, using a toothpick as before, to one side of a test-arena. The test-arena (diameter: 60 mm, height: 40 mm) was split in two halves by a separation wall, and was lined with fresh filter paper for each trial, thus, we excluded deposits of markings such as the trail pheromone. Over time, we transferred single workers successively from a social group, thereby reducing the number of workers in the group, and we stopped worker transfer when 20 workers were left in the manipulation-arena. This restriction ensured that all workers of social groups had frequent interactions before they were tested. Due to the small size of the manipulation-arena, workers in social groups had a high number of interactions. We decided on such a restricted space for the workers to promote interactions that maintain the social context for the workers, and this experimental design precluded us from quantifying individual interactions even within short time periods.

For testing, a single worker was directly transferred from one of the colonies to the other half of the test arena, and both workers were allowed to briefly acclimate to the new arena before the separation wall was removed (sliding sideways). The workers that came from the manipulation-arena were observed in detail (for a period of three minutes), and these workers are termed focal-workers in the following, while the other workers in each test are termed encounter-workers. All focal-workers came from colony 1 and encounter-workers either came from colony 2 (non-nestmates) or colony 1 (nestmates). The pairs of focal- and encounter-workers were size matched.
3.3 Materials and Methods

Fig. 3.1 Experimental procedure. Workers were collected from the colony and transferred, first to one of the manipulation-arenas (red arrows) for either social or isolated manipulation. Later, after a separation time (green arrows), these focal-workers were transferred to one half of the test-arena (red arrows). Before the test started, a retractable separation wall (W) divided the test-arena into two halves. Encounter-workers were transferred to the other half of the test-arena, and either workers from the same colony (NM; red arrows) or a different colony (NNM; blue arrows) were used. Video recording and test started by sliding out the separation wall.

by visual inspection in order to reduce size-related bias in behavioral responses (Batchelor and Briffa, 2010).

All individual data are independent from each other because workers were sacrificed after being tested. A total of 240 trials, consisting of pairs of focal- and encounter-workers were analyzed. The pairings for the tests (groupings) were: social focal-workers versus nestmates (social-FW vs NM), social focal-workers versus non-nestmates (social-FW vs NNM) and isolated-FW vs NNM (n=80 for each group), and tests were done in parallel on 19 days between 5th June to 11th July in the room where the colonies were kept. Interactions between the two workers are defined by physical contact, such as antennation or targeted behavior of the focal-workers towards the encounter-worker (Table 3.1). In the vast majority of encounters, the focal-workers made antennal contact (in 2621 of a total of 2815 encounters in 240 trials), and encounters are terminated when the two workers separated spatially by at least one body length. Encounters that occurred after a worker (unsuccessfully) tried to escape by climbing the wall of the test-arena were excluded from the analysis.

The two workers were video recorded, using a high-speed camera (Casio, Exilim F1) at a sampling rate of 300 frames/sec for three minutes, starting with the removal of the separation wall. The videos were analyzed, using free video-editor software (Avidemux 2.5, Mean, fixounet.free.fr) and the behaviors of focal-workers were scored separately for all encounters throughout the trial (whole recording
Table 3.1 Behaviors of focal-workers while interacting with the encounter-worker during a trial.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>antennation</td>
<td>antennae of focal-worker touch the encounter-worker at any body part</td>
</tr>
<tr>
<td>body rising</td>
<td>focal-worker raises its body while moving its front legs in the air</td>
</tr>
<tr>
<td>opening mandibles</td>
<td>mandibles of focal-worker are wide open and distance between opponents is less than one body length</td>
</tr>
<tr>
<td>biting</td>
<td>focal-worker grasps any body part of the opponent with its mandibles</td>
</tr>
<tr>
<td>flexing gaster</td>
<td>focal-worker bends its gaster forward, possibly spraying formic acid</td>
</tr>
<tr>
<td>fighting</td>
<td>both workers are entangled with each other, such that no further specific behaviors can be discriminated</td>
</tr>
</tbody>
</table>

Our aim was to classify the behavior of focal-workers as being aggressive or non-aggressive, rather than describing aggressive behavior itself in detail. To this aim, we only scored unambiguous behaviors and abstained from an arbitrary renaming the video files for an observer-blind analysis. We built our classification scheme on focal-workers as being aggressive when they showed at least one of the clearly aggressive behaviors that are potentially harmful: 'biting', 'flexing gaster', 'fighting' in any of their encounters during a trial (Figure 3.2). The rational of this experimental procedure and scoring of behavior is to allow focal workers to experience the encounter-workers long enough to decide on aggression or not. Quantifying the odds of being aggressive based on our classification was 0.19 for social-FW vs NM, 1.58 for social-FW vs NNMM and 0.38 for isolated-FW vs NNMM. The low odds for social-FW vs NM confirms that our classification depicts specific aggression against NNMM.

Data analysis

First, we quantified potential differences between the groups in how many encounters (number of interactions) occurred. For comparisons, we used a quasi-Poisson generalized linear model (GLM), with groupings (social-FW vs NM, social-FW vs NNMM, isolated-FW vs NNMM) and the separation time as explanatory variable, and we also included an interaction of both variables (model I). In order to draw inferences about differences between groups, we used a Bayesian framework by calculating 10 000 values that are random draws from the posterior distribution of the model I estimates. We compared the number of interactions at the shortest and longest separation time (20 min and 245 min) by the proportion of simulated values
Fig. 3.2 Behavioral scores of focal-workers for later binomial classification as being aggressive or not. Social focal-workers were examined with respect to behaviors that mainly or exclusively occurred in encounters with non-nestmates. If one of the following behaviors: 'biting', 'flexing gaster' or 'fighting' occurred at least once during any encounter, the focal-worker was classified as being aggressive. Otherwise, the focal-worker was classified as non-aggressive. Scale bar: 100% prevalence of a behavior in at least one of the encounters during a trial.
from the posterior distribution that are larger in one group compared to another group (measure of certainty). We specifically analyzed whether the isolation of focal-workers results in a disproportionate decrease in number of interactions which, in case, might impact our measure of aggression, for example: if the number of interactions decreases over time, this may lead to a lower number of focal-workers being classified as being aggressive.

Second, we tested whether the number of interactions impacts our measure of aggression. We used a binomial GLM (model II), with a logit link function to quantify differences in aggression of focal-workers in response to NM and NNM. To the binary response variable of aggression, we included number of interactions as well as grouping and their interactions as explanatory variables. As in the Bayesian framework above, we calculated 10 000 values that are random draws from the posterior distribution of the model II estimates for the description of the credible intervals.

Third, in the following model we excluded the number of interactions since they had no impact on our measure of aggressiveness (model II). We used a binomial GLM (model IIIA), with a logit link function to quantify differences in aggression of focal-workers in response to NM and NNM. To the binary response variable of aggression, we included separation time (log-transformed), grouping and their interactions as explanatory variables. Based on the model estimates, we calculated the probability of being aggressive at short (20 min) and long (245 min) separation times, and we calculated the change in odds over time for being aggressive. As in the Bayesian framework above, we calculated 10 000 values that are random draws from the posterior distribution of the model IIIA estimates. We compared the probability of aggression at the shortest and longest separation time (20 min and 245 min) by the proportion of simulated values from the posterior distribution that are larger in one group compared to another group (measure of certainty).

Finally, using the same approach, we compared the change over time in being aggressive between isolated-FW and social-FW vs NNM and between social-FW vs NM and NNM. In addition, we estimate the effect of separation time on the probability of workers being aggressive by using a linear regression (model IIIB) on the simulated data from model IIIA.

In the Bayesian statistics, we always used the 2.5% and the 97.5% quantiles as the lower and the upper limits of the 95% credible interval. All statistical analyses were done using R (3.3.3) (R Core Team, 2017) in RStudio, including the package arm with the sim function to draw random samples from the posterior distribution of model parameters (Gelman and Hill, 2007).

**Results**

Focal-workers of all three groups interacted frequently with their encounter-workers (Figure 3.3). Irrespective of separation time, the median number of interactions was very similar across the groups (social-FW vs NM: 8.5, quartile range (QR):
5-15; social-FW vs NNM: 10, QR: 7-18; isolated-FW vs NNM: 12, QR: 6-16). We calculated the correlation between separation time and number of interactions, using the GLM model I (S1 Table 1 on page 70). At the beginning of our observation period, at a separation time of 20 min, the calculated numbers of interactions are 16 for social-FW vs NM, 17 for social-FW vs NNM and 14 for isolated-FW vs NNM (Figure 3.3). At longer separation time, the number of interactions decreased significantly in focal-workers from social groups, irrespectively whether they encounter a NM or a NNM (decrease by 28% and 24% for one log-unit increase in time, respectively). The focal-workers that previously were kept in isolation also had fewer interactions at longer separation time, however, this decrease by 13% does not differ significantly from no change (S1 Table 1 on page 70). Bayesian statistics revealed low certainty of differences between number of interactions at short separation times (20 min; 65% between the two groups of social-FW and 82% for isolated-FW vs NNM, having fewer interactions compared to social-FW vs NNM). We find no indication that after long separation time (245 min) isolated-FW have fewer interactions compared to the two social groups. Rather the opposite seems to be the case: with certainties of 98% and 83% (social-FW vs NM and vs NNM, respectively), isolated-FW had more interactions at longest separation time.

The decrease in number of interactions (slope) is very similar between the two social groups, social-FW vs NM and social-FW vs NNM (certainty of difference is only 69%), and it is very unlikely that the decrease in number of interactions is higher in isolated-FW vs NNM compared to the two groups of social-FW (14% and 5% for social-FW vs NNM and vs NM, respectively). We therefore are confident that our measure of aggression is not biased, thus not favoring our hypothesis by the number of interactions at different separation time.

Since the number of interactions relates to separation time in some of the groups tested, we further analyzed this parameter as explanatory variable for aggression. In all three groups, the number of interactions has no significant predictive value for the probability of an aggressive response in focal-workers. A larger number of interaction is not related to a higher probability for being classified as aggressive, since we found no significant change in odds. The calculated change in odds from the GLM (model II) are: 2% for social-FW, irrespective of the type of encounter (NM or NNM) and 4% for isolated-FW against NNM for each additional interaction (S1 Figure on page 69 and S1 Table 2 on page 71), and we found large and overlapping credible intervals.

Based on the results that: i) number of interactions does not relate to the probability of isolated-FW being classified as aggressive (model I) and ii) isolated-FW have the lowest decrease in number of interactions with longer separation time (model II), we decided to exclude the number of interactions as explanatory variable in the following model. We calculated the correlation between separation time and probability of being aggressive, using the GLM model IIIA. Over separation time, the probability of being aggressive changes (Figure 3.4). The calculated change in odds for being aggressive from model IIIA are: -33% for social-FW vs NM;
Fig. 3.3 Effect of separation time (min) on number of interactions. In two of the groups (social-FW vs NM in blue: -28% and social-FW vs NNM in red: -24%), the number of interactions decreases significantly with increasing separation time. For the group of isolated-FW vs NNM (black: -13%), this decrease is lower and statistically not significant different from no change. Dots represent independent measures of number of interactions. Solid lines represent fitted values from model I and shaded areas represent the 95% credible intervals of Bayesian statistics.

-29% for social-FW vs NNM and -62% for isolated-FW vs NNM for one log-unit increase in time. Only the change in odds of isolated-FW is significantly different from zero (S1 Table 3 on page 72).

The estimates for the intercepts (probability of aggression at separation time equal zero) are imprecise because of missing data during the period of 0-20 min of separation time. Only the (predicted) aggression at a separation time of 0 min of isolated-FW vs NNM are significantly different from no aggression. At a separation time of 20 min, we calculated the lowest probability of being aggressive for social-FW vs NM and the highest probability of being aggressive for social-FW vs NNM (24% and 71%, respectively, and 54% for isolated-FW vs NNM). At long separation times (245 minutes), the probability of being aggressive drops down in isolated-FW vs NNM and is comparably low in social-FW vs NM (9% and 10%, respectively) but remains high in social-FW vs NNM (51%).

Bayesian statistics revealed high certainty of differences between social-FW vs NM and social-FW vs NNM at separation time 20 min (99.9%), thus, the type of encounter worker determines the focal-worker’s response. This is also supported by the certainty of difference between social-FW vs NM and isolated-FW vs NNM (97%). The certainty of difference between social-FW vs NNM and isolated-FW vs NNM is less than 90%, indicating that these two groups do not differ considerably when the manipulation (social/isolated) lasted only 20 min.

