The olfactory system of leaf-cutting ants: neuroanatomy and the correlation to social organization

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I. Zusammenfassung

Die Arbeiterinnenkaste der Blattschneideameisen (Gattungen *Atta* und *Acromyrmex*) zeigt einen ausgeprägten Größenpolymorphismus. Man findet hier einen Alloethismus; unterschiedlich große Arbeiterinnen führen verschiedene Arbeiten im Stock durch. Verschiedene Verhaltensversuche haben gezeigt, dass viele Verhaltensweisen der Arbeiterinnen olfaktorisch gesteuert werden und dass das olfaktorische System hoch entwickelt und sehr sensitiv ist. Es ist wahrscheinlich, dass sich im olfaktorischen System verschieden großer Arbeiterinnen Anpassungen finden lassen, die gut abgestimmte Verhaltensantworten auf die verschiedenen Aufgaben der Tiere ermöglichen. Und tatsächlich zeigt eine aktuelle Studie, dass zwei verschiedene Phänotypen des Antennallobus der Arbeiterin bei *Atta vollenweideri* existieren, der MG- und der RG-Phänotyp (mit oder ohne Makroglomerulus, MG). Die Existenz des Makroglomerulus kann mit der Körpergröße der Tiere korreliert werden: bei kleinen Arbeiterinnen findet man den RG-Phänotyp, bei großen den MG-Phänotyp. Im Makroglomerulus wird die olfaktorische Information über den verhaltensauslösenden Bestandteil des Spurpheromons verarbeitet.

Im ersten Tel meiner Doktorarbeit versuche ich, Verhaltensunterschiede verschieden großer *Atta vollenweideri* Arbeiterinnen zu quantifizieren. Dazu konzentriere ich mich auf das Spurfolgeverhalten, dass bei Arbeiterinnen jeder Größe beobachtet werden kann. Um die Spurfolgeleistung einzelner Arbeiterinnen zu testen, wurde eine künstlich gelegte Spur mit abnehmender Konzentration des verhaltensauslösenden Bestandteils des Spurpheromons verwendet. Die Spurfolgeleistung der Arbeiterinnen hängt von der Existenz des Makroglomerulus im Antennallobus ab. Arbeiterinnen, die den MG-Phänotyp zeigten, konnten die Spur in signifikant geringeren Konzentrationen wahrnehmen als Arbeiterinnen, die den RG-Phänotyp vorwiesen.

Im zweiten Teil meiner Doktorarbeit untersuche ich die Neuroanatomie des olfaktorischen Systems bei verschieden großen *Atta vollenweideri* Arbeiterinnen auf eventuelle weitere anatomische Unterschiede neben dem Makroglomerulus – im Besonderen ob die Anzahl an Glomeruli bei verschieden großen Tieren unterschiedlich ist. Die Antennalloben kleiner Arbeiterinnen beinhalten cirka 390 Glomeruli (geringe Anzahl, LN-Phänotyp), die Antennalloben großer Arbeiterinnen dagegen cirka 440 Glomeruli (hohe Anzahl, HN-

Phänotyp). Alle Arbeiterinnen mit dem LN-Phänotyp und einige mit dem HN-Phänotyp besitzen keinen Makroglomerulus (LN-RG-Phänotyp und HN-RG-Phänotyp). Die meisten Tiere mit HN-Phänotyp besitzen jedoch einen Makroglomerulus (HN-MG-Phänotyp). Massenfärbungen der olfaktorischen Rezeptorneuron-Axone zeigen, dass der Antennennerv sich in sechs Trakte teilt und so die Glomeruli in sechs verschiedene Glomerulicluster unterteilt werden können (T1-T6). Bei den Arbeiterinnen mit LN-Phänotyp fehlen cirka 50 Glomeruli im T4-Cluster. Einzelsensillenfärbungen zeigen, dass die Rezeptorneuronen der olfaktorischen Sensilla trichodea curvata alle sechs Cluster, also auch das T4-Cluster innervieren. Ein weiterer Sensillentyp, die Sensilla basiconica, innerviert ausschließlich Glomeruli im T6-Cluster. Quantitative Analysen ergeben eine Korrelation zwischen der Anzahl der Sensilla basiconica auf der Arbeiterinnenantenne und des durchschnittlichen Volumens der T6-Glomeruli bei verschieden großen Tieren. Die Ergebnisse der Verhaltensversuche und der neuroanatomischen Studien könnten darauf hinweisen, dass Unterschiede im Verhalten auf olfaktorische Reize möglicherweise durch die Entwicklungsplastizität der Antenallobus-Phänotypen ausgelöst werden. Dies könnte innerhalb der Kolonie die Grundlage der Spezialisierung von Arbeiterinnen auf bestimmte Arbeiten sein.

Den letzten Teil meiner Doktorarbeit nimmt eine Untersuchung über den evolutionären Ursprung des Makroglomerulus und der Anzahl der Glomeruli im Antennallobus ein. Dazu verglich ich in den Antennalloben 25 verschiedener Arten aus den drei Attini-Gruppen (basale, höhere und blattschneidende Attini) die Anzahl, das Volumen und die Position der Glomeruli. Die Antennalloben aller untersuchten Arten bestehen aus sehr vielen Glomeruli (257-630). Die meisten Glomeruli wurden bei einer basalen Art, nämlich bei *Apterostigma cf. mayri* gefunden. Eine große Anzahl an Glomeruli könnte für die Evolution der hochentwickelten olfaktorischen Systeme der Attini vorteilhaft gewesen sein. Der Makroglomerulus findet sich in allen untersuchten blattschneidenden Attini-Arten, aber nie in den untersuchten basalen und höheren Attini-Arten. Er findet sich nur bei größeren Arbeiterinnen und befindet sich immer in der Nähe des Antennennerveingangs. Dies bedeutet, dass es sich bei der Existenz des Makroglomerulus in den großen Blattschneidearbeiterinnen um eine abgeleitete Überexpression eines Merkmals innerhalb der polymorphen blattschneidenden Attini-Arten handelt. Der Makroglomerulus ist

wahrscheinlich eine olfaktorische Anpassung an das hoch entwickelte Fouragier- und Rekrutiersystem dieser Arten. Er ist ein Baustein der komplexen Arbeitsteilung und der komplexen sozialen Organisation, die für die Arten dieser Gruppe bekannt sind.

II. Abstract

In leaf-cutting ants (genera *Atta* and *Acromyrmex*), the worker caste exhibits a pronounced size-polymorphism, and division of labor is largely dependent on worker size (alloethism). Behavioral studies have shown a rich diversity of olfactory-guided behaviors, and the olfactory system seems to be highly developed and very sensitive. To allow fine-tuned behavioral responses to different tasks, adaptations within the olfactory system of different sized workers are expected. In a recent study, two different phenotypes of the antennal lobe of *Atta vollenweideri* workers were found: MG- and RG-phenotype (with and without a macroglomerulus, MG). The existence of the macroglomerulus is correlated to the body size of workers, with small workers showing the RG-phenotype and large workers showing the MG-phenotype. In the MG, the information about the releaser component of the trail-pheromone is processed.

In the first part of my PhD-project, I focus on quantifying behavioral differences between different sized workers in *Atta vollenweideri*. The study analyzes the trail following behavior; which can be generally performed by all workers. An artificial trail consisting of the releaser component of the trail-pheromone in decreasing concentration was used to test the trail-following performance of individual workers. The trail-following performance of the polymorphic workers is depended of the existence of the MG in the antennal lobe. Workers possessing the MG-phenotype were significantly better in following a decreasing trail then workers showing the RG-phenotype.

In the second part I address the question if there are more structural differences, besides the MG, in the olfactory system of different sized workers. Therefore I analyze whether the glomerular numbers are related to worker size. The antennal lobes of small workers contain ~390 glomeruli (low-number; LN-phenotype), and in large workers I found a substantially higher number of ~440 glomeruli (high-number; HN-phenotype). All LN-phenotype workers and some of the small HN-phenotype workers do not possess an MG (LN-RG-phenotype and

HN-RG-phenotype) at all, whereas the remaining majority of HN-phenotype workers do possess an MG (HN-MG-phenotype). Mass-stainings of antennal olfactory receptor neurons revealed that the sensory tracts divide the antennal lobe into six clusters of glomeruli (T1-T6). In the T4-cluster ~50 glomeruli are missing in the LN-phenotype workers. Selective staining of single sensilla and their associated receptor neurons showed that T4-glomeruli are innervated by receptor neurons from the main type of olfactory sensilla, the Sensilla trichodea curvata which are also projecting to glomeruli in all other clusters. The other type of olfactory sensilla, the Sensilla basiconica, exclusively innervates T6-glomeruli. Quantitative analyses revealed a correlation between the number of Sensilla basiconica and the volume of T6 glomeruli in different sized workers. The results of both behavioral and neuroanatomical studies in *Atta vollenweideri* suggest that developmental plasticity of antennal-lobe phenotypes promotes differences in olfactory-guided behavior which may underlie task specialization within ant colonies.

The last part of my project focuses on the evolutionary origin of the macroglomerulus and the number of glomeruli in the antennal lobe. I compared the number, volumes and position of the glomeruli of the antennal lobe of 25 different species from all three major Attini groups (lower, higher and leaf-cutting Attini). The antennal lobes of all investigated Attini comprise a high number of glomeruli (257-630). The highest number was found in *Apterostigma cf. mayri.* This species is at a basal position within the Attini phylogeny, and a high number of glomeruli might have been advantageous in the evolution of the advanced olfactory systems of this Taxa.

The macroglomerulus can be found in all investigated leaf-cutting Attini, but in none of the lower and higher Attini species. It is found only in large workers, and is located close to the entrance of the antennal nerve in all investigated species. The results indicate that the presence of a macroglomerulus in large workers of leaf-cutting Attini is a derived overexpression of a trait in the polymorphic leaf-cutting species. It presumably represents an olfactory adaptation to elaborate foraging and mass recruitment systems, and adds to the complexity of division of labor and social organization known for this group.

1. General introduction

1.1. Eusociality in insects

What are the characteristics of social insects? The generally accepted definition of eusociality refers to three factors. First, there has to be an overlap of generations within the insect community. Second, cooperative brood-care is necessary. Third, different castes can be found, from which some are specialized on reproduction, others consist of sterile individuals [Wilson, 1971]. Eusociality occurs in three insect orders, in Hymenoptera (wasps, ants and bees), Isoptera (termites) and Homoptera (aphids) [Krebs and Davies, 1993]. All known ant species are eusocial. Important for the ecological success of the eusocial Hymenoptera are a highly developed pheromonal communication system and a complex system of division of labor within the colony.

1.2. Division of labor in social insects

An important character of social organization is task allocation, leading to division of labor, which is fundamental for efficient insect colonies [Wilson, 1971; Hölldobler and Wilson, 1990; Seeley, 1997]. One proposed mechanism of task allocation is that individuals of a colony have different response thresholds for different tasks, and some individuals perform a particular task more often than others [Robinson and Page, 1988; Bonabeau et al., 1998]. Response thresholds may be fixed or may change over live time resulting in age-polyethism. Age-polyethism has been well described in the honeybee *Apis mellifera*, but also in different ant species [Seeley, 1982; Wilson and Hölldobler, 1988; Hölldobler and Wilson, 1990; Kühn-Buhlmann and Wehner, 2006]. A well studied ant species which shows an age-polyethism is the desert ant *Cataglyphis bicolor*. Workers of this species stay within their nests for about a month, where they perform indoor tasks like brood care. After their indoor stage they become outdoor foragers for a mean period of six days [Wehner et al., 1972; Schmid-Hempel and Schmid-Hempel, 1984].

A third form of division of labor is the alloethism. Here, the members of a colony show a pronounced size-polymorphism, and division of labor is structured to a great extent according to the different sizes of individuals. We find an alloethism in the worker caste of bumblebees, different ant species and also slightly in different stingless bees [Fowler, 1983; Fowler, 1985; Goulson, 2003; Goulson et al., 2005].

1.2.1. Alloethism in ants

Division of labor according to different sized workers leads to the existence of physical worker sub-castes. In ants we can find the phenomenon of different sized worker castes in diverse genera, but if we consider the high number of ant genera, it is the exception rather than the common form of division of labor [Oster and Wilson, 1978; Hölldobler and Wilson, 2009]. In species with size polymorphism in worker castes, mostly the largest or second largest workers are fulfilling special tasks. For example in different driver ant (*Dorylus*) and army ant (*Eciton*) species, large workers equipped with long mandibles are specialized on the hunting of large pray, but also on defending the nest. In some *Acanthomyrmex* and *Pheidole* species, majors with large heads and large adductor muscles can be found. These workers are specialized in "milling", which means in breaking open seeds [Hölldobler and Wilson, 2009]. There is even a species known (*Crematogaster smithi*), with large workers producing trophic eggs, that are fed to the queen and the brood [Heinze et al., 1999].

1.2.2. Alloethism in the leaf-cutting Attini

The ants with the presumably most investigated system of size polymorphism and alloethism are the leaf-cutting Attini (*Acromyrmex* and *Atta*). Most of the existing studies deal with the labor system of large *Atta* colonies.

Atta workers show a broad array of size variation (Fig. 1). For example in *Atta sexdens*, the head width oft the largest workers is eight times larger than those of the smallest ones. The dry weight of the large workers is 200 times larger than the dry weight of the smallest workers [Hölldobler and Wilson, 2009]. Although small workers are found on the trails in different leaf-cutting Attini species [Stradling, 1978; Wilson, 1980b; Hughes and Goulson, 2001], their main tasks - like the maintenance of the brood and the fungus - are performed inside the colony. The large workers are specialized in leaf-cutting and transport of the leaf fragments back to the nest.

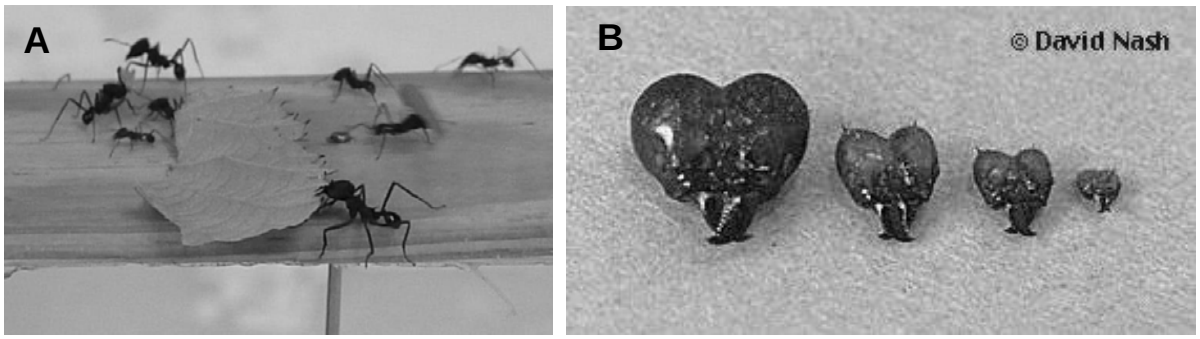

Figure 1: Size-polymorphism in the leaf-cutting *Atta* species. **A:** Large and small *Atta vollenweideri* workers on the trail (laboratory colony). The large workers transport leaf fragments back to the nest as a substrate for the fungus. **B:** Different sized *Atta* worker heads. Shown from left to right are a bold soldier, a large, a medium sized and a small worker (Photo: David Nash).