After a long separation time (245 min), the difference between the two groups of social-FW vs NM and NNM is maintained (certainty of 99.8%). Now also
Fig. 3.4 Effect of separation time (min) on probability of being aggressive. Social-FW differ with high certainty (>99.9%) in their probability of being aggressive at short separation time (20 min), depending on the type of encounter ant (vs NM in blue: 24% and vs NNM in red: 71%). The change in odds decreases with increasing separation time, and this decrease is only significant for isolated-FW (vs NNM in black: -62%). Dots represent binary, independent data of focal-workers that were classified either as being aggressive (upper) or non-aggressive (lower). Solid lines represent fitted values from the binomial GLM (model IIIA) and shaded areas represent the 95% credible interval (based on Bayesian statistics). Dotted lines are linear regressions on log-transformed separation times (model IIIB).
the isolated-FW differ with high certainty (99.9%) from the social-FW vs NNM and this marked difference is the result of our manipulation. Following a long separation time, isolated-FW have a similar probability of being aggressive against NNM as social-FW against NM (certainty of difference only 55%). Further support for our hypothesis that isolation reduces the probability of being aggressive against NNM is provided by the comparison of the change in aggression over time. The decrease in the probability of being aggression is, with a certainty of more than 90%, stronger in isolated-FW compared to social-FW against NNM.

In order to estimate the change in probability of being aggressive in the separation time-range we investigated, we calculated a linear regression (LM; model IIIB), using simulated values from Bayesian statistics that are based on data from the previous GLM (model IIIA). The decrease in probability of an aggressive response is at least two times higher in isolated-FW compared to social-FW (social-FW vs NM: -5%, social-FW vs NNM: -8%, isolated-FW vs NNM: -17% change for one log-unit increase in time).

**Discussion**

The behavior of individual workers within an insect colony depends strongly on their social environment. In this study, we investigated how the social environment modulates aggression, and we show that a social environment maintains or generates a worker’s context that facilitates an aggressive response against potential competitors. Without a social environment, e.g. isolated from nestmates, workers have a reduced propensity for an aggressive response against workers from a different colony. We propose that nestmate recognition, a system primarily functioning for colony coherence, modulates individual aggressiveness. Aggregations of workers, for example at highly profitable food sources after mass recruitment along with frequent social interactions can promote a switch from foraging to defense behavior. The defense of highly profitable food sources is a well-established phenomenon in ants (Boulay et al., 2010; Skinner, 1980), and in our reductionistic approach we excluded any other cues that may occur at a feeding site, and focused on the effect of the presence or absence of a social environment for different time spans, and how this effects the probability of being aggressive to a non-nestmate. We based our classification on behaviors that are obviously threatening, and this conservative classification of aggression excludes the ‘spreading the mandibles’ that is used in many other studies as indicative for recognition of a non-nestmate (Guerrieri and d’Ettorre, 2008). Since our focal observations were long, we observed the ‘spreading the mandibles’ in almost all trials at least once, and this behavior was not necessarily triggered by the encounter-workers.

Aggressive interactions during an encounter depend on both workers participating. An aggressive behavior by one of the workers may lead to either an escalation, where both workers are engaged in aggression, or retraction, where one worker may disengage from the interaction. Thus, the encounter-workers in our tests probably
contribute to the focal-worker’s response by initiating aggressive interactions. This may lead to an overestimation of focal-workers’ aggressiveness against NNM encounter-workers and, if amicable behavior also is influential, to an even lower aggressiveness towards NM encounter-workers (false positive response). The main result of our study is based on the separation time of the focal-workers and the corresponding NNM encounter-workers are expected to contribute in the same way to the focal-worker’s behavior, irrespective of their separation time since encounter-workers were all treated equally.

The CHC-profiles of workers possibly change while foraging, similar to what has been described for patrolling harvester ants (Greene and Gordon, 2003; Greene et al., 2013; Wagner et al., 1998). However, it is clearly not rendered to a non-nestmate CHC-profile, since homecoming foragers are readily accepted by their nestmates even after longer absences. In our (control) experiments, even a separation from the colony for 4 hours did not impair nestmate recognition to a greater extent. We cannot rule out that during separation slight changes in the CHC-profile might occur, but it is highly unlikely that these changes could contribute significantly to reduced aggression in encounter-workers, and in turn in focal-workers. Thus, we are confident that the differences in behavior we observed in focal-workers are due to differences in their own social context rather than due to changes in their CHC-profile and corresponding behavioral changes in encounter-workers.

Other forms of communication, e.g., pheromonal signals and chemical markings have received much more attention in the field of task allocation in social insects than the social environment and interactions. Chemical markings are used in many ant species to spatially extend the nest environment with a home range or a territory (Cammaerts, 2004; Cammaerts and Cammaerts, 2000; Mayade et al., 1993; Wenseleers et al., 2002). For example, Tetramorium species mark their foraging area (i.e. home range), resulting in an increased probability of aggressive behavior against heterospecific, but not against conspecific non-nestmate workers (Cammaerts, 2004). In contrast to home range markings, territorial markings can promote aggression against non-nestmates (Hölldobler and Wilson, 1990). Another fascinating example of competition among conspecific colonies is the ritualized combat in Myrmecocystus mimicus. During display tournaments that may last for several days, in which almost no physical fights occur, workers of two different colonies gauge each other and assess group size by ‘head-counting’, ‘caste polling’, and ‘presence of opponents uninvolved in display fights’ have been proposed (Hölldobler and Lumsden, 1980; Lumsden and Hölldobler, 1983). The underlying mechanisms for such an assessment are unknown. Based on our results, the absolute and relative rate of interactions with nestmates and non-nestmates probably contributes considerably to the modulation of a worker’s propensity for an aggressive response. Interestingly, ‘body rising’ occurred more often in our experiments with non-nestmates and it is also the common behavioral display of workers engaged in the tournament. Further support for the idea that beside the absolute number of nestmates also the relative number of nestmates
Social interactions promote adaptive resource defense in ants vs. non-nestmates and interactions among them has an influence on the outcome in competitions comes from studies on *Azteca* ants when territories are formed (Adams, 1990).

Accumulations of large numbers of workers do not only occur within and close to the nest or in display tournaments, but also at valuable food sources as a consequence of mass recruitment along pheromone trails (Beckers et al., 1992; 1990). In a previous study, in which workers were collected from foraging trails and tested against heterospecific workers, the authors already speculated that recent experience with nestmates may modulate the workers’ aggressiveness (Sakata and Katayama, 2001). Frequent social interactions (e.g. antennation, trophallaxis, or allogrooming) occur wherever many workers aggregate. During encounters with nestmates, workers are exposed to and perceive the CHC-profile of nestmates. For *Formica xerophila*, the exposure to nestmate CHC-profiles (presented on glass beads) maintains a high aggressiveness against heterospecific workers (Tanner, 2006; 2008; Tanner and Adler, 2009). Our study extends this finding by demonstrating that the workers’ context is modulated such that task allocation for defense in general (not only against heterospecifics) is facilitated.

In solitary foraging species, e.g. in the desert ant *Cataglyphis fortis*, the individuals’ homing vector modulates the worker’s context, with long homing vectors relating to low and short homing vectors relating to high propensity of aggression (Knaden and Wehner, 2004). Desert ants have to rely on their (egocentric) homing vector as an indication for vicinity to their home, as odor cues can be too volatile in the extreme desert habitat. Indeed, social interactions are not necessary to maintain aggressiveness against non-nestmates in the desert ant *C. niger*; whereas learning the CHC-profiles of familiar, neighboring conspecific colonies impacts aggressiveness (Nowbahari, 2007). Such colony level ‘dear enemy’ effects as well as ‘nasty neighbor’ effects have also been described in other ant species. Genetic, as well as spatial and chemical differences can lead to various, causal relationships among colonies and their aggressiveness (Fournier et al., 2016; Frizzi et al., 2015; Jutsum et al., 1979; Langen et al., 2000).

Although conclusions from reductionistic experimental approaches might suggest something else, egocentric, geocentric and sociocentric modulation of social context probably occurs in parallel at all times with the consequence of a fine-tuned task allocation within the colony. Except for desert ants that rarely deploy trail pheromones or other chemical markings and rarely encounter nestmates while foraging, other ants most likely use all these available cues that reliably indicate a social environment. An egocentric modulation of context, based solely on the homing vector will not lead to a collective defense at remote food sources, while a geocentric modulation with chemical markings can induce this (Figure 3.5). Along a pheromone trail, the modulation is directly related to recruitment and aggregation of workers, and it may even support protection of resources while transported. Marking of a territory rather reflects the colony’s experience in the past, which might have predictive value for upcoming resources in the same area.
A sociocentric modulation, based on interacting workers is a direct measure of the social environment and its momentariness makes it highly flexible. Species might be biased towards one available cue over another, depending on adaptations to different habitats, colony organization and life history.

![Diagram of worker's context promoting aggressive responses](image)

**Fig. 3.5 Worker’s context promoting aggressive responses.** The nest environment provides various cues that might modulate a worker’s context and eventually promotes aggression against competing con- and heterospecifics. A sociocentric modulation of defense of highly profitable food sources is plausible, when many foraging workers are gathering at the source. Some ant species use chemical markings, indicating the home range and areas with potentially valuable resources, which are defended mainly against heterospecifics (Wenseleers et al., 2002). Such a geocentric modulation of context is more stable over time and less flexible than a sociocentric modulation. Solitary foragers like the desert ant *Cataglyphis* can access the homing vector of their path integration, and the length of the homing vector is negatively correlated with the social context of the forager for an aggressive response (Knaden and Wehner, 2003; 2004). Such egocentric modulation of context seems most important for species without mass recruitment but might also act together with geocentric and sociocentric modulation in other species.

In general, context is a neurophysiological state and any modulation of the context is an internal process leading to a different state. The classical candidates for neuromodulation are biogenic amines, like dopamine, octopamine and serotonin (Roeder, 1994; Stevenson et al., 2005; Szczuka et al., 2013). In order to fill our gap in understanding the link between perception (e.g. non-nestmate) and action (e.g. aggressive response), we need to investigate the underlying neural mechanisms that lead to individual decision making. The ease of manipulating an individual’s social context and assessing aggressive behavior makes the nestmate recognition system of ants a highly promising system for experimentally addressing both, proximate mechanisms and the consequences of individual behavior on the social organization in insect colonies.
Funding

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The original video files can be accessed at: DOI: 10.12751/g-node.f53069
Supplemental Materials

Results of GLM model I: correlation between separation time and number of interactions

```r
glm(formula = NoInt + grouping + time.log + time.log:grouping, family = quasipoisson, data = data.glm)
```

Estimates can be back-transformed using `exp()`.

**S1 Figure: Effect of number of interactions on probability of being aggressive.**

With larger numbers of interactions, there is no significant increase in the probability of being aggressive (flaring credible intervals). When having few interactions within a trial, social-FW were more likely to act aggressively against NNM compared to encounters with NM (significant difference in intercepts). This indicates that only very few interactions are necessary to discriminate NNM from NM. Dots represent binary, independent data of focal-workers that were classified either as being aggressive (upper) or non-aggressive (lower). Solid lines represent fitted values from model II and shaded areas represent the 95% credible intervals of Bayesian statistics.
Supplemental Table 1. Results of GLM model I: correlation between separation time and number of interactions glm(formula = NoInt ~ grouping + time.log + time.log:grouping, family = quasipoisson, data = data(glm))

Estimates can be back-transformed using exp().

| Estimate                      | Std. Error | t value | Pr(>|t|) |
|-------------------------------|------------|---------|----------|
| intercept social-FW vs NNM    | 3.6241     | 0.3625  | 9.9971   | 0        |
| slope social-FW vs NNM        | -0.2673    | 0.0865  | -3.0896  | 0.0022   |
| intercept isolated-FW vs NNM  | 3.0749     | 0.3487  | 8.8185   | 0        |
| slope isolated-FW vs NNM      | -0.1359    | 0.0814  | -1.6697  | 0.0963   |
| intercept social-FW vs NM     | 3.7539     | 0.3948  | 9.5081   | 0        |
| slope social-FW vs NM         | -0.3329    | 0.0953  | -3.4945  | 0.0006   |

Contrast estimates of the reference group (social vs NM) compared to the other two groups

| Estimate                      | Std. Error | t value | Pr(>|t|) |
|-------------------------------|------------|---------|----------|
| Intercept social-FW vs NM     | 3.7539     | 0.3948  | 9.5081   | 0        |
| to intercept social-FW vs NNM | -0.1298    | 0.536   | -0.2422  | 0.8089   |
| to intercept isolated-FW vs NNM | -0.679    | 0.5267  | -1.2891  | 0.1986   |
| slope social-FW vs NM         | -0.3329    | 0.0953  | -3.4945  | 0.0006   |
| to slope social-FW vs NM      | 0.0656     | 0.1287  | 0.5099   | 0.6106   |
| to slope isolated-FW vs NNM   | 0.197      | 0.1253  | 1.5726   | 0.1172   |

Deviance Residuals

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(Dispersion parameter for quasipoisson family taken to be 4.499142)
Null deviance: 1182.5 on 239 degrees of freedom
Residual deviance: 1058.2 on 234 degrees of freedom
AIC: NA
Number of Fisher Scoring iterations: 5
Supplemental Table 2. Results of GLM model II: correlation between number of interactions and probability of being aggressive.

\[
\text{glm(formula = aggression} \sim \text{ NoInt } \ast \text{ grouping, family = binomial(link = } \text{ 'logit')}, \text{ data = data.glm)}
\]

Estimates can be back-transformed using \text{plogis}().