Large colonies necessitate more appropriate substrate for the fungus than small colonies do. Fresh materials like leaves or grasses are available in a much larger amount than dead plant material, and they are renewable resources. But for the cutting and harvesting of fresh plant material, an ant worker has to have a minimum of size. A single large worker could be able to harvest more plant material than a whole group of small workers [Beshers and Traniello, 1996]. At the other hand, quite small workers are still needed inside the colony to take care of the fungus garden and the brood. This situation probably resulted in the development of alloethism and the leaf-cutting abilities of large workers.

1.3. The tribe Attini

The tribe Attini consists of 13 genera and over 230 species of ants, restricted to the new world. All attine species are characterized by their obligate cultivation of symbiotic fungi. The tribe Attini seems to be a monophyletic group [Schultz and Meier, 1995], but there are great differences between the attine ant species in colony size, food sources and life ecology, turning them to a well suited group for comparative studies. According to studies on life ecology [Weber, 1956; Wilson, 1971; Hölldobler and Wilson, 1990], larval characteristics [Schultz and Meier, 1995] and also based on mDNA sequences [Wetterer et al., 1998], at least three subgroups can be distinguished (Fig. 2).

Lower (formerly basal) attine ant species live in small colonies which do normally contain about several hundred workers. The workers are all monomorph and in general very small (about 0.5 mm – 1.0 mm head width). Their fungus is reared with animal material and also dead plant parts. Exemplary for the fungus diet in basal Attini are workers of *Mycocepurus smithi*, feeding their fungus with bat feces, caterpillar frass and dead leaf parts [Mackay et al., 2004] or *Cyphomyrmex longiscapus* workers, feeding their fungus with seed, seed pods and also with dead leaf parts [Mueller and Wcislo, 1998]. A special fungus diet can be found in several *Apterostigma* species, their fungus is reared with dead wood [Hölldobler and Wilson, 1990]. The small body size and the small mandibles of basal Attini are not suited to cut fresh leaves [Wilson, 1980b; Wilson, 1980a; Beshers and Traniello, 1996]. Instead of using mass recruitment for foraging, their workers forage in small groups or individually [Blum and Wilson, 1964; Beckers et al., 1989].

In contrast to the lower Attini, the higher (formerly intermediate) Attini (genera *Sericomyrmex* and *Trachymyrmex*) feed their fungus with dead plant material, but also with fresh plant parts if they could reach them. The colony size is about several thousand individuals and the workers show no pronounced size polymorphism [Beshers and Traniello, 1996]. It is shown that some *Trachymyrmex* species have the ability to recruit other workers and to use a trail system for foraging, but normally they do not use mass recruitment [Jaffe and Villegas, 1985].

Because of their huge colony size (up to millions of individuals in some *Atta* species) and due to their extreme need of fresh leaves, the leaf-cutting Attini (*Acromyrmex* and *Atta*) are a serious problem for the agriculture in south and middle America [Hölldobler and Wilson, 1990]. Both genera show a polymorphism in workers, but only *Atta* species exhibit an extreme polymorphism with large soldier workers. In most *Atta* and *Acromyrmex* species, we find a very high developed system of mass recruitment for the foraging of fresh plant material.

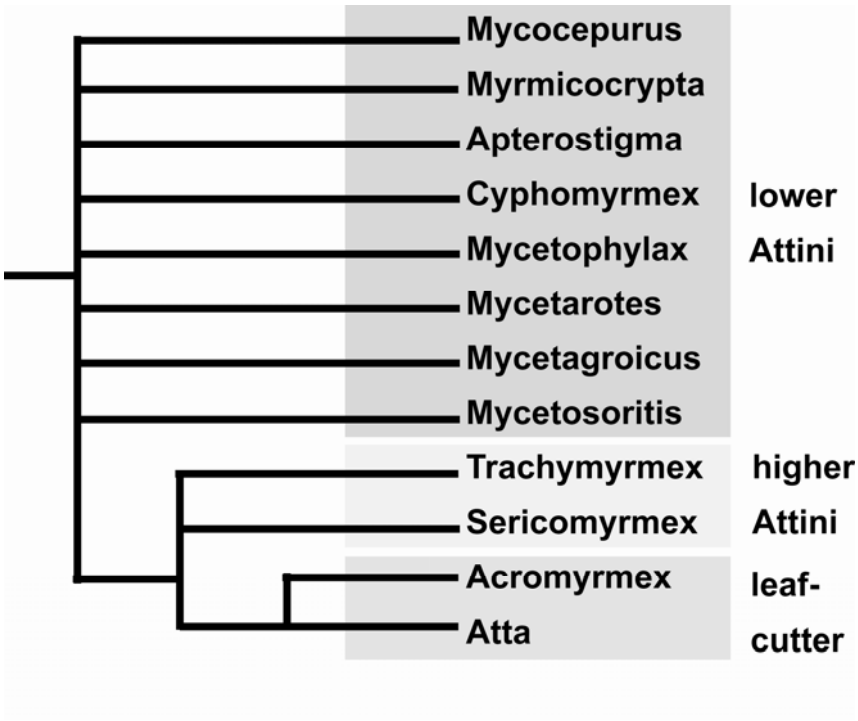

Figure 2: Phylogeny of the tribe Attini. The tribe Attini is divided into three groups according to life ecology, larval characteristics and mDNA sequences. Eight genera belong to the lower Attini. The higher Attini contain the two genera *Sericomyrmex* and *Trachymyrmex*. The two genera *Atta* and *Acromyrmex* belong to the third group, the leaf-cutting Attini.

1.4. Pheromone communication in ants

A pheromone is defined as a chemical substance, which is produced and used for communication within a species. Traditionally, pheromones are divided into two groups: Releaser pheromones which elicit a quick specific behavior and primer pheromones which induce long lasting physiological changes [Wilson and Bossert, 1963]. In ants, a high number of pheromones of both types are used for the social organization and communication within the colony. Ants produce several substances used as pheromones in different exocrine gland like the mandibular glands, the Doufour´s glands or the postpharyngal glands (Fig. 3). Each pheromone is usually a mixture of different substances. The substances used as a specific signal vary in different species [Hölldobler and Wilson, 1990]. Some of the most common pheromones used in ant colonies are queen pheromones, which function as primer pheromones preventing the reproduction of workers and sexual pheromones, in order to enable the males finding and fertilizing virgin queens. Other common pheromone types are alarm pheromones which inform other workers of potential danger and trail pheromones, used to enable other workers to find food resources.

The usage of trail pheromones is known from a high number of ant species belonging to the subfamilies of Ponerinae, Formicinae and also Myrmicinae [Hölldobler and Wilson, 1990]. Trail laying has been defined as follow: an insect marks a route with a scent or an odor trace that other insects of the same community are able to follow [Sudd, 1959]. The usage of a trail begins with a scout and finding food, after that, the scout deposits a trail and returns to the nest [Morgan, 2009]. Single, several or large numbers of workers follow the trail to the food resource and can re-enforce the trail-pheromone trace until the resource is exploited.

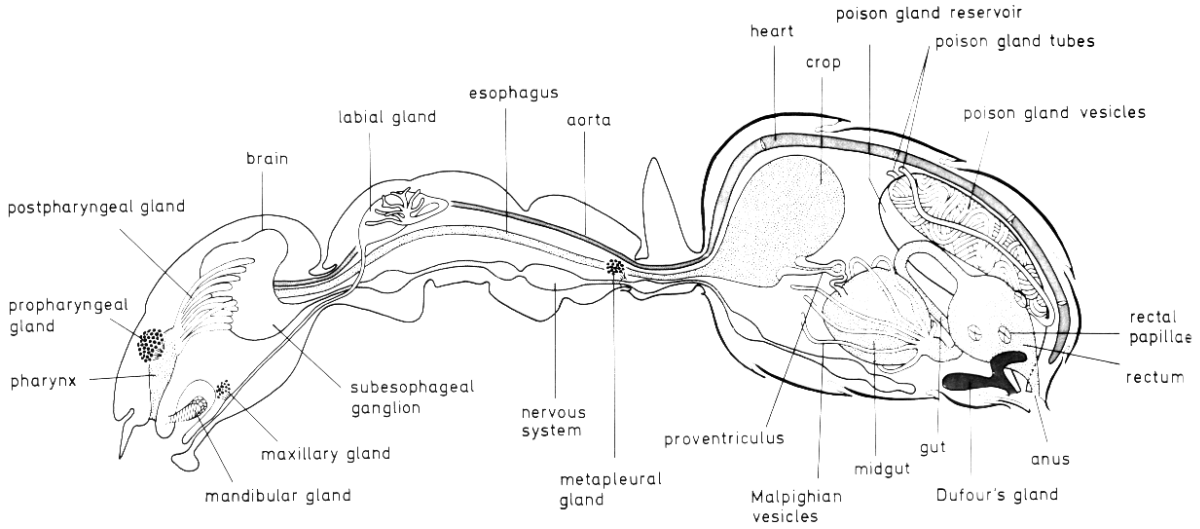

Figure 3: The positions of several glands are shown in a schematic drawing of a section through an ant worker. Pro- and postpharyngal gland, mandibular and maxillary glands can be found in the head, the labial and the metapleural gland are positioned in the thorax and Dufour´s gland and the poison gland can be found in the abdomen [Otto, 1962].

In the leaf-cutting Attini *Atta* and *Acromyrmex*, the trail pheromone is used in huge amount. The large colonies need fresh plant material as a substrate for the fungus. Therefore the trail traces in most species are broad, cleaned from obstacles and are real “highways”, used by thousands of workers. The first trail pheromone substance identified in leaf-cutting *Atta* species was methyl-4-methylpyrrole-2-carboxylate (M4MPC) [Tumlinson et al., 1971]. It was also identified as a pheromone in *Atta cephalotes* [Riley et al., 1974], *Acromyrmex octospinosus* [Cross et al., 1982], and *Acromyrmex subterraneus* [Nascimento et al., 1994].

Another substance, 2-ethyl-3,6-dimethylpyrazine (2E3,6DMP), was identified as trail pheromone of *Atta rubropilosa* [Cross et al., 1979] and *Atta sexdens* [Evershed and Morgan, 1983]. Both substances can be found in different ratios in different *Atta* species. In *Atta vollenweideri*, which is the main species in focus of my behavioral and neuroanatomical studies, both M4MP2C and 2E3,6DMP can be found. It seems that M4MP2C is the releaser component of A*tta vollenweideri*, while 2E3,6DMP alone does not release trail-following behavior [Kleineidam et al., 2007].

1.5. The olfactory sense

The olfactory sense is an important source for environmental information in most species. In many cases, the behavior of animals is guided by olfactory cues and most species have developed an olfactory system with a high sensitivity for the detection of diverse odors. Both vertebrates and insects show an interface for detecting odors in their environment. In insects, this interface is the antenna with its numerous olfactory sensilla. In vertebrates, the interface is the nasal mucosa. In both, vertebrates and insects, the odor information is led to several neuropiles in the brain, where the information is processed. Many common principles in the odor detection and the information processing can be found across phyla [Hildebrand and Shepherd, 1997; Strausfeld and Hildebrand, 1999]. The following chapters summarize the knowledge about the olfactory system of vertebrates and insects.

1.5.1. The olfactory sense in Vertebrates

The olfactory system of vertebrates is able to discriminate a broad array of structurally diverse odorants. This perceptual acuity derives from a series of information-processing events, starting in the nose epithelia and ending in the higher integration centers in the brain [Buck, 1996]. The detection of odors take place in the nose, where olfactory receptor neurons (ORNs) are embedded in an epithelium. This epithelium contains three major cell types: ORNs, supporting cells and olfactory stem cells [Graziadei and Montigraziadei, 1979]. Odorants bind to specific odorant receptors on the ORNs and induce a cascade of transduction events that lead to the generation of action potentials, which are led to the first olfactory neuropile in the vertebrate brain, the olfactory bulb (bulbus olfactorius). The ORNs of one side of the nose project to the ipsilateral olfactory bulb. In the olfactory bulb, the axons of the ORNs synapse in the functional units of the bulb, the glomeruli [Harrison

and Scott, 1986; Shepherd, 1993]. Here, the ORNs form synapses with the dendrites of periglomerular interneurons and with the dendrites of mitral and tufted secondary neurons, which sent the olfactory information to the olfactory cortex [Buck, 1996]. The olfactory cortex is divided into five main areas: the anterior olfactory nucleus; the piriform cortex, the olfactory tubercle, the amygdala and the entorhinal area [Scott et al., 1985; Brunjes et al., 2005; Ashwell and Phillips, 2006].

1.5.2. The olfactory sense in Insects

The olfactory system of insects can be divided into three sections. The first part of this chapter deals with the interface between the insect and its environment, the insect antenna. Here the detection and discrimination of odors take place. From the antenna, the olfactory information is led to the first olfactory neuropil, the antennal lobe, where the primary steps of odor processing occurs. The neuroanatomy and the physiology of the antennal lobe are the main theme of the second paragraph, due to the importance of the antennal lobe anatomy for this study. From the antennal lobe, the olfactory information is transmitted via the projection neurons into the higher integration centers of the brain, where learning and memory takes place. Although these higher integration centers are not part of this study, the knowledge about their anatomy and function is shortly summarized in the third part of this chapter.

1.5.2.1. The insect antenna and its sensilla

The insect antennae are packed with a high number of sensilla, from which only several types are olfactory sensilla. We also find sensilla e.g. for the reception of taste, temperature or humidity. Different types of olfactory sensilla can be found within the insect species. Frequently found sensillar types are placoid sensilla (sensilla placodea) or hair sensilla (Sensilla trichodea curvata, sensilla basiconica). In related beetle species (Coleoptera, Lamellicornia), three different types of olfactory sensilla have been found, sensilla trichodea, placodea and basiconica, and also several intermediate forms of sensilla [Meinecke, 1975]. In the honeybee, we find a high number of sensilla placodea on the antennae [Frisch, 1921; Schneider, 1964]. In ants, hair shaped sensilla seem to be common [Renthal et al., 2003; Nakanishi et al., 2009]. In all insects, the olfactory sensilla are equipped with olfactory receptor neurons (ORNs). For the ORNs, the specificity to an odor is given by their receptor

molecules and binding proteins. There is a great variance in the number of ORNs belonging to a single sensillum in the different insect species. In most species, like flies or moths, the olfactory sensilla are equipped with one to three ORNs. In contrast, the olfactory sensilla of social hymenoptera are equipped with a high number of ORNs [Schneider and Steinbrecht, 1968; Esslen and Kaissling, 1976; Kelber et al., 2006]. The advantages of these multiple ORNs are not yet clear.