**Estimates of intercepts and slopes, separately for each of the three groups**

| Estimate | Std. Error | z value | Pr(>|z|) |
|----------|------------|---------|----------|
| intercept social-FW vs NNM | 0.2352 | 0.4474 | 0.5257 | 0.5991 |
| slope social-FW vs NNM | 0.0184 | 0.0321 | 0.5744 | 0.5657 |
| intercept isolated-FW vs NNM | -1.4934 | 0.498 | -2.9985 | 0.0027 |
| slope isolated-FW vs NNM | 0.0414 | 0.0327 | 1.2661 | 0.2055 |
| intercept social-FW vs NM | -1.8757 | 0.5247 | -3.5748 | 0.0004 |
| slope social-FW vs NM | 0.0213 | 0.0372 | 0.5733 | 0.5665 |

**Contrast estimates of the reference group (social vs NM) compared to the other two groups**

| Estimate | Std. Error | z value | Pr(>|z|) |
|----------|------------|---------|----------|
| intercept social-FW vs NM | -1.8757 | 0.5247 | -3.575 | 0.0004 |
| to intercept social-FW vs NNM | 2.1109 | 0.6896 | 3.061 | 0.0022 |
| to intercept isolated-FW vs NNM | 0.3823 | 0.7234 | 0.528 | 0.5972 |
| slope social-FW vs NM | 0.0213 | 0.0372 | 0.573 | 0.5665 |
| to slope social-FW vs NNM | -0.0029 | 0.0491 | -0.059 | 0.9531 |
| to slope isolated-FW vs NNM | 0.0201 | 0.0495 | 0.406 | 0.6846 |

**Deviance Residuals**

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(Dispersion parameter for binomial family taken to be 1)
Null deviance: 310.77 on 239 degrees of freedom
Residual deviance: 269.67 on 234 degrees of freedom
AIC: 281.67
Number of Fisher Scoring iterations: 4
**Supplemental Table 3.** Results of GLM model IIA: correlation between separation time and probability of being aggressive.

```r
glm(formula = aggression ~ grouping + time.log + time.log:grouping, family = binomial(link = "logit"), data = data.glm)
```

Estimates can be back-transformed using `plogis()`.

### Estimates of intercepts and slopes, separately for each of the three groups

|                           | Estimate | Std. Error | z value | Pr(>|z|) |
|---------------------------|----------|------------|---------|----------|
| intercept social-FW vs NNM| 1.9318   | 1.2784     | 1.5111  | 0.1308   |
| slope social-FW vs NNM    | -0.342   | 0.2901     | -1.1789 | 0.2384   |
| intercept isolated-FW vs NNM| 3.0602  | 1.4353     | 2.1321  | 0.033    |
| slope isolated-FW vs NNM  | -0.9719  | 0.3524     | -2.7576 | 0.0058   |
| intercept social-FW vs NM | 0.0133   | 1.6612     | 0.008   | 0.9936   |
| slope social-FW vs NM     | -0.3941  | 0.3971     | -0.9926 | 0.3209   |

### Contrast estimates of the reference group (social vs NM) compared to the other two groups

|                           | Estimate | Std. Error | z value | Pr(>|z|) |
|---------------------------|----------|------------|---------|----------|
| Intercept social-FW vs NM | 0.0133   | 1.6612     | 0.008   | 0.9936   |
| to intercept social-FW vs NNM | 1.9186  | 2.0962     | 0.9153  | 0.36     |
| to intercept isolated-FW vs NNM | 3.0469  | 2.1954     | 1.3879  | 0.1652   |
| slope social-FW vs NM     | -0.3941  | 0.3971     | -0.9926 | 0.3209   |
| to slope social-FW vs NNM | 0.0521   | 0.4917     | 0.106   | 0.9156   |
| to slope isolated-FW vs NNM| -0.5778 | 0.5309     | -1.0882 | 0.2765   |

### Deviance Residuals

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(Dispersion parameter for binomial family taken to be 1)

Null deviance: 310.77 on 239 degrees of freedom
Residual deviance: 260.51 on 234 degrees of freedom
AIC: 272.51

Number of Fisher Scoring iterations: 4
Investigating the neural encoding of colony odors with calcium imaging experiments

Stefanie Neupert and Christoph J. Kleineidam

Abstract

Ants vigorously defend their colony against strangers. Colony defense requires efficient discrimination between members from the own colony (nestmates) and members from foreign colonies (non-nestmates). It is well established that ants use olfactory cues to discriminate between nestmates and non-nestmates. The body surface of each ant is covered with species- and colony-specific mixtures of low-volatile components. Even though the chemical profiles of many ant species are known, the neuronal mechanism of nestmate recognition is still unknown. A previous calcium imaging study on the neural encoding of colony odors in the antennal lobe of *Camponotus floridanus* found no difference in the spatial activity patterns across glomeruli between nestmate and non-nestmate extracts. However, repeated stimulations with nestmate extracts elicited more variable glomerular activity patterns than repeated stimulations with non-nestmate extracts.

In the first part of this chapter, we test the hypothesis that the difference between nestmate and non-nestmate extracts is encoded by the sequence of activation of glomeruli. To measure the sequence of activated glomeruli, we used high temporal resolution calcium imaging. In the second part of this chapter, we reassess the previous finding of a higher trial-to-trial variability for glomerular activity patterns evoked by nestmate extracts. To measure the variability of glomerular activity patterns, we used calcium imaging via high-speed and frame recording of calcium signals. Moreover, we developed a behavioral monitor to reveal how individual ants react to olfactory stimuli during calcium imaging.
Introduction

In eusocial insects, the female worker’s behavior altruistically by sacrificing their own reproduction in order to help their mother (queen) to produce offspring (workers, new queens and males). Since it is beneficial for the own inclusive fitness to only direct altruistic acts to closely related individuals, ants vigorously defend their colony (including their queen, brood, and food storage) against individuals of other con- and heterospecific colonies. Therefore, workers need to know who is member of their own colony (a nestmate) and who is member of a foreign colony (non-nestmates). In ants, it is well established that the discrimination is based on chemical mixtures of long-chained hydrocarbons of each individual’s exoskeleton (Lahav et al., 1999; Wagner et al., 2000). Those cuticular hydrocarbons (CHCs) are species-specific and are distributed and exchanged between colony members by trophallaxis and grooming (Dahbi et al., 1999; Lenoir et al., 2001a). This results in uniformation of the CHC profiles between individuals from the same colony. However, there are differences between colony members in the CHC profile (Kaib et al., 2000) and ants are able to discriminate between nestmates that perform different tasks (Greene and Gordon, 2003). Colony odors differ between neighboring colonies of the same species in quantitative differences of the compounds in the mixture (d’Ettorre and Lenoir, 2010). It is assumed that when an ant encounters another individual, it compares the perceived CHC profile from the encounter ant (label) with an internal representation of its own colony odor (template), a process which is referred to as label-template matching. When the difference between the label and the template is above a specific threshold, the encountered ant is perceived as a non-nestmate and an aggressive response may follow.

Previous behavioral experiments demonstrated that ants can discriminate between dummies loaded with nestmate extracts and dummies loaded with non-nestmate extracts (Brandstaetter et al., 2011). Mandible treat was used as a proxy for aggression. Ants mostly did not respond with aggression toward nestmate extracts, but responded aggressively against non-nestmate extracts in most cases (Brandstaetter et al., 2011). The neuronal basis of this nestmate recognition capacity is not known yet. As other insects, ants detect odorants with their antennae. Many different types of olfactory receptor neurons (ORNs) are located on the antenna and housed in sensilla. A single sensillum can house one or several different ORNs. Each ORN expresses a specific type of receptors. Depending on the receptor type, ORNs have diverse response profiles that can be narrowly tuned to detect one or only a few components (pheromone receptors) and that can be broadly tuned to many different components (Hallem and Carlson, 2006). According to their receptor type, ORNs are sorted by converging in the antennal lobe (AL) into corresponding functional units, called glomeruli. In ants, the AL contains several hundred glomeruli (Kelber et al., 2010; Zube and Roessler, 2008). Olfactory information is processed in the AL by a network of different cell types (Hansson and Anton, 2000). Glomeruli are connected with each other via local (mostly inhibitory) interneurons (LNs)
which allows cross-talk between glomeruli. The olfactory information is further transmitted to higher brain centers, like the mushroom bodies (MB) and the lateral horn (LH), via project neurons (PNs). The MB is known to be important for learning and memory (Giurfa, 2007; Heisenberg, 2003) and the LH is considered as an important structure for evaluating the biological significance of odors (Sandoz, 2011). Upon stimulation with an odorant, the connectivity in the AL results in a spatio-temporal activity pattern, carrying information about stimulus identity. Comparing activity patterns with odor discrimination abilities revealed that odors perceived as being similar induce similar spatial patterns in the AL (Guerrieri et al., 2005).

From an imaging study, which investigated the PN responses in the AL of *Camponotus floridanus* workers, it is known that colony odors induce activity in the whole antennal lobe (Brandstaetter and Kleineidam, 2011). A further result of this study is that the spatial activity patterns in the AL is not predictive for colony odor identity, since nestmate and non-nestmate extracts induced indistinguishable overlapping spatial activity. Overlapping patterns are not surprising, since CHC profiles of nestmates and neighboring non-nestmates consist of the same CHCs, and only the concentrations of the single CHCs is different between colonies of the same species (especially when raised in the laboratory under the same conditions and fed with the same diet). Thus, the same ORNs should be activated during stimulation with nestmate and non-nestmate extracts. However, nestmate and non-nestmate extracts differ in the concentrations of the single CHCs. These concentration differences should be visible in the strength of ORN responses and hence the across-glomerular PN response strengths should differ between nestmate and non-nestmate extracts. The lack of such difference in the study by Brandstaetter and Kleineidam (2011) could be due to a limited dynamic range of the odorant induced changes in cytosolic calcium concentration or of the calcium sensor (Fura2). However, an increase in ORN response strength is also visible as a shortening in its response latency (Szyszka et al., 2014). We therefore tested the hypothesis that the temporal patterns of activated glomeruli differ between nestmate and non-nestmate extracts due to the differences in concentration of the CHCs. To this end, we used high-speed calcium imaging (using line scan recordings instead of frame recordings) in PNs in the AL of the carpenter ant *Camponotus floridanus*.

Furthermore, a surprising finding from the study by Brandstaetter and Kleineidam (2011) is that repeated stimulations with nestmate extracts revealed higher variability in glomerular response patterns than repeated stimulations with non-nestmate extracts. As a possible explanation for this phenomenon, the authors suggested that ants need to update their template of their own colony odor and therefore, plasticity is needed and the plasticity may be seen as variable activity patterns when nestmate extracts are presented. Inspired from the findings of the study by Brandstaetter and Kleineidam (2011), we tested the hypotheses that the higher variability of glomerular response patterns to nestmate extracts origins from one or a combination of the following scenarios: i) the samples of nestmate extracts
are more variable than the samples of the non-nestmate extracts, ii) recognition of
a nestmate extracts in the AL or a higher brain area induces rapid feedback which
alters glomerular response patterns, or iii) the AL circuit is optimized to detect
the nestmate labels and due to this optimized circuitry, differences in the nestmate
CHC profiles are more amplified than differences in non-nestmate CHC profiles. In
order to test these hypotheses, we did calcium imaging (using frame recordings) in
individuals of *C. floridanus* from two different colonies and repeatedly presented
nestmate and non-nestmate extracts.

Additionally, we developed a monitor for a behavioral response of the ants during
calcium imaging experiments to provide the possibility to relate neural activity to
the perception of a particular odorant or CHC profile. During calcium imaging
experiments, it is important to prevent body movements of the tethered ant,
otherwise the brain moves as well. Therefore, a common behavioral observation is
not possible. In honey bees, extracellular recordings from the protractor muscle
of the labium (M17) were used to monitor the behavioral response related to
olfactory learning while the bees were imaged (Haehnel et al., 2009). In the current
study, we used a similar behavioral monitor, using extracellular recordings of the
mandible muscles while we image their AL. We assume that ants, which try to
behave aggressively, try to open their mandibles, since opening mandibles is the
first behavioral indication of aggression.

**Material and Methods**

**Study animals**

For the imaging experiments, we used mayor workers of the carpenter ant species
*Camponotus floridanus*. We used three different lab colonies (Cf2, D544 and D360).
Colony Cf2 was collected at Sugarloaf Shores, Florida by A. Endler in July 2003.
Colony D360 was collected in September 2008 at Grassy Key, Florida by D. Moore
and colony D544 was collected in August 2009 at an unknown key by people from
the laboratory of J. Liebig. The three lab colonies were reared in plaster nests in a
climate chamber with a temperature of 25-26°C, a relative humidity of 40-60% and
a light-dark cycle of 12h/12h. Colonies were fed twice a week with honey water
and dead locusts and water was provided ad libitum.