1.5.2.2. The first olfactory center: the antennal lobe

The axons of the ORNs project to the first olfactory neuropil, the antennal lobe (AL) in the brain and terminate in the functional units of the AL, the glomeruli (Fig 4A). It is assumed that axons from ORNs that express the same odorant receptor gene converge onto the same glomerulus [Rodrigues, 1988]. This is supported by a good match in the number of functional odorant receptor genes and the number of glomeruli found in the AL, for example as shown in the honeybee or in *Drosophila* [Vosshall et al., 2000; Robertson and Wanner, 2006]. This organization results in a spatial representation of odors in the glomeruli. This can be shown with the help of functional imaging techniques. Different aspects of the glomerular activity have been studied by calcium-imaging studies, mainly in the honeybee *Apis mellifera*, but also in other insect species like *Drosophila* or *Manduca* [Joerges et al., 1997; Galizia et al., 1999b; Sachse et al., 1999; Fiala et al., 2002; Hansson et al., 2003].

There is a great variance in the number of glomeruli found in different insect species. In most insects the number of glomeruli in the AL does not exceed 100. About 43 glomeruli have been found in *Drosophila melanogaster*, about 50 glomeruli in the mosquito *Aedes aegypti,* and about 65 in *Manduca sexta* [Stocker, 1994; Laissue et al., 1999; Huetteroth and Schachtner, 2005; Ignell et al., 2005]. In solitary Hymenoptera, 160 glomeruli are located in the AL in the female beewolf *Philantus triangulum* [Rybak et al., 2003], and 186-189 glomeruli have been found in the female wasp *Cortesia glomerata* [Smid et al., 2003]. In social Hymenoptera the number of glomeruli found so far range from about 164 in the honeybee *Apis mellifera* to up to 460 in the Carpenter ant *Camponotus floridanus* [Arnold et al., 1985; Flanagan and Mercer, 1989; Galizia et al., 1999a; Zube et al., 2008].

When the axons of all ORNs terminate in the AL, they are sorted according to their odor specificity. There are two possibilities of sorting. In species with only a low number of glomeruli, like in moths, the ORNs are normally sorted at the sorting zone, which is positioned at the entrance of the antennal nerve in the AL. From here, ORNs with the same odor specificity move to their target glomerulus [Rössler et al., 1999]. In species with a higher number of glomeruli, the incoming ORNs are sorted into several sensory tracts, which innervate different clusters of glomeruli. For example, in the honeybee *Apis mellifera*, the antennal nerve divides into four sensory tracts (T1-T4) entering the AL and consequently all glomeruli can be sorted into four clusters according to their belonging to a sensory tract [Arnold et al., 1985; Flanagan and Mercer, 1989; Galizia et al., 1999a]. In ants, *Camponotus floridanus* is so far the only species where the sensory tracts have been investigated, and seven sensory tracts have been found [Zube et al., 2008]. The functional significance of the different clusters of glomeruli is not yet clear. One possible hypothesis for the existence of the sensory tracts is that due to logistic reasons the sorting via different tracts is more efficient when a high number of glomeruli have to be innervated.

1.5.2.3. Higher integration areas

The glomeruli are interconnected via local interneurons. These are multiglomerular neurons which innervate many different glomeruli and here, the olfactory information is processed. For further integration, but also for the learning of odors, the information is transferred to the higher integration centers of the insect brain, the mushroom bodies and other structures in the protocerebrum. In social Hymenoptera, the information transfer in the different neuropils is well studied in the honeybee. Here, a “dual olfactory pathway” was found (Fig. 4B). About 800 projection neurons build the two main output tracts of the AL [Abel et al., 2001; Kirschner et al., 2006] and innervate either first the mushroom body and than the lateral horn (medial antenno-cerebral tract), or first the lateral horn and than the mushroom body (lateral antenno-cerebral tract). In the mushroom bodies, the projection neurons synapse onto about 170.000 Kenyon cells. The mushroom bodies integrate olfactory information with other modalities and are the scene of memory consolidation [Menzel et al., 1994; Strausfeld et al., 1998; Heisenberg, 2003].

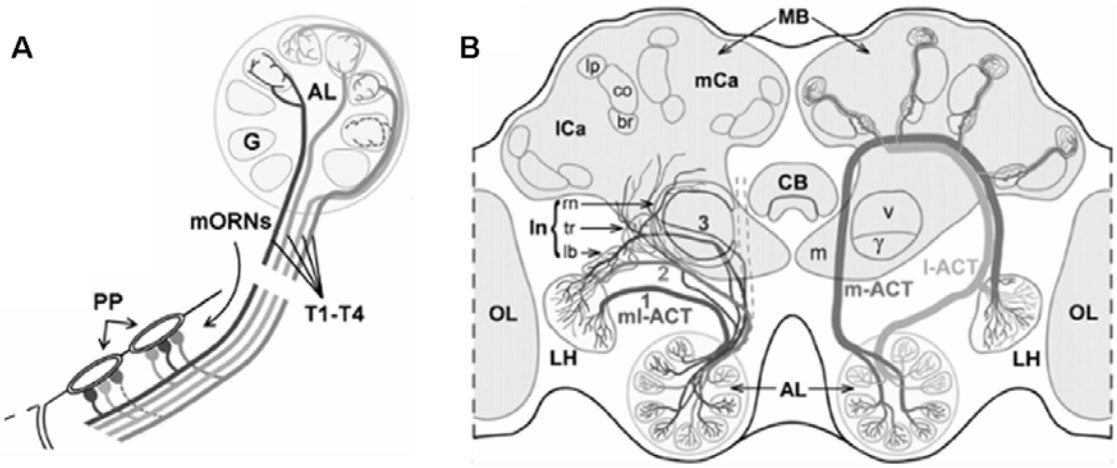

Figure 4: Schematic overview of the olfactory pathway of Hymenoptera. **A:** Olfactory sensilla (pore plates) of the honeybee, equipped with multiple-ORNs. The ORNs project to the functional units (glomeruli) of the antennal lobe. **B:** Projection neurons connect the antennal lobe with the higher integration areas, the mushroom body and the lateral horn. On the left side, the projection of the multiglomerular projection neurons (ml-ACT) is shown. The right side shows the two other projection neurons tracts, the medial (m-ACT) and the lateral (l-ACT) antenno-cerebral tract. AL = antennal lobe; G = glomerulus; PP = poreplate sensilla, T1-T4 = sensory tracts; mORNs = multiple olfactory neurons; OL = optic lobes; LH = lateral horn, CB = central body; MB = mushroom body; lCa = lateral calyx; mCa = medial calyx [Kleineidam and Rössler, 2009].

1.6. Goals of this study

My PhD-project focuses on the neuroanatomical basics of social organization in the polymorph worker caste of leaf-cutting Attini and on behavioral differences in different sized *Atta vollenweideri* workers. Additionally, the neuroanatomy of the whole tribe of Attini was studied, in order to find the origin of different neuroanatomical characters. The high level of socialization in the leaf-cutting Attini and the obvious ecological success of these species bring up the question, how this success is achieved and what the underlying mechanisms at the level of workers are. Three main subjects were investigated:

1.6.1. Behavioral differences in polymorph workers

Do different sized workers behave differently?

Differences in olfactory guided behavior were quantified for the polymorph worker caste in *A. vollenweideri*. The study focuses on the trail-following behavior, which is one of the most important tasks a leaf-cutting worker has to perform. This task can generally be performed by all workers. When testing the trail following behavior of different sized workers, it has to be taken into account that eventually found differences are not only based on advantages that larger workers have according to their size (larger legs, larger antennae) but on their abilities to detect and follow the trail pheromone. An artificial trail of the releaser component of the trail-pheromone in decreasing concentration was used to test the trail-following performance of individual workers.

1.6.2. Neuroanatomical differences in polymorph workers

What are the neuroanatomical differences of different sized workers of the polymorph worker caste in *Atta vollenweideri*?

To answer this question I focused on neuroanatomical studies to find, beside the macroglomerulus, more differences in the olfactory system of different sized workers. Therefore I analyzed whether the glomerular numbers are related to worker size.
For further investigations of the olfactory system, I also analyzed mass stainings of all ORNs and stainings of single olfactory sensilla as well as the number of olfactory sensilla. For the

analyses of anatomical differences, all sizes of workers have been investigated, ranging from smallest fungus gardening workers to large leaf-cutting individuals.

This part of my PhD-thesis is based on the following manuscript:
Kelber C, Rössler W, Kleineidam CJ: Phenotypic plasticity in number of glomeruli and sensory innervation of the antennal lobe in leaf-cutting ant workers (*A. vollenweideri*); Developmental Neurobiology; submitted.

1.6.3. Evolutionary origin of neuroanatomical differences

What is the origin of different characters in the olfactory system of the Attini?

This part of my project concentrates on the evolutionary origin of the macroglomerulus and the number of glomeruli in the antennal lobe. I compared the number, volumes and position of the glomeruli in the antennal lobe of 25 different species from all three major Attini groups (lower, higher and leaf-cutting Attini).

This part of my PhD-thesis is based on the following manuscript:
Kelber C, Rössler W, Roces F, Kleineidam C (2009): The antennal lobes of fungus-growing ants (Attini): neuroanatomical traits and evolutionary trends; Brain Behav Evol 73:273-284.

2. Behavioral plasticity and the correlation to neuroanatomical differences in the polymorph worker caste of leaf-cutting ants (*Atta vollenweideri*)

2.1. Introduction

The ecological success of ant colonies is based on their highly developed social organization and communication between the colony members is fundamental. Within the worker caste different pheromones are used as signals for the nestmates.
In many ant species the ability to follow chemical trails can be observed. Workers can use a trail-pheromone to recruit their nestmates to go to a specific place, for example to a food resource. Several different recruitment systems have been observed, e.g. tandem running or mass recruitment [Hölldobler and Wilson, 1990]. A trail-pheromone system can be found in a high number of ant species in different peculiarity, for example in fire ants, carpenter ants

or harvester ants [Hölldobler and Wilson, 1990]. One of the best developed trail-pheromone systems with trunk trails and clearly visible “highways” from the colony to herbal food resources can be found in leaf-cutting *Atta*. On these trails, thousands and hundred thousands of individuals can be found in some *Atta* species [Hölldobler and Wilson, 1990]. For the detection and processing of pheromonal information, the ant workers need a good olfactory system and indeed, the olfactory sense seems to be a highly developed sense [Kleineidam et al., 2005; Kleineidam et al., 2007; Kelber et al., 2009; Kuebler et al., 2009].

In *Atta* species, most of the workers on the trail are large enough to cut leafs, but also the smallest sized workers can be found outside the nest. It is assumed that the small leaf-cutting ant workers also have the ability to detect the trail-pheromone and follow the trails [Hughes and Goulson, 2001; Morgan et al., 2006; Evison et al., 2008]. Recent experiments showed that the trail-following performance of small workers differs from those of large workers. Different sized *A. vollenweideri* workers were tested on a discrimination test, in which both the conspecific and a heterospecific trail-pheromone was tested on a y-maze. Small workers preferred the conspecific trail over the heterospecific trail. Large *A. vollenweideri* workers showed no significant preference and it is thought, that those large workers primarily respond to the releaser component present in both trails, whereas small workers focus more on the conspecific traits provided by the blend of components [Kleineidam et al., 2007]. Additionally, neuroanatomical differences have been found in the trail-pheromone processing structures of the different sized *A. vollenweideri* workers. An enlarged glomerulus (macroglomerulus, MG) was found in the antennal lobe (AL) of large workers (head width larger than 1 mm) only. Therefore, two different antennal lobe phenotypes exist, with MG (MG-phenotype), and with only a regular glomerulus (RG-phenotype) [Kuebler et al., 2009]. Calcium imaging experiments showed, that in this MG, the releaser component of the *A. vollenweideri* trail pheromone (methyl-4-methylpyrrole-2-carboxylate M4MP2C) was processed [Kleineidam et al., 2005; Kuebler et al., 2009].

In this study I ask the question, if there are differences in the trail-following performance of large and small workers according to the trail-pheromone concentration. Are larger workers, which are specialized on leaving the colony and using the trails to the food resources able to

detect the trail-pheromone in lower concentrations than small workers? I tested the trail-following performance of different sized workers on artificial trails.

2.2. Methods

2.2.1. Animals

I used *A. vollenweideri* workers from a laboratory colony. The founding queen was collected in Formosa, Argentina in 2005 from M. Bollazzi. The colony was reared in an environmental chamber at 26°C and at a humidity of 50%. Five plastic boxes (19x19x19 cm) interconnected with plastic tubes provided an artificial nest. The workers were allowed to forage fresh leaf material (*Rosa canina*) across a wooden bridge (~1m long, 5 cm width) to an additional feeding box (19x19x19 cm). Each morning, the wooden bridge was connected with the colony and the workers had two hours to establish the trail from the nest to the feeding box. Single ants were branched of the trail with a wooden stick (5 cm long), which was laid on the trail before and was treated by the ants as substrate on which they presumably deposited the trail pheromone. The single workers where then transported to the starting point of two different experiments on the experimental area (Fig. 5).

2.2.2. Experimental area

The behavioral experiments were conducted on a DIN A4 sized piece of paper. On one edge of the paper, a piece of toothpick (2 cm) was fixed; this was the starting point for each worker. On the piece of paper, a trace of trail-pheromone was laid in different forms. I used the releaser component of the *A. vollenweideri* trail-pheromone, M4MP2C, diluted in different concentrations in hexane. All trails were laid using a Hamilton syringe, and 10 µl dilution was used for ~9 cm trail. The syringe was washed carefully with pure hexane between each step. I used a new piece of paper with a fresh laid trace for every single worker in the experiments.

2.2.3. Trail-following experiments

The first experiment was used to analyze differences in the trail following behavior due to concentration-dependent reasons. For this experiment, the artificial trail was laid on the paper in a zigzag way, so that the trail consisted of 7 equal sized sections, 4.5 cm long and six 90 degree turnings (Fig. 5A). For the group which was tested on equal concentration

(Con_{equal}), the trail was laid at a concentration of 10^{-5}. For the group which was tested on a trail with decreasing concentration (Con_{dec}), the start concentration was 10^{-5} and at each turning, the concentration decreased by a factor of 10. After the test, the head width of the workers was measured between the outer edges of the eyes.