**Creating CHC profile extracts**

For stimulation with nestmate and non-nestmate CHC profiles, we used cuticle
washes of single ants. Minor workers were selected from the three lab colonies and
cooled in a freezer at -20°C for at least 2 hours. Each ant was transferred to a
separate 2 ml glass vial and 400 µl of pentane was added to each vial. The vials
were gently manually shaken for 2 minutes and then the ants were removed and
the pentane remained in the vials. Vial containing the extracts were stored in a freezer at -20°C until imaging experiments.

**Animal preparation for calcium imaging**

Mayor workers were randomly selected from colony D544 or D360 and shortly cooled on ice until immobilization. A single ant was put into a holder and the head was fixed with hard wax (Deiberit, Dr. Böhme and Schöps) to the holder. The antennae were fixed with soft dental wax so that they pointed forwards. We opened the head by cutting a rectangular window into the head capsule. Glands and trachea were removed so that the dye injection side became visible. Supernatant hemolymph was removed with a tissue. A glass needle coated with a dried droplet of dye (diameter approximately 50 µm) was injected between the two mushroom body calyces and kept there for a few seconds to let the dye dissolve from the needle. We used the calcium sensitive dye Oregon green® 488 BAPTA-1 dextrane, potassium salt, 10.000 MW, anionic, 498 nm (Mobitec, Göttingen). Then the piece of cuticle was put back onto its original position and sealed with eicosane in order to prevent dessication. The ant was kept in a dark humidified box for at least 4h. Before imaging, the head capsule was removed again and the open window was enlarged so that the antennal lobes were accessible. We removed glands and trachea covering the antennal lobes. We removed supernatant hemolymph and covered the brain with transparent silicone (KwikSil, World Precision Instruments) to stabilize the brain. In some cases, we squeezed the ant’s body with some soft foam into the ant holder in order to prevent pumping and to reduced movement of the ant’s brain.

**Calcium imaging**

Calcium imaging data were acquired using an upright microscope (LSM 510 Meta; Carl ZEISS) with a water-immersion lens (Apochromat 20x, NA 1, VIS-IR, Carl ZEISS). For excitation, we used an argon laser (488nm) and adjusted the laser power depending on the preparation and its fluorescence. During line scan recordings and stimulations with CHC extracts, we used a sampling rate of 65 Hz, recorded in total for 10 seconds and each scanned line consisted of 256 pixels. During frame recordings, we used a sampling rate of 10 Hz and recorded in total for 7 seconds. Each frame had a resolution of 64x64 pixels.

**Stimulus delivery**

In order to test, whether a preparation was suited for experiments, we presented the general odorant octanol (diluted to 10⁻² in mineral oil). We incorporated the head space of the octanol solution in a constant air stream and controlled the stimulus application with a custom made odor delivery device in which three-way solenoid valves (LFAA1200118H, The Lee company, Essex, CT) switched between...
Calcium Imaging in the antennal lobe of ants

odor-loaded air and clean air. Since CHCs have a low volatility, we used a dummy-delivered stimulation setup described and successfully used in previous studies (Brandstaetter and Kleineidam, 2011; Brandstaetter et al., 2010). In order to increase the concentration of the CHCs in the volatile phase (head space), we heated the metal dummy to 28°C. The forward movement of the dummy was achieved through a servo-motor. Both the servo-motor for dummy stimulation and the solenoid valve for stimulation with the general odorant were controlled by a re-programmable compact RIO system (NI-9074) equipped with a digital IO module NI-9403 via a graphical user interface (software ‘BDOS – Build digital output sequence’, written in LabVIEW 2011 SP1, National Instruments, by myself).

Stimulation protocols

We used two different stimulation protocols during experiments.

a) Protocol 1: Sequence of activation (high-speed calcium imaging)
We used repeated stimulations via a dummy loaded with nestmate or non-nestmate extracts in the following pattern: We started with 10x nestmate stimulations (same colony as imaged ant), followed by 10 stimulations with extracts from a non-nestmate colony and 10x stimulations with extracts from another non-nestmate colony. Each extract was applied once on the dummy and then used for the whole set of stimulations. An advantage of this stimulus protocol is that always the same stimulus of one colony was presented to an ant, which allowed minimizing the differences in presented chemical profiles.

b) Protocol 2: Variability in encoding of nestmate colony odors (frame recordings)
We did repeated stimulations with nestmate and non-nestmate extracts. However, for each stimulation, we used extracts from different nestmates and different non-nestmates. We always started with nestmate (NM) stimulations and presented nestmate and non-nestmate (nNM) extracts in alternating order: NM1, nNM1, NM2, nNM2, NM3, nNM3, . . ., NM12, nNM12. In between, we gave control stimulation with only pentane (the solvent) on the extract. The pentane stimulations in between served as a control for neuronal responses to other stimulus components than nestmate or non-nestmate extracts (e.g., mechanical, temperature or olfactory stimuli). Between stimulations, we rinsed the dummy with pure pentane and after loading the dummy with extract or control, we waited 2 min until the pentane was evaporated at room temperature. The dummy was heated to 28°C and 30 seconds after reaching 28°C, it was presented for 2 seconds. Start of stimulation was 2 seconds after start of frame recording.
4.3 Material and Methods

Recording of mandible muscle activity during frame recordings

Extracellular recordings of the mandible muscles were done in order to monitor CHC extract-evoked behavioral responses. We cut a slit into one eye and inserted an enamelled copper wire (outer diameter: 0.081mm, Elektrisola, Eckenhagen), using it as the reference electrode. For the recording electrode, we cut a slit posterior the opposite eye and inserted a second enamel-insulated copper wire. Both electrodes were sealed to the head of the ant with KwikSil. We used a custom-build amplifier with a gain of factor 500 and a high-pass filter of 20 Hz. The electro-physiological data were then digitalized by an AD-converter (NI-6009, National Instruments) and recorded with digital oscilloscope software ‘Osci 2.0’ (software written in LabVIEW 2011 SP1, National Instruments, by myself). Mandible muscle activity was recorded for always 5 seconds. The Oscilloscope was triggered one second before stimulus presentation. Additionally to the recordings, we installed a loudspeaker for direct monitoring the neuronal activity. Pilot experiments revealed that we are able to make extracellular recordings from the mandible muscles for more than 24 hours.

Analysis of line scan recordings

In order to correct for differences in the raw fluorescence, we calculated for each pixel separately a mean baseline before stimulation onset and subtracted this value from the whole time trace. Furthermore, we divided by the baseline values, to get the relative fluorescence change for each pixel. We then calculated correlation coefficients for neighboring pixels. For each pixel separately, we additionally selected a specific number of pixels before and after the single pixel. The number of selected pixels was adjusted depending on the scan line resolution. We calculated the mean correlation coefficient between the selected pixels. Adjoining pixels with correlation values above a specific threshold were defined as belonging to one region of interest (ROI). Those ROIs corresponded to clearly visible fibers in the raw fluorescence images. For each ROI, we calculated the mean time traces of relative fluorescence change for each recording. Response latencies of selected ROIs were exemplary calculated for stimulations with 3 pulses of octanol. We compared the response latencies of glomeruli between different recordings. For the response latencies of the glomeruli to the first pulse, we extracted the time point between stimulus onset and maximum fluorescence change at which the fluorescence was higher than 2 times standard deviation before stimulus onset. Since the octanol pulses had an inter-stimulus interval of only 1 s, the fluorescence was not back to baseline before the stimulus arrived at the antennae. Therefore, we calculated for the 2nd and 3rd octanol pulses the time points at which 95% of maximum fluorescence change was reached.
Analysis of frame recordings

ROI extraction of calcium imaging signals during frame recordings was done by an automated algorithm for KNIME (Berthold et al., 2008) using the ImageBee plugin (Strauch et al., 2013). A data pre-processing step for correcting for animal movement in x- and y-direction was also done with the ImageBee plugin. Each extracted ROI consists of spatially neighboring pixels with correlated behavior over time. Each ROI now presumably refers to one glomerulus. We calculated for each ROI and each of the three stimulus types (nestmate extract, non-nestmate extract, control stimulation with only pentane), the mean fluorescence values over time, pooling several recordings with the same stimulus type. We also extracted for each recording a single values as a measure for activity. To this end, we calculated for each recording separately, the difference between the relative fluorescence change of a ROI during (mean of frame 26 and 27) and before stimulation (mean of frame 21 and 22). For dimension reduction and for visualization purpose, calcium imaging data are often analyzed using principal component analysis (PCA) and calculations of the Euclidean distance (ED) is often then used as a measure for similarity between different neural response patterns. However, there are also other methods that examine data from a different point of view and that might be more appropriate than PCA and ED. Here, we used beside the common PCA also local linear embedding (LLE), metric multi-dimensional scaling (metric MDS) and isomap. All methods reduce the dimensionality in the original data set, but use different algorithms. For LLE and isomap, we used the RDRToolbox package, for PCA we used the R function prcomp and for metric MDS we used the cmdscale function with k set to 2, in order to create 2-dimensional plots of the results.

Analysis of mandible activity

Mandible activity was auralized with loudspeakers and during recordings, it was directly noted, whether mandible activity was increased during stimulation with CHC extract. For demonstration purpose, we analyzed the extracellular recordings by calculating a running median with window size 9. Recordings with median values above a specific threshold (in the presented example 0.023 V) after stimulation with a CHC extract were classified as increased mandible activity upon stimulation. The data analysis was done in R v3.4.3 (R Core Team, 2017).

Analysis of colony odor extracts

In order to find out, if the chemical variability is different for the three used lab colonies, we tried to chemically analyzed the CHC profile extracts which we used for stimulations during experiments. We analyzed them via gas-chromatography mass-spectrometry (GC-MS). We added single droplets of pentane to the empty glass vials, where small amounts of CHCs should still stick to the inner wall. Using a vortex machine on a low speed level, we resolved the remaining CHCs in pentane.
Then we concentrated the sample under a constant stream of nitrogen so that only approximately 10µl remained. From this amount, we manually injected 1µl of the samples into a GC (Trace GC Ultra coupled to a DSO II mass spectrometer, Thermo Scientific). The CHCs in the samples were separated on a fused silica column (Optima-5-MS - 0.25µm, 30m x 0.25mm, Macherey-Nagel GmbH & Co. KG) with helium as carrier gas (1.2ml/min). Chromatograms were recorded with Xcalibur software 1.4 SR1 (Thermo Scientific) using the following temperature program: 1) constant temperature of 70°C for 1 min, 2) increasing temperature at 30°C/min to 200°C, 3) increasing temperature at 3°C/min to 290°C, 4) constant temperature of 290°C for 5 min.

**Results**

In total, we prepared 383 ants and from these, 181 had a well stained antennal lobe (Figure 4.1). We saw spontaneous activity in 54 ants and from these 37 had stimulus related responses. We recorded changes in cytosolic calcium concentration in PNs of 16 ants, however only 2 individuals could be analyzed.

![Fig. 4.1 Example of a left AL of a worker ant *Camponotus floridanus*. Single stained glomeruli are visible as bright oval structures.](image)
Investigating the sequence of stimulus-evoked activation based on line scan recordings

In order to illustrate, how line scan recordings while stimulations with odorants should look like, we here first present recordings with octanol stimulations where we focused only on a small part of the antennal lobe (a line through several visible glomeruli). Figure 4.2a shows three example recordings during octanol stimulations and the vertical pixel line is the measured line through the antennal lobe and the time runs from top to bottom on the y-axis. The pixel intensity illustrates the fluorescence intensity and the brighter a pixel, the higher the fluorescence. Fluorescence change of a pixel over time indicates neuronal activity.

Fig. 4.2 Three example recordings for three repeated stimulations with octanol \((10^{-2})\). Each of the 1270 horizontal lines represent the intensity values of the specified pixel line at a certain time point. The brighter a pixel the higher the fluorescence intensity. An increase of brightness over time indicates an increase in cytosolic calcium concentration. (a) Raw Fluorescence values for the three different recordings. (b) Relative changes of fluorescence for the same recordings as in a. Note: the y-axis (time) runs from top to bottom. Red vertical bars indicate the time points of octanol stimulations. The sampling rate for these recordings was 165 Hz.
Based on our calculation of correlation coefficients on these three recordings, we reveal six distinct ROIs, because in several separated regions we find neighboring pixels with correlation coefficients above 0.2 (Figure 4.3). Each ROI presumably corresponds to one glomerulus. The extracted time traces from these glomeruli are depicted in Figure 4.4. Two glomeruli show stimulus related responses, whereas the other glomeruli show spontaneous activity, but no response to the presented odorant.

**Fig. 4.3 Identification of ROIs.** Correlation coefficients for neighboring pixels can range between 0 and 1. A value above 0.15 was used as a threshold to identify pixels that belong to the same responding unit. The mean correlation coefficient was calculated from 15 pixels before and 15 pixels after the current pixel. Consecutive pixels with high correlation coefficient values above 0.15 are considered as one ROI. ROIs with a pixel length of more than 50 were manually checked and split into several parts. Here, we have 6 distinct ROIs and their ranges are indicated by the blue vertical bars.
Fig. 4.4 Time traces of six glomeruli over three recordings during stimulation with 3 pulses of octanol (10^{-2}). Only glomerulus 3 and 4 show stimulus related responses (increasing fluorescence shortly after the onset of stimulation with octanol), while the other glomeruli show only spontaneous activity. Red horizontal bars indicate stimulus onset of the octanol pulses.

Since we only focused on a small part of the AL, we only imaged from few glomeruli. From these glomeruli, two responded consistently to the presented octanol pulses. The extracted response latencies for the two glomeruli towards each of the octanol pulses are variable (Figure 4.5). For the three example recordings with 3 pulses of octanol each, we find no fixed sequence of the two glomeruli, however, when focusing only on the first pulse per recording, we find that ROI 3 is responding earlier than ROI 4. The response latency of ROI 3 towards the first octanol pulse is relatively constant with less than 250ms after stimulus onset. In contrast, the response latency of ROI 4 towards the first octanol pulse is variable ranging from almost 750 ms to a bit more than 250 ms.
Fig. 4.5 Response latencies of the two selected glomeruli vary. (a) Response latency above the threshold of 2 times standard deviation from baseline activity of 2 different glomeruli to the first out of three pulses of octanol. ROI 3 is always responding earlier than ROI 4. (b) Response latencies of the same glomeruli as in (a), but based towards the second and third octanol pulse. Here, latencies were calculated based on 95% maximum fluorescence change. In only 3 out of 6 cases, ROI 3 is responding earlier than ROI 4. Note: Shown are the response latencies of ROI 3 (orange) and ROI 4 (blue) from Figure 4.4.
For the recordings during stimulation with nestmate and non-nestmate extracts, we imaged the fluorescence intensity from many glomeruli. The 512 pixels are spread among many glomeruli stained with different intensities (Figure 4.6a). A correction for the different baseline intensity removes the differences between pixel intensities and makes fluorescence changes better visible (Figure 4.6b).

**Fig. 4.6 An example line scan recording during stimulation with a dummy loaded with non-nestmate extract.** In total 512 pixels were continuously recorded (x-axis) over 10 seconds (y-axis) with a sampling rate of 65Hz. Red line indicates stimulation time. (a) The raw data visualizes the differently strong fluorescence of glomeruli. The brighter the pixel intensity, the higher the fluorescence. (b) Same recording as in (a), but baseline corrected and adjusted to relative fluorescence change. Here, the gray values reflect changes in fluorescence. However, also movements becomes visible, for example at around time of 4 seconds.
Based on our calculation of correlation coefficients on this recording, we reveal 14 distinct ROIs, because in several separated regions we find neighboring pixels with correlation coefficients above 0.37 (Figure 4.7a). Each ROI presumably corresponds to one glomerulus. The extracted time trace from one of those glomeruli is depicted in Figure 4.7b. Shortly after dummy presentation, the glomerulus shows an increase in cytosolic calcium concentration.

Fig. 4.7 Identification of ROIs and time trace of one glomerulus during stimulation with nestmate extract. (a) Correlation coefficients for neighboring pixels can range between 0 and 1. A value above 0.37 was used as a threshold to identify pixels that belong to the same responding unit. The mean correlation coefficient was calculated from 12 pixels before and 12 pixels after the current pixel. Consecutive pixels with high correlation coefficient values above 0.37 are considered as one ROI. ROIs with a pixel length of more than 50 were manually checked and split into several parts. Here, we have 14 distinct ROIs and their ranges are indicated by the blue vertical bars. (b) Time trace of a single ROI (ROI 12 which is likely a single glomerulus). The upper graph shows the raw (unfiltered) data and the bottom plot shows smoothed data (via running median, k=20). The red lines indicate the time of dummy stimulation.
Although, the microscope settings remained the same during the whole imaging session, the regions that are represented by the imaged pixel line shifted. This is illustrated in Figure 4.8, where 4.8a shows the second and 4.8b shows the 34th line scan recording. Since we could not get rid of the shift, we were not able to compare the recordings without impact of the shift. We focus on another research question and change the recording method (from line scans to frame recordings).

Fig. 4.8 Comparison of an early and a late recording (2nd vs 34th) from the same calcium imaging session reveals changes of intensity values over time. (a) Raw data of the first line scan recording taken during stimulation with nestmate extract. (b) Raw data of the last line scan recording taken during stimulation with non-nestmate extract. Red lines indicate the dummy controlling digital output pulses. Note: Some slight movements are visible in both of the recordings.
4.4 Results

Variability in response patterns

Here we present physiological and data analysis approaches to investigate behavioral and neuronal responses to stimulations with nestmate and non-nestmate CHC profiles. Figure 4.9a shows the result of glomerulus segmentation based on calcium imaging data using the automated algorithm for KNIME (Berthold et al., 2008) using the ImageBee plugin (Strauch et al., 2013). Glomeruli differed in their response properties (Figure 4.9b). In some glomeruli, the cytosolic calcium concentration increased only in response to a stimulation with nestmate or non-nestmate extract (green and blue traces, respectively), but not in response to the control stimulation with pentane on the dummy (red traces), demonstrating that single glomeruli respond to the components of the extract and not to other olfactory, thermal or mechanical stimulus components of the dummy.

Fig. 4.9 Automated segmentation of imaging data and corresponding time traces for different stimuli. (a) Antennal lobe overview map based on neighboring pixels that highly correlate over time. Pixels with the same color represent regions with equal response properties which likely correspond to glomeruli. (b) Calcium signals in PNs of different glomeruli over time in response to a CHC profile presented via a movable dummy to the antennae of an ant. Each plot represents the mean activity responses of a glomeruli during stimulation with different nestmate extracts (green traces; n=12 stimulus repetitions), different non-nestmate extracts (blue traces; n=12 stimulus repetitions) and pentane control (red traces; n=4 stimulus repetitions). Traces show mean and shaded areas show the standard deviation. Red horizontal bars indicate the presentation of the dummy. Glomerulus numbers are depicted in gray boxes on the right side of each plot. Glomeruli 7 and 20, for example, respond to the CHC extracts, while there is no response to a dummy with pentane.
To compare the glomerular response patterns evoke by the different stimulations, shown in Figure 4.9b, we used different techniques to reduce the many dimensions resulting from the 20 glomeruli (Figure 4.10). Each single stimulus presentation is now reduced to a single point in a 2-dimensional space. In all methods, but PCA, the 2nd to 4th control stimulation (pentane) evoke more similar response patterns than the 1st control stimulation. In metric MDS, LLE and isomap, the areas of points for stimulations with nestmate and non-nestmate extracts are spatially overlapping, while in the PCA, the areas are almost distinct. Hence, for the given data set of one ant, PCA seemed to separate stimulations with nestmate from non-nestmate extracts best in comparison to metric MDS, LLE and isomap.

Fig. 4.10 Dimensionality reduction techniques reveal differences between activity patterns evoked by nestmate and non-nestmate extracts. All plots are based on the same data set during stimulation with different nestmate extracts (green; n=12 stimulus repetitions), different non-nestmate extracts (blue; n=12 stimulus repetitions) and pentane control (red; n=4 stimulus repetitions). (a) Principal component analysis (PCA), (b) metric multidimensional scaling (metric MDS), (c) local linear embedding (LLE), (d) and isomap. Stimulations with nestmate (NM) and non-nestmate (nNM) extracts are depicted in green and blue, respectively. The control stimulation with a black dummy (only pentane) is depicted in red. Numbers after extract names indicate trial number of the specific stimulus.
Furthermore, we wanted to know, if we can still analyze the chemical composition and quantify the components of the CHC extracts, although we used the whole extract for stimulation. This would open the possibility to compare the chemical variability of the CHC extracts which are used during calcium imaging. Indeed, we revealed some chromatograms in such a quality that it would be possible to investigate the quantity of the single peaks. For illustration, we present for two different lab colonies an example chromatogram (Figure 4.11). The CHC extracts contain mainly the low-volatile CHCs, which in our case elute after more than 25 min. Each peak presumably corresponds to one or a few similar hydrocarbons.

Fig. 4.11 Chromatograms of cuticular washes from single workers from two different colonies. Depending on the size of the molecules, different components have a different retention time. The chemical profiles of ants are composed mainly of CHCs, which are low-volatile and elute after more than 25 min. The relative concentrations of single CHCs differ between the two samples. Samples are from two different single worker of colony D544 (a) and colony D360 (b), respectively.
Electrical recordings of mandible muscles during calcium imaging

We used electro-physiological recordings of the mandible muscles as a monitor for a behavioral response of the ants due to a presentation of a CHC extract. During pilot experiments, fixed ants in a holder, but with free mandibles, reacted aggressively with mandible opening when stimulated with hexanol. This mandible opening induced muscle spikes. For the imaging preparation, mandibles needed to be immobilized. After fixating the mandibles, the ants could not move their mandibles anymore, however, hexanol stimulations still evoked muscle spikes. Thus, mandible muscle recordings are suited to monitor intended mandible opening in fixed ants. Therefore, we recorded mandible muscle activity during calcium imaging. However, only for one ant, we recorded both mandible muscle activity and neuronal activity in the AL. During the imaging recordings of that ant, an increase in mandible muscle activity while stimulation with nestmate extracts was measured in 3 out of 12 cases (Figure 4.12a), but in none out of 11 cases while stimulation with non-nestmate extracts (Figure 4.12b).

Fig. 4.12 Recordings of mandible muscle activity during CHC extract presentations. Mandible muscle activity was recorded in a single ant during simultaneous calcium imaging and stimulation with nestmate (NM) extracts (a) and with non-nestmate (nNM) extracts (b). The corresponding calcium imaging data are presented in Figure 4.9. Red vertical bars indicate muscle spikes. The dummy loaded with CHC extract was presented 1 s after start of electrical recording and lasted for 2 s (red horizontal line).
Discussion

In the present study, we investigated whether the temporal patterns of activated glomeruli in the AL is different for nestmate and non-nestmate extracts. Therefore, we used high temporal resolution calcium imaging, which is achieved through recording of a single line instead of frames. Furthermore, we investigated the variability in glomerular activity patterns during repeated nestmate and non-nestmate extract stimulations. Simultaneously, we recorded mandible muscle activity in order to have a behavioral read-out for the imaged ant.

We only presented preliminary data, because most recordings could not be analyzed due to shift in the preparation and movement of the animal, both which we were not able to eliminate so far during the restricted time of the study. Therefore, this chapter is meant to help designing physiological studies on the neuronal basis of nestmate recognition in ants.

However, we successfully recorded from mandible muscles during calcium imaging, and find that increased mandible activity may not be related to aggression.

Temporal coding for colony odors

Differences in CHC concentrations between different colonies should be visible in the strength of ORN responses and hence a higher concentration of a component should be also visible as a shortened response latency for ORNs that detect that component (Szyszka et al., 2014). Accordingly, PNs may also respond with a specific temporal pattern depending on the colony origin of the CHC profile. However, in the study by Brandstaetter and Kleineidam (2011), no such investigation was possible, since the sampling rate was only 4Hz. Unfortunately, we could not analyze our line scan recordings due to a shift of recorded tissue over the time of experiments. Stable line scan recording would enable us to search for a sequence of activated glomeruli that is typical for recordings during nestmate stimulations. However, we would assume that the few fastest responding PNs are sufficient for the olfactory system to extract the important information of the stimulus. Support for this comes from a study in honey bees, where it was shown that the mushroom body output neurons (MBONs) already reached a maximum of separation of different stimuli between 84-120 ms after stimulus onset, which is 26 to 133 ms earlier than the maximum separation at the AL output ensembles (PNs) two synapses earlier in processing (Strube-Bloss et al., 2012). This would be also in-line with the fast behavioral response towards non-nestmates found in ants. In C. floridanus, workers respond with opening mandibles within 200 ms (Fischer, 2013) after start of presentation of a non-nestmate extract. In a related species, in C. aethiops, workers respond similarly fast (Stroeymeyt et al., 2010).
We had difficulties with the preparations due to movements in the brain of the prepared ants. Over time the brain shifted so that we recorded in different regions during the time course of the experiments of single ants. Hence, potential differences between stimulations could arise from differences in the glomerular activity patterns or rather due to the fact that we recorded from slightly different areas. When recording line scans and if there is movement or a shift over time in the brain, we acquire data from different regions in the antennal lobe and it is impossible to reveal the fluorescence changes of the original imaged region.