A second trail following experiment was used to detect differences between workers with or without an MG (with a range from 0.5 to 1.69 mm head width). For this experiment I used a sinusoidal trail form to follow, which seems to be an easier task for the very small workers than 90 degree turnings. Each segment of the wave was 12 cm long. The start concentration was 10^{-5} and at the middle of each wave (sees lines in Fig. 5B) the concentration decreased by a factor of 10. The concentration changed not on the turning points, but on even parts of trail, this also alleviates the task compared to the zigzag way. The trail consisted of five waves. After the test, the head width of the workers was measured between the outer edges of the eyes.
The data of both experiments were analyzed with the help of Statistika 7.1 (StatSoft, Tulsa, USA).

2.2.4. Preparation

After the second experiment, I analyzed if the workers had a MG in the antennal lobe. Therefore, the heads were cut open and the brains were dissected in trehalose ringer (127 mM NaCl, 7 mM KCl, 1.5 mM $CaCl_2$, 0.8 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.8 mM TES, 3.2 mM Trehalose). The brains were dissected and then immediately transferred to ice-cold Fix-Mix (2% Formaldehyde/2% Glutaraldehyde) and stored for 5-7 days at 4°C. Brains were then rinsed in PBS (3 times 10 minutes), dehydrated in an ascending series of ethanol (50, 70, 80, 90, and 95% and 2 times 100%, 10 minutes each) and finally transferred to methylsalicylic acid (M-2047, Sigma-Aldrich, Steinheim, Germany). Glutaraldehyde fixation intensifies the autofluorescence of the brain and allows confocal analyses and 3D-reconstructions of neuropiles without any additional staining. The brains were examined with a laser-scanning confocal microscope (20x 0.7, Leica TCS SP2 AOBS, Leica Microsystems AG, Wetzlar, Germany).

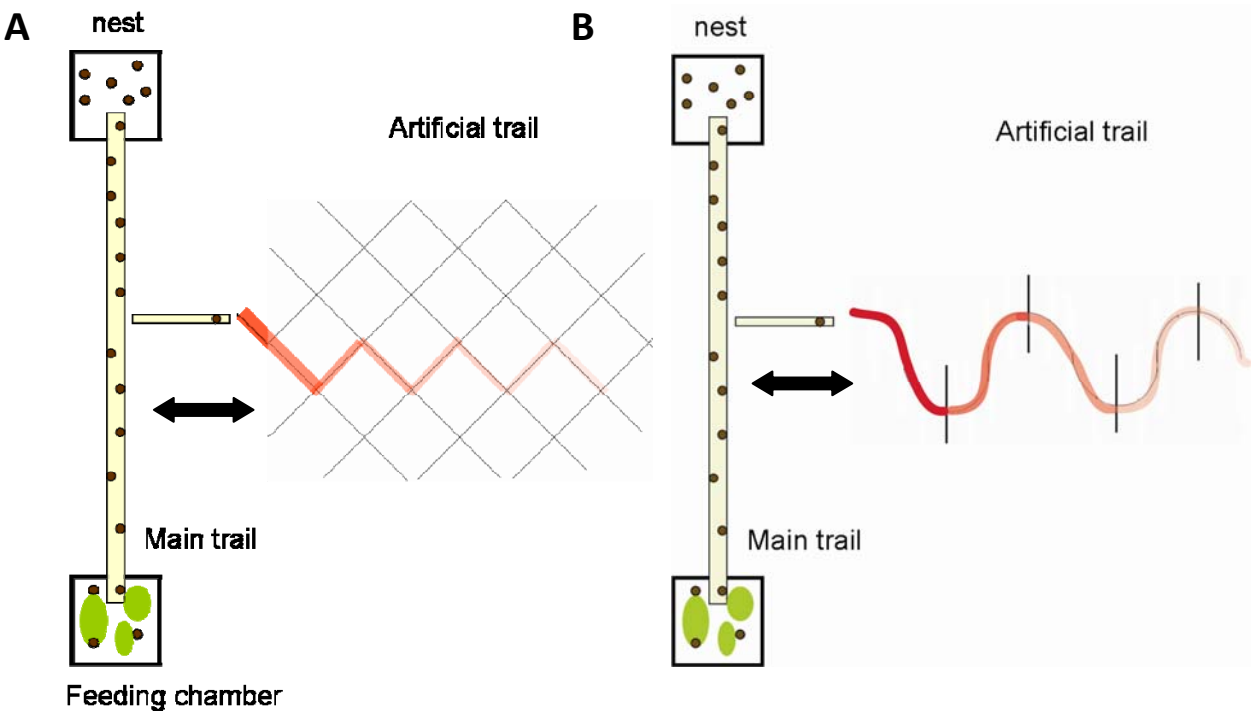

Figure 5: Setup of two different trail-following experiments. A laboratory colony of *Atta vollenweideri* (nest) was connected via a wooden, ~1 meter long bridge (main trail) with the feeding chamber, where fresh dog rose leafs where presented. The main trail was newly established every day and after two hours, single workers of different size were transported to the testing area with the artificial trails. **A**: In the first experiment, the artificial trail of the releaser component (M4MP2C) of the *A. vollenweideri* trail-pheromone was laid in a zigzag way. One group was tested with an equal concentration; one group was tested with a trail in decreasing concentration. **B**: In the second experiment, different sized *A. vollenweideri* workers were tested on a sinusoidal trail with a decreasing concentration of the releaser component of the trail pheromone. For all experiments, the trail was newly laid on a fresh piece of paper for every single ant.

2.3. Results

2.3.1. First experiment: Trail-following performance is dependent on trail-pheromone concentration

Workers of different sizes can be found on the trail. The trail-pheromone has to be on the ground in a adequate concentration that it can be detected from the workers, I tested different sized workers on a trail with constant (Con_{equal}) and on a trail with decreasing

(Con_{dec}) concentration of the releaser component of the trail-pheromone, to find out if the trail-following performance is dependent on the concentration of trail-pheromone. For two groups I tested 69 individual workers with a head width from 1.43 to 2.76 mm. Figure 6 shows that the trail-following performance is significantly lower (Mann-Whitney-U-test, $p<0.001$) in the Con_{dec} group. The median way which was covered by the Con_{equal} group was six turnings while in the Con_{dec} group, only a median way of three turnings was covered. This experiment shows clearly that the concentration of the trail-pheromone trace is important for the detection.