Similar problems with shift were also encountered in experiments with *Drosophila melanogaster* (G. Raiser, pers. comm., Jan 2018), and to our knowledge, could not be resolved so far. What we call shift, is actually a change of the raw fluorescence intensity pattern of one brain over the time course of experiments. The shift may result from slight brain movement in x- and y-direction and/or in z-direction. The latter are up or down movement of the brain with respect to the objective of the microscope. A possible reason for shift in general is that the brain may dessicate and shrink. However, we used KwikSil in order to fixate the ants’ brains, which should minimize or even suppress dessication. In a study in honey bees, doing also line scan recordings, no problems with shift were encountered during calcium imaging (Paoli, pers. comm., Jan 2018). In this bee study, another microscope system was used than in the present study and it might be that the shift in our study was due to technical issues, where the scan line was perhaps shifting and not the preparation itself.

We recommend to solve the problem with the shift first, for example, by testing another microscope system or, if this is not the reason, by fixing the AL in a specific position so that even when parts of the brain sag, the AL remains in the same position and distance to the objective of the microscope. Alternatively, line scan recordings should be only done when comparing between recordings that are timely close to each other (about 5 min), but not between recordings that were recorded over several hours. The line scan recording is also a tool well suited for research questions that do not compare between different recordings, but within recordings. An exemplary question could be, whether the response in a glomerulus is in each part similarly or some parts respond earlier and others later.

Due to the mentioned problems and due to the restricted time for the study, we did not further try to record line scans, but rather used frame recordings and focused on the variability of response patterns.
Variable glomerular activity patterns based on stimulations with CHC profiles

Repeated stimulations with nestmate and non-nestmate CHC profiles reveal highly variable spatial patterns (Brandstaetter and Kleineidam, 2011), which is not common for encoding of general odorants in other insects (Carlsson et al., 2002; Joerges et al., 1997) and in ants (Zube et al., 2008). The higher variability in encoding of nestmate extracts compared to non-nestmate extracts observed in the study by Brandstaetter and Kleineidam (2011) may result from a different variability in the chemical profiles of the the colonies. In general, colonies of the species C. floridanus consist of one single mated queen (Gadau et al., 1996). As mentioned above, our laboratory colonies are feed with the same diet and had the same nesting material. Hence, the difference in their CHC profiles should be mainly based on the genes and the current composition of the colony. We assume that within our lab colonies the variability in CHC profiles is lower than in colonies in the natural environment. In behavioral experiments with the same C. floridanus laboratory colonies as in the present study, we find that differences in CHC profiles between workers from different colonies, are sufficient for individuals to behaviorally discriminate between nestmates and non-nestmates (Pink, 2016). We wanted to exclude the differences in the chemical variability between different lab colonies as possible reason for the different variability in encoding of the colony specific CHC extracts by using ants from two different colonies as the imaged ants. Ants from the colony of origin for each imaged ant served for creating nestmate extracts and ants from the other colony served for creating non-nestmate colony. In the present study, we show that even though, we used up the whole CHC extracts for stimulations during the experiments, the remaining CHCs on the inner glass walls were not always, but sometimes sufficient to reveal nice chromatograms. If a tiny amount of extract would remain in the glass vials, presumable always good chromatograms could be revealed. Hence, a chemical analysis of the CHC extracts used for stimulations in calcium imaging experiments could be used to relate different variabilities in the neuronal encoding of nestmate and non-nestmate CHC profiles to differences in quantitative variability of the stimulus samples.

We assume that the CHC profiles within our three laboratory colonies are chemically similar variable and that the high variability in encoding of nestmate extracts arises due to specific features of the neuronal network. Higher order feedback neurons, such as the centrifugal neurons, innervate the AL and are thought to have feedback and modulatory functions and biogenic amines have been found in centrifugal neurons in several species, such as locusts, moths, bees (Hansson and Anton, 2000). From a study in honey bees, the authors concluded that the mushroom body output neurons may be fast enough to provide feedback to the ongoing computations in the AL (Strube-Bloss et al., 2012). Hence, the higher variability in the encoding of nestmate extracts observed in the study by Brandstaetter and Kleineidam (2011) may be a result from rapid feedback induced by
AL neurons or by modulatory neurons of higher brain areas. Accordingly, already an early stimulus-evoke response in the AL may lead in a higher brain area to the recognition of either nestmate or non-nestmate. If a nestmate is recognized, the nestmate odor may elicit an internal modulation driving antennal lobe network into a specific state, leading to a refinement of the recognition. The late AL response after the recognition may reflect such refinement, allowing to discriminate also between different nestmates. If line scan recordings would have worked in the current study, we would be able to actually investigate, when the difference in variability in nestmate and non-nestmate encoding arises. If a higher variability in encoding of nestmate extracts would arise 'late' after stimulus onset (after more than 150ms), it supports that feedback is inducing the variability.

Future experiments, addressing if a late and variable stimulus related activity in the AL has a biological significance, such as being important for template acquisition, late stimulus related responses could be masked by presentation of another (neutral) odorant which is not related to nestmate recognition. If this infers with the individuals’ ability to become familiarized to previously foreign CHC profiles, it supports that a late stimulus related activity in the AL is important for template reformation.

In psychology there is an effect called the ‘out-group homogeneity effect’. It refers to the fact that out-group members are perceived more similar to another than in-group members to another (Mullen and Hu, 2010). It could be similar in ants, where the AL circuitry may be optimized to detect the nestmate CHC profiles and as a result, differences in the nestmate CHC profiles may be more amplified than differences in non-nestmate CHC profiles. This could be possible, since ants usually only encounter their nestmates (in-group members) and have frequent contact with them. This in turn possibly increases the representational space for the frequently experienced (familiar) CHC profiles and accordingly, individual ants may be able to also distinguish between different nestmates.

**Movements during frame recordings**

Although, we could used the movement correction for frame recordings (Strauch et al., 2013), when there was movement in x- and/or y-direction, we could only record a small data set. The main reason was that we had many preparations with pumping-like movements also in z-direction, which resulted in acquiring data from different layers during the movements. Hence, fluorescence changes due to the movement and due to a stimulation cannot be distinguished. It seemed that as we got more and more experience in preparation of the ants, the more lively the ants became and we experienced stronger movements during imaging. Calcium imaging in general only works in alive individuals. In order to avoid that the imaged animals move during imaging sessions, philanthotoxin, a poison from the bee-killer wasp *Philanthus triangulum* (Fabricius, 1775), was used in a previous imaging study (Fiala et al., 2002). Philanthotoxin is an antagonist of ionotropic glutamate and nicotinic acetylcholine receptors and could be also used to reduce
brain movement in ants during imaging. However, it is important to verify that Philanthotoxin does not interfere with the olfactory processing in the ant’s brain.

**Analysis tools for calcium imaging data**

Calcium imaging data are often analyzed using Principal component analysis (PCA), a technique for dimensionality reduction. PCA is a linear transformation method converting the data into a new coordinate system with the greatest variance of the data being on the first coordinate (principal component 1), the second greatest variance on the second coordinate orthogonal to the first coordinate and so on. Since the new coordinates are no real-system produced variables anymore, the interpretability of the data is lost. Here, we also used other methods that do not project the data onto an ’intelligent’ set of directions, as in PCA, but look for a manifold close to the data, project the data onto the manifold and unfold the manifold for interpreting the data representation (Sorzano et al., 2014). As such, we used MDS (a linear technique) as well as LLE and Isomap (both non-linear dimensionality reduction techniques), each resulting in a different representation of the imaging data (Figure 4.10). Based on the limited set of data we recorded, we will not draw conclusions about, which of the methods is best suited to analyze the data. We rather want to emphasize that different kinds of analysis tools may give us insight into the black box of the processing of odorants and their mixtures.

**Recording of mandible muscle activity as measure for behavior**

We have shown an example of recorded mandible muscle activity during the presentation of nestmate and non-nestmate CHC extracts, while we conducted an imaging session in an ant. Interestingly, even if this is just from one individual ant, we recorded increased mandible muscle activity only during presentations of nestmate CHC extracts. This is contradictory to our original idea to record mandible muscle activity as a measure for aggression. As a possible explanation for this, we suggest that the ants during the imaging process are far away from being in a social context. Ants prepared for calcium imaging experiment, did not have normal social contact to nestmates since several hours. As a possible result, they might have a very low propensity to show aggressive behavior in general as was shown for freely moving ants (Kleineidam et al., 2017). The question arises how a freely moving ant would behave, when it is stimulated with nestmates and non-nestmates several times in an alternating manner as we did during the imaging session? The results of an experiment investigating this question is that the ants are mostly aggressive against non-nestmates and almost always not aggressive against nestmates. Over time, the probability of aggressive responses against non-nestmates decreased, while the probability of aggressive responses against nestmates remained low (Pink, 2016). Furthermore, unpublished experiments with
tethered ants having different degrees of freedom to move, also show differences in discrimination behavior between nestmate and non-nestmate extracts (Karl & Kleineidam, unpublished). The absence of a social context and the fixation of the ant in a holder for several hours may lead to low propensity of an aggressive response towards non-nestmate extracts. The reason, why the mandible activity was increased during some of the stimulations with nestmate extract, but never with non-nestmate extracts may be that the perceived nestmate odor triggered a behavioral response, such as trying to escape from the situation for a reunion with its nestmate. Hence, the mandible muscle activity may not be opening mandibles per se, but rather straining of the individual. We show that the behavioral monitor generally works, however, it seems to be not suited in combination with nestmate and non-nestmate discrimination in restrained conditions and in an non-social context.

Conclusion

In this chapter, we investigated the sensory processing of olfactory information about colony membership in the AL of the carpenter ant *C. floridanus*. Although we were not able to gain a sufficient data set that allows a data analysis with respect to our hypotheses, we are confident that future work will solve the major issue with movement. In our experiments, the ants survive for several hours and we also had no issues with bleaching of the dye. We predict that 50 and more recordings of one ant are feasible.

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In this doctoral thesis, I investigated different levels of nestmate recognition in ants. 1) Chemical strategy by a parasite to circumvent the nestmate recognition of its host: In chapter 1, I investigated the CHC profile produced by a social parasite (*Megalomyrmex symmetochus*) and revealed the chemical strategy of how the parasite integrates into host ant colonies. The results indicate that *M. symmetochus* uses mainly offensive chemicals to maintain an association with its host colony. 2) Perception of the label: In chapter 2, I asked, whether *Camponotus floridanus* learns a new colony odor by a widening of the template or by the generation of multiple templates. I found that these ants have multiple, experience-dependent templates and I provide a testable hypothesis on how label-template matching could be implemented in the brain. 3) Decision for action: In chapter 3, I investigated how the current social context affects the propensity of individuals of *Formica rufa* to respond aggressively. I could show that recent social contact maintains a high propensity of aggression in workers, while the aggression propensity of socially isolated workers decreases with time. 4) Sensory processing in the AL: In chapter 4, I propose an opto-physiological approach to investigate the neural coding of nestmate and non-nestmate CHC profiles. Using calcium imaging, I recorded spatio-temporal activity patterns in the AL elicited by nestmate and non-nestmate CHC profiles and I developed a method to observe a behavioral response of fixed ants during calcium imaging.

Considerations on the analysis of CHC profiles

In the past, the chemical composition of the upper layer from the cuticle has been investigated in many ant species (Martin and Drijfhout, 2009a). However, the complete compositions of ants’ CHC layers may still be unknown, as until a few years ago GC-MS could not detect CHCs with more than 34 C atoms. Only recently, high-temperature columns for GC-MS have become available which allow to detect hydrocarbons with chain lengths of more than 34 C atoms (Akino, 2006). As an example, in *Formica truncorum* more than 50% of the overall CHCs have more than 34 C atoms (Akino, 2006), contradicting a previous study which claimed that *F. truncorum* contains no CHCs longer than 31 C atoms (Boomsma et al., 2003). Therefore, re-investigating the CHC profiles of the already investigated ant species may reveal so far unknown CHCs. Accordingly, the CHCs of the host
Sericomyrmex amabilis and its social parasite Megalomyrmex symmetochus that we studied in chapter 1, could have more CHCs that we did not detect. The largest CHC that we detected, had a chain length of 37 C atoms in the host *S. amabilis* and 39 C atoms in the parasite ant *M. symmetochus*. Although we used a high-temperature column, it is possible that there are CHCs in the parasite profile that we did not detect with our temperature regime. However, even if there would be longer chained CHCs in the profile, I assume that these CHCs are so low-volatile that they are likely not detected by a receiver ant’s antennae. Additionally, a ‘sponge’ function has previously been suggested to long chained alkenes which themselves may not be detected, but may absorb traces of lighter CHCs (Lambardi et al., 2007).

**Circumventing nestmate recognition system**

Studying social parasites and their host ant colonies (chapter 1) or studying (artificially) mixed-species colonies provides insight into the cues used in nestmate recognition. Social parasites are able to enter social insects colonies and live in their nests without being recognized as non-nestmates. Some parasites present the same CHCs as their host by synthesizing host-specific CHCs (Akino, 2008) or they acquire host CHCs by passive or active contact with nest material and host ants (Akino et al., 1999). Other parasites produce a cuticular layer which is poor in CHCs (in quality and quantity), thereby presenting only few recognition cues (Nehring et al., 2015). This strategy is referred to as the olfactory insignificant strategy. We find support for the olfactory insignificant strategy in the host parasite system (chapter 1) because the number of CHC types and their quantity is significantly lower in parasites than in host individuals. However, critical evidence for the olfactory insignificant strategy is still lacking (see below).

Parasites which would be recognized by the host due to non-matching CHC profiles, may use offensive chemicals to integrate into the host colony. This strategy is referred to as weaponry strategy. Offensive chemicals are used by the parasites to distract the nestmate recognition system of the host, acting as appeasement or repellent substance (d’Ettorre et al., 2000; Mori et al., 2000) or eliciting submission (chapter 1). Hence, the chemical profiles of individuals are composed not only of the low-volatile CHCs, but also of more volatile compounds. In the ant *Formica selysi*, head-space samples reveal the volatile Dufour’s glands constituents (in particular undecane) (Errard et al., 2008). In the host-social parasite system used in our study (chapter 1), head space samples from the parasite *M. symmetochus* also reveal volatile components (alkaloids) (Adams et al., 2013). Hence, in our experiments, *S. amabilis* workers from non-parasitized host colonies may detect the volatile alkaloids and/or the parasite derived CHCs. In the following, I propose two complementary experiments in order to test why *S. amabilis* workers from non-parasitized host colonies are aggressive against the parasite *M. symmetochus*, while workers from parasitized colonies are not aggressive neither to their own nor to foreign parasites.
1) The first experiment tests the hypothesis that the CHC profile of the social parasite *M. symmetochus* is insignificant. In the proposed experiment, the parasites will be prevented to spread their alkaloids in the surrounding and on their own body surface. This could be achieved, e.g. by sealing their abdomen. After a sufficient amount of time, where all the remaining volatile alkaloids are evaporated, the parasites could be presented to workers from non-parasitized host colonies. The insignificance-hypothesis would be supported, if the workers from non-parasitized host colonies do not show aggression against the manipulated parasites. If workers from non-parasitized host colonies would show aggression against the manipulated parasites, it would support the alternative hypothesis that the CHC profile is not insignificant, but shows sufficient olfactory cues to be detected by a host.

2) The second experiment tests the hypothesis that *S. amabilis* worker from parasitized host colonies are familiarized to the alkaloids and only *S. amabilis* workers from non-parasitized colonies (not familiarized to alkaloids) react with aggression when detecting alkaloids. In the proposed experiment, workers from non-parasitized host colonies will be artificially familiarized to the parasite-specific alkaloids by presenting them the alkaloids in their nest environment over several weeks. Then, the *S. amabilis* workers from these pre-exposed colonies will be tested against real parasites *M. symmetochus* workers. The familiarization hypothesis would be supported, if the pre-exposed *S. amabilis* workers are not aggressive against parasites.

**One or several templates for nestmate labels?**

Freshly emerged adult ants (callows) learn during their first days how their nestmates smell and accordingly develop a template for their own colony odor (Lenoir et al., 1999). Newey (2011) proposed that individuals have two templates, one for their own CHC profile (by self referent matching) and another one for their nestmates’ CHC profiles. Presumably, individuals from the same colony have different templates based on their individual experience (Esponda and Gordon, 2015). Based on their template(s), ants recognize other ants as nestmates or as non-nestmates. Since the colony odor can change over time (Lambardi et al., 2004; Vander Meer et al., 1989), the templates need to be updated accordingly. Several studies showed that the template indeed can be updated and that the current template is either widened or several templates are generated (Guerrieri et al., 2009; Leonhardt et al., 2007; chapter 2). In our study of the host-social parasite system, it is likely that workers of parasitized host colonies have generated an additional template for own parasites in the nest (chapter 1), because host ants of parasitized colonies did not show discrimination between nestmates and own parasites based on aggression behavior. However, after several years of amiable association, the host workers sometimes kill the parasites (Boudinot et al., 2013; Bruner et al., 2014). The causes and mechanisms for this change from tolerance to resistance behavior against their parasites are so far unknown (Bruner et al., 2014). I propose the following, possible explanation: The recent cohort of host
ants (or parts of it) did not develop a template for parasites due to a spatial separation. Correspondingly, some host ants may not have been exposed to the parasite-specific labels or volatile alkaloids. Alternatively, the host ants’ response threshold may have changed, e.g. due to changes in the colony reproductive state (d’Ettorre et al., 2004), resulting in a shifted response threshold and eventually leading to aggression towards their parasites.

New nestmate templates can be generated and used for nestmate recognition (chapter 2). Can a formed nestmate template also disappear? There are indications that templates can last for a long time. In experiments with two different ant species, which were raised together, but separated after three months, individuals were still not aggressive against the other species even after one year of separation (Errard, 1994). In lab colonies, workers can become old (more than a year). However, workers from colonies in the natural environment, may only live for several weeks or a few months. Hence, the template for former nestmate labels would disappear due to the turn-over of workers within several months. Alternatively, a template, when not reinforced by current experience, may disappear by forgetting or extinction. So far, it remains to be investigated to what extent forgetting or extinction of former templates influences the acceptance range on the level of individuals and on the colony level.

Mechanism for label-template matching and learning of novel labels

Individual ants frequently encounter nestmate labels and only rarely encounter non-nestmate labels. Hence, individuals may learn nestmate-specific labels and everything that smells similar - but not identical (e.g. conspecific non-nestmate labels), is recognized as foreign. Two hypotheses arise on how ants are able to respond aggressively against non-nestmate CHC profiles without the need to learn how non-nestmates smell:

1) CHC profiles may have innately an aversive meaning, and experiencing nestmate CHC profiles renders their meaning specifically to something positive. Support for an innate negative meaning of CHC profiles comes from the observation that encounters between heterospecific non-nestmates induce aggression and heterospecifics carry labels that are distinct from nestmate labels which are different in quantity and quality of CHCs, (Errard, 1994; Stuart, 1991; Tanner, 2008; Tanner and Adler, 2009). Moreover, artificial mixtures of synthetic hydrocarbons induce aggression (Akino et al., 2004; Greene and Gordon, 2007).

2) Alternatively, CHC profiles may have innately a neutral meaning and nestmate CHC profiles become positive through learning, while similar, but not identical CHC profiles receive a negative meaning. Support for this hypothesis comes from the observation that callow workers are not aggressive against workers from any colony (Carlin and Hölldobler, 1983; Errard, 1994; Fielde, 1903) and become aggressive when they are several days old and have learned the own colony odor.
It is not known which of the two hypotheses is true, but we can make some assumptions on what modifications in the ant’s olfactory processing might happen due to a frequent exposure to nestmate labels:

1) It may lead to sensory adaptation which would result in no or reduced nestmate label-evoked response in the corresponding ORNs and in their post-synaptic partners in the AL. A sensory adaptation was previously proposed for the ant *Camponotus japonicus* as mechanism for nestmate recognition (Ozaki et al., 2005). A particular sensillum (basiconic sensillum) was described which is only active when non-nestmate CHC profiles are presented, but is silent when nestmate CHC profiles are presented. Hence, only information about non-nestmate labels would be transmitted to the brain. Adaptation can occur on a time scale of hundreds of milliseconds (Martelli et al., 2013), but can also be slow, requiring an exposure to the odorant prolonged or repeatedly over seconds to minutes (Guo and Smith, 2017). However, in behavioral experiments, where ants were consistently exposed to non-nestmate odors, individuals showed reduced aggression only after more than 2 hours, indicating that the mechanism is no adaptation but rather a process in the central nervous system (Leonhardt et al., 2007). Additionally, results from another study in ants showed that sensilla basiconica actually respond to both extracts of non-nestmate but also of nestmate CHC profiles (Sharma et al., 2015). The ‘nestmate sensillum’ hypothesis would not be able to explain the ants’ ability to discriminate between nestmates of different tasks (Bonavita-Cougourdan et al., 1993; Greene and Gordon, 2003). Hence, the hypothesized ‘nestmate sensillum’ is unlikely and the results of the study by Ozaki et al. (2005) could be explained by an accidental recording of muscle spikes that corresponded to behavioral rather than to sensory (ORN) responses.

2) Frequent exposure to nestmate CHC profile may lead to non-associative learning during which a repeated or prolonged exposure to the stimulus (e.g. nestmate CHC profile) is not reinforced by a reward or a punishment. Non-associate effects are habituation, the most basic form of learning, and latent inhibition. In *Drosophila*, a mechanism for short- and long-term odorant-specific habituation was found, in which a reduced behavioral response to an odorant is a result from an increased GABAergic transmission from LNs to PNs (increased inhibition) which reduces the PN responses (Das et al., 2011). However, important to note, in ants, extracts of nestmate CHC profiles induce similar strong overall PN responses as extracts with non-nestmate CHC profiles (Brandstaetter and Kleineidam, 2011). Hence, I reason that the model for short- and long-term odorant-specific habituation is not likely for nestmate recognition, however, other non-associative effects may be possible (see below).

3) Frequent exposure to nestmate labels may also lead to associative learning, when the stimulus (e.g. nestmate CHC profile) is reinforced by a reward (e.g. being groomed, fed or tactically stimulated at the antennae). Associative learning, where an odorant (the conditioned stimulus: CS) is trained to be associated with sucrose, a rewarding reinforcer (the unconditioned stimulus: US), is possible in
ants (Guerrieri and d’Ettorre, 2010). Even though, a positive or negative reinforcer is not needed for template reformation when there is a prolonged exposure to a non-nestmate CHC profile (Leonhardt et al., 2007), associative learning may be important for fast acquisition of new templates during time as a callow ant or when the colony odor changes.

Both, associative and non-associative learning result in changes of odorant-evoked response patterns in the AL (Daly et al., 2004; Faber et al., 1999; Fernandez et al., 2009; Locatelli et al., 2013; Rath et al., 2011), which may lead to an optimization in the encoding of variable and changing nestmate CHC profiles leading to an enhance discriminatory ability in higher brain centers. This would also support, why a higher variability in activity patterns evoked by nestmate labels has been found (Brandstaetter and Kleineidam, 2011). Plasticity due to non-associative and associative learning has also been found in higher order neurons in the brain of honey bees (Strube-Bloss et al., 2011; Szyszka et al., 2005; 2008).

Is there a dedicated circuitry for nestmate label detection in the ants’ AL? In Drosophila, a dedicated circuitry is described by the labeled line model for the CO\(_2\) detection (Suh et al., 2004). When CO\(_2\) is presented, a specific glomerulus is activated, and this glomerulus was shown to be responsible for the innate CO\(_2\) avoidance behavior. However, there are several evidences against the existence of such a dedicated circuitry in the AL for the nestmate recognition system in ants: 1) Ants do not innately recognize their nestmates (Lenoir et al., 1999). Based on their experience during their first days as an adult, they accept individuals with familiar labels as nestmates, which can even be hetero-specific, hence non-sisters with another set of CHCs than their own (Carlin and Hölldobler, 1983). 2) CHC profiles are composed of many different components, activating different glomeruli and CHC profiles from nestmates and non-nestmates elicit spatially overlapping glomerular response patterns (Brandstaetter and Kleineidam, 2011). 3) Different nestmates can carry various labels and are still recognized as nestmates (Greene and Gordon, 2003; Kaib et al., 2000; Wagner et al., 2001; chapter 2). Hence, I reason that a labeled line system is unlikely for the nestmate recognition system.

Another model proposed that a specific cluster of glomeruli in the AL (the so-called T6 cluster) is a dedicated subsystem for the recognition of nestmate labels. The special characteristics of the T6 cluster are that there is seemingly no exchange of local information via LNs with other clusters in the AL (Nishikawa et al., 2012; Zube and Roessler, 2008) and PNs from the T6 cluster innervate segregated areas within the higher brain centers, MB and LH (Nishikawa et al., 2012; Zube and Roessler, 2008). However, both CHCs and general odorants are encoded in the T6, and information of CHCs is not exclusively encoded in the T6 but also in the other parts of the AL (Brandstaetter and Kleineidam, 2011; Sharma et al., 2015; Slone et al., 2017) arguing against such a dedicated subsystem in the AL for nestmate recognition. Therefore, as has been proposed before by others (Brandstaetter and Kleineidam, 2011; d’Ettorre et al., 2017), I assume that there is no dedicated
circuitry for nestmate label detecting in the AL, but rather that the capacity of the whole AL is used to encode CHC profiles.

The mushroom body (MB) is the brain center, where the olfactory information is associated with a meaning. Kenyon cells (KCs) are the intrinsic cells of the MB and their dendrites receive input from the PN axons. There is a vast divergence from a few hundred PNs onto several thousand KCs (>170,000 KCs in each MB in honey bees; Rybak and Menzel (1993)). Always only a small subset of all KCs is responding to a given odorant (sparse coding; Perez-Orive et al. (2002); Szyszka et al. (2005)). KCs converge onto fewer extrinsic cell of the MB (mushroom body output neuron: MBONs), which output probably to pre-motor centers. In honey bees there are about 400 MBONs (Rybak and Menzel, 1993) and I would assume a similar high number of MBONs in ants as in honey bees (compared to only 34 in Drosophila; Tanaka et al. (2008)), due to the eusocial organization and the high behavioral repertoire in both groups of Hymenoptera.

As argued in chapter 2, acquiring a template for a nestmate label could be a form of associative learning that uses the same mechanisms of associative learning during classical olfactory conditioning because nestmates often exchange food with and groom each other, both may represent a reward for the receiver individual. When the frequently encountered CHC profiles of nestmates are once in a while paired with receiving food or being groomed, the receiving individual will learn to associate the CHC profiles of nestmates with a positive reward. Associative learning takes place in the mushroom body (Heisenberg, 2003). From studies on Drosophila, it is known that reward (e.g. sucrose) and punishment (e.g. electric shock) is encoded by different dopaminergic neurons (DANs) that form synapses with KC in distinct compartments of the MB (Aso et al., 2010). Each compartment of the MB presumably receives input from all KCs and conveys the olfactory information to another MBON. Behaviorally neutral odorants activate MBONs which transmit positive and negative valence equally, hence mediating neutral meaning. Associative odor learning alters the relative output strength of the positive and negative MBONs (Hige et al., 2015a). This is because coincident activity in KCs (activated by the olfactory stimulus) and DANs (e.g. activated by a reward) induces synaptic depression at the synapses between KCs and MBONs (Hige et al., 2015a). In Drosophila, aversive odor learning leads to synaptic depression between KCs and MBONs that mediate attraction. Following the same logic, I propose that learning the association between a nestmate label and the rewarding encounter with a nestmate leads to synaptic depression between KCs and MBONs that mediate aversion. Thus, after this learning process, another presentation of the label leads to a weaker output of the aversion-mediating MBON compared to the acceptance-mediating MBON. As a result, the overall drive for 'acceptance' dominates the label. Hence, a nestmate template may be a weaker synaptic strength between the nestmate label activated KCs and MBON in the 'aversion compartment' compared to the 'acceptance compartment'.

The above described mechanism is a working hypothesis about how the template
could be implemented in the brain and remains to be tested. This hypothesis could be tested to block MBONs by injecting a blocker of neural activity into the lobes of the mushroom body similar as has been done in honey bees (Devaud et al., 2007). If this hypothesis is true then treated ants should not react aggressively to nestmates.

**Influence of internal state on behavioral response towards non-nestmates**

A behavioral response of an ant towards an encounter ant depends on the external stimulus itself, e.g. the CHC label of the encounter ant, and on the internal (physiological) state of the individual perceiving the external stimulus. In general, the internal state of an individual depends on many different internal and external factors (Sayin et al., 2018), such as nutritional state, current behavior, distance to the nest, presence of pheromones or the presence of surrounding nestmates (chapter 3). The internal state itself is also influenced by the external stimulus, e.g. if the encounter ant is a non-nestmate the individuals internal state may change. Hence, if there is no aggression response towards a non-nestmate (chapter 4), it could have several reasons. On the one hand, the evaluating individual might not have recognized the encounter as a non-nestmate, for example because the encounter ant did not present any recognition cues (e.g. a callow or a social parasite with an insignificant CHC profile; Morel et al. (1988); Nehring et al. (2015)) or the evaluating ant might have confused the encounter ant as nestmate because the encounter ant presents CHCs which mimic the recognition cues of a nestmate (e.g. a social parasite mimicking the host CHC profile; Lambardi et al. (2007)). On the other hand, a missing aggression response towards a non-nestmate might arise from the individuals' internal state which may act on one or several specific neural levels of olfactory processing and behavioral output.

In the following, I discuss different possible mechanisms of how the internal state of an individual may influence the behavioral response in such a way that an individual would not respond with aggression when encountering a non-nestmates. The internal state of the evaluating individual may affect the processing of the sensory information which then leads to a modified percept such as something neutral and finally results in non-aggressive behavior. Alternatively, the internal state of the evaluating individual may affect the evaluation of the perception of a non-nestmate. This may also lead to a non-aggressive behavior.

A potential mechanism of how the internal state could affect the evaluation of the percept comes from work on the fruit fly *Drosophila melanogaster*. The MB has an essential role in context-dependent behavior in flies (Bräcker et al., 2013; Siju et al., 2014). Cohn et al. (2015) propose that the *Drosophila* mushroom body functions like a switch board in which dopaminergic input reroutes the same odor signal (the same KC encoding) to different MBONs leading to a different MBON...
activity pattern, which in turn drive different appetitive or aversive behaviors. That means that depending on the internal state of the individual, a different behavioral circuit is activated (Cohn et al., 2015). Similarly, in ants information about non-nestmate CHC profiles could be context-dependently rerouted in the MB by dopaminergic neurons. In one situation, an ant may be in a social context, close to the nest, and upon perceiving a non-nestmate, the individual reacts with aggression. While in another situation, where the same individual also perceives a non-nestmate, but this time is far away from the nest and recently did not encounter many nestmates. Now the individual would not be aggressive but rather retreats from the non-nestmate. The same individual may respond to the same stimulus in different manners due to a different internal state, which might be encoded by different activity levels of the different dopaminergic neurons or via other neurons that release biogenic amines, such as octopamine and serotonin. Biogenic amines are known to modulate aggression in different species (lobster: Kravitz (1988), crickets: Hofmann and Stevenson (2000); Stevenson et al. (2005), ants: Szczuka et al. (2013)). Artificially increased serotonin (5-HT) levels in the brain increase, for example, the aggressiveness in stalk-eyed flies (Bubak et al., 2014). In the pavement ant (*Tetramorium caespitum*), social isolation modulates serotonin and octopamine levels in the brain, whereas dopamine levels in the brain are changed, when individuals were fighting against non-nestmates (Bubak et al., 2016). The neural mechanism of these biogenic amine-mediated modulations of behavior are not known. A possible mechanism is that - depending on the animal’s internal state - the biogenic amines have a specific concentration in the brain and may modulate neuronal circuits by extra-synaptic receptors. For example, in another eusocial hymenoptera, the honey bee, an octopamine receptors (AmOA1) is expressed in GABAergic neurons located in several neuropiles (AL, MB, central complex) (Sinakevitch et al., 2011). Some of those GABAergic neurons do not have an octopaminergic presynaptic partner. Hence, these receptors may respond to amines released from distant release sites, so that octopamine acts as a neurohormon (Sinakevitch et al., 2011). This may be also true for octopamine, dopamine and serotonin receptors in ants. I hypothesize that dopamine, octopamine and/or serotonin modulate aggression in ants for an adaptive modulation of the behavior.

**Future directions**

The first step to test whether octopamine, dopamine and serotonin play a role in modulation of aggression in ants, is to measure the biogenic amine levels in brains of individuals that are in a social context and showed recent aggressive behavior (encounter with a non-nestmate) and compare them to individuals that are either in a non-social context (isolated from their nestmates for 1 h) or in social context, but did not show recent aggressive behavior (encounter with a nestmate). Investigations with similar settings have been done before in ants using high performance liquid chromatography (HPLC) (Bubak et al., 2016; Wada-Katsumata et al., 2011) and the results indicate that the levels of biogenic amines in the ant brain are
indeed influenced by and/or influencing the social context and aggressive behavior in ants. However, often brains of several individuals need to be pooled to reveal sufficient high concentrations of the substance of interest to be detected with HPLC. Furthermore, each brain can be measured only once, revealing only one snapshot of biogenic amine levels before dissection. An alternative technique, which is more sensitive in detecting biogenic amines, is the fast scan cyclic voltametry (Cooper and Venton, 2009). In a study on Drosophila melanogaster, Fang et al. (2011) investigated the levels of biogenic amines in single dissected larval brains. Using fast scan cyclic voltametry in alive ants that are in a social/non-social context and measuring the change in biogenic amine level due to encounters with a nestmate or a non-nestmate will reveal which of the biogenic amines are involved in modulation of aggression or the potential to show aggressive behavior. The second step of testing the hypothesis that biogenic amines are modulating aggression in ants is to interfere with amine signaling. An interference can be achieved by artificially changing the amine levels in individuals and study the effect on aggression, which was done before e.g. in crickets (Stevenson et al., 2005). Furthermore, a blocking of the amine receptors interferes with amines signaling (Rillich and Stevenson, 2015), which could be done by specific receptor-agonists and -antagonists. An investigation of biogenic amine levels within the same individual over a time period of minutes or even hours could reveal how and how fast the biogenic amine levels change, when the individual’s internal state is changing or when a non-nestmate is presented. In the experiment I propose, the Buchner ball (a method, where a tethered animal can freely move on an air floating Styrofoam ball; Buchner (1976)) could be combined with the above mentioned fast scan cyclic voltametry in brains of alive, freely behaving ants. The internal state of the individual could be changed by mimicking surrounding nestmates by frequently presenting nestmates or keeping it without nestmate presentation. The proposed combination of techniques may help to understand the causal relationship between biogenic amines, internal state and behavior.

An interesting finding in our study with manipulated colony odors (chapter 2) is that workers from manipulated sub-colonies had a lower probability to respond with aggressive behavior towards strongly manipulated worker, with an unfamiliar label compared to workers from the sham-treated sub-colony, which had a high probability of aggression. There are two possible explanation for this phenomenon: 1) it is only a temporal phenomenon, which would enable workers to be more tolerant to nestmates, when the colony odor recently changed or 2) workers of the manipulated sub-colonies generally perceive these unfamiliar profiles as more similar to their own profiles.

In order to test these hypotheses, I propose the following experiment: After manipulation of the colony odor from different sub-colonies, by adding synthetic hydrocarbons (similar as in chapter 2), the workers in these sub-colonies are tested against workers from other sub-colonies and also against conspecific non-
nestmates after different periods of time after manipulation (between 1 day and 1 month). If the probability of aggressiveness is still low after 1 month, also against non-nestmates, it would support the hypothesis that workers are generally less aggressive against workers with an unfamiliar label. However, if the workers’ probability of aggression response against unfamiliar labels is only decreased for a short time period after, it would support the hypothesis that a recent change in colony odor may modulate the response threshold of workers perhaps, in order to not falsely reject a nestmate that has a slightly different label.

These before mentioned experiments will shed light on the proximate mechanism of the modulation of behavioral responses and of label-template matching in the ant’s brain. Together with my data on the naturally occurring chemical labels of host and social parasite ants, on manipulated chemical labels, on the modulation of behavioral responses and the preliminary physiological data, the proposed experiments will help to understand how ants can so amazingly fast and reliably discriminate between very complex (multi-component), but also very similar CHC profiles. Moreover, revealing the behavioral and physiological mechanisms of nestmate recognition in ants is also relevant for a broad range of neuroethological questions, because similar mechanisms might underlie other forms of odor recognition and decision making.
Author Contributions

Chapter 1: Host colony integration: *Megalomyrmex* guest ant parasites maintain peace with host ants through weaponry

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**Study Design** SN and RMMA designed the experiments.

**Experiments** SN performed the behavioral experiments. RMMA and SN did the chemical extractions.

**Data Analyses** SN and AD analyzed the behavioural data. All authors analyzed the chemical data.

**Manuscript** SN wrote the first draft. SN and RMMA edited the manuscript. All authors read and commented on the manuscript.

Chapter 2: Learning distinct chemical labels of nestmates in ants

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**Study Design** CJK, MH, and SN designed the behavioural experiments.

**Experiments** MH performed the behavioral experiments and did the chemical extractions.

**Data Analyses** SN analyzed the chemical data. SN and CJK performed the statistical analysis of the chemical and the behavioral data.

**Manuscript** SN and CJK wrote the manuscript. All authors read and commented on the manuscript.
Chapter 3: Social interactions promote adaptive resource defense in ants

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Study Design  CJK and ELH designed the behavioural experiments.

Experiments  ELH performed the behavioral experiments.

Data Analyses  ELH analyzed the videos files. SN and CJK did the statistical analysis.

Manuscript  CJK and SN wrote the manuscript. All authors read and commented on the manuscript.

Chapter 4: Investigating the neural encoding of colony odors with calcium imaging experiments

Study Design  SN and CJK designed the experiments.

Experiments  SN performed the experiments.

Data Analyses  SN analyzed the data.

Manuscript  SN wrote the chapter.
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