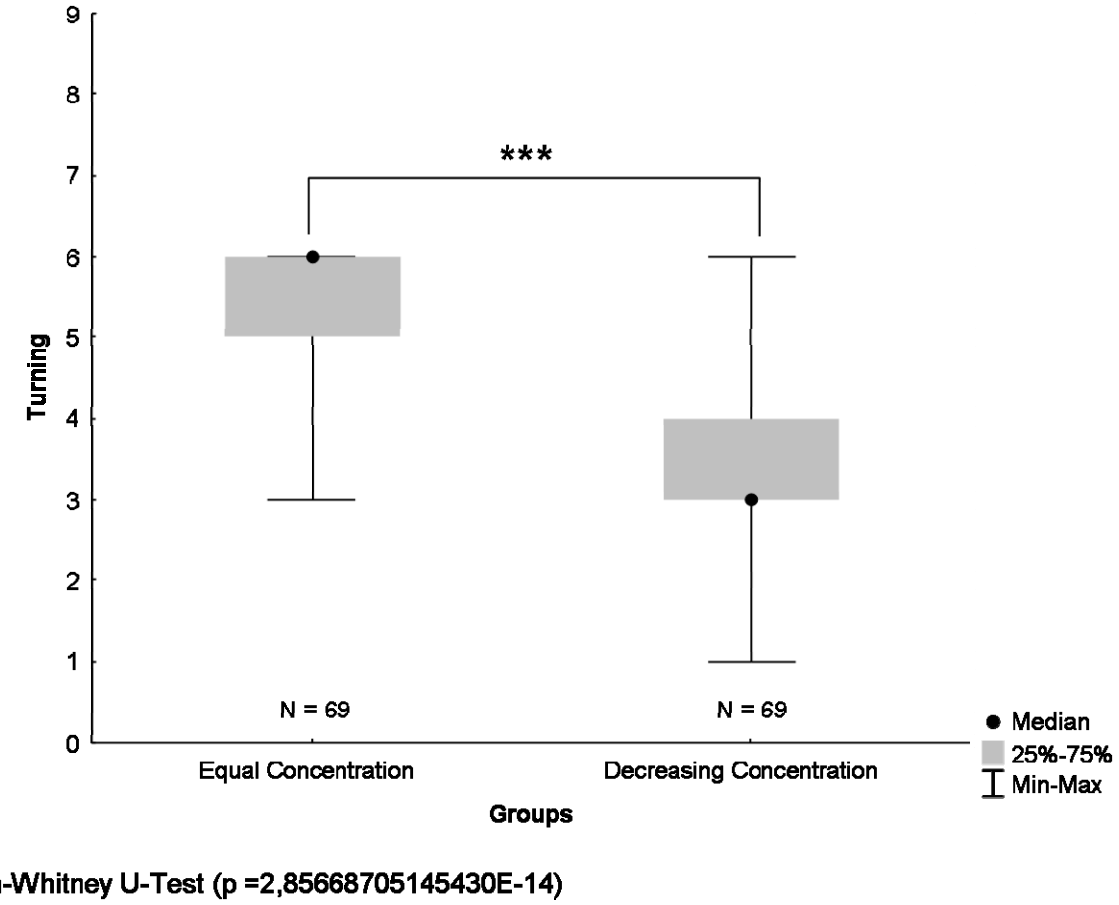

Figure 6: In the first experiment, the trail-following performance of workers on a trail with equal trail-pheromone concentration was compared to workers following a trail with decreasing trail pheromone concentration. 69 Workers of a broad range of sizes were tested for both groups on a zigzag trail, using the releaser component (M4MP2C) of the trail pheromone. The trail-following performance was significantly better (median 6 turnings), when the concentration was constant along the trail (Mann-Whitney-U-test, $p<0.001$). When

the concentration was decreasing with the factor of 10 each 90 degree turning, the trail-following performance was much weaker (median 3 turnings).

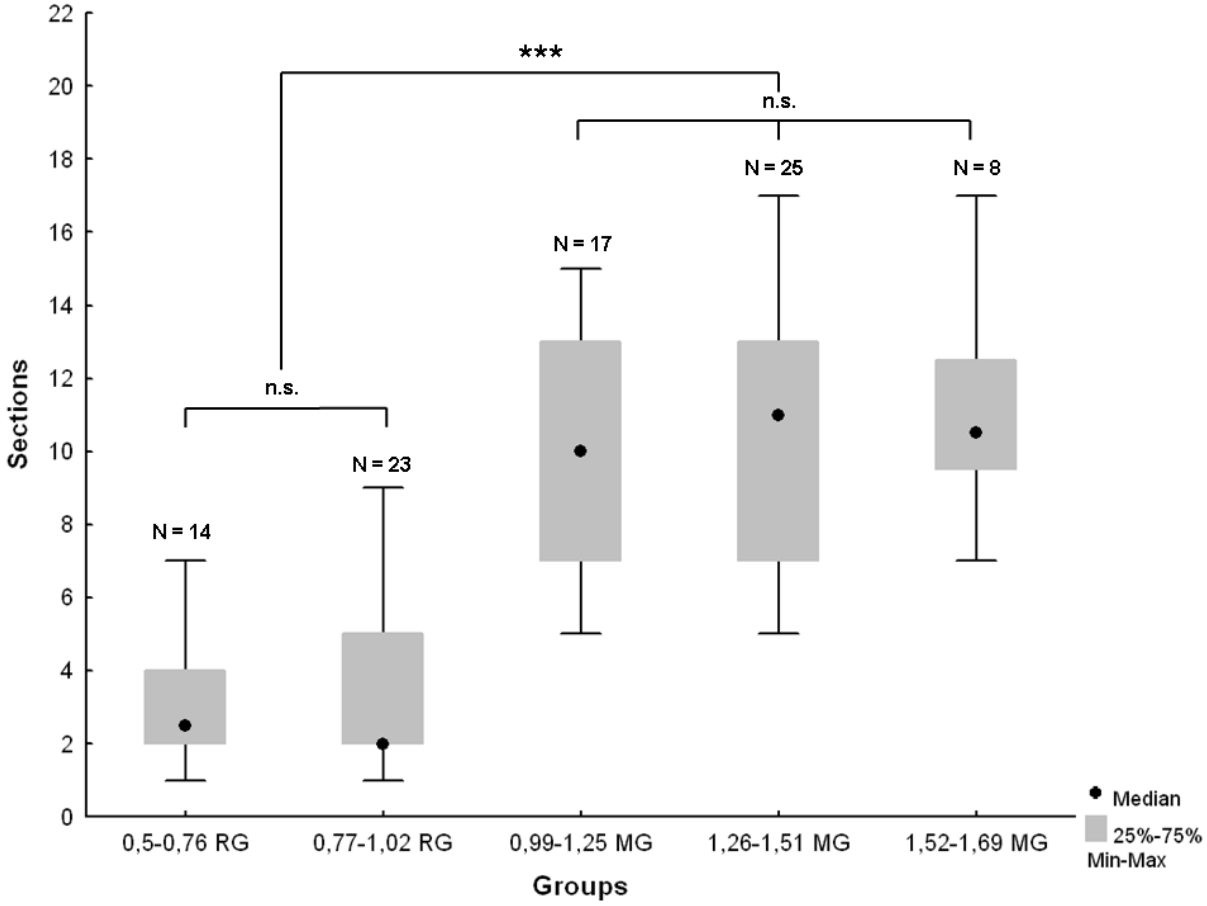

Figure 7: In the second experiment, the trail-following performance of workers of different size and different neuroanatomy was tested on a trail with decreasing trail-pheromone. I used a sinusoidal trail to test, if the size of the worker or the existence of an enlarged glomerulus for the processing of trail-pheromone information (macroglomerulus, MG) has an influence on the trail-following performance. I tested 87 workers in 5 different groups according to their size and according to the MG- or RG-phenotype. The size classes and the phenotype (RG/MG) are given on the x-axis. In the two groups of smallest workers, the workers don't have an MG and these workers have a significantly lower trail-following performance than the workers of the three other groups which have an MG (Kruskal-Wallis-test, $p < 0.001$). The trail following performance of the workers is related to body size; however the occurrence of an MG seems to have a greater impact than the body size.

2.3.2. Second experiment: Trail-following performance is dependent on the existence of a macroglomerulus

The 90 degree turning in the first experiment is a task which is difficult to perform for the workers of *A. vollenweideri*. Therefore I used another trail form, a sinusoidal curve for the next experiment. I analyzed, if there are differences in the trail-following performance in different sized workers. I also investigated, if these differences are dependent on the size or on the AL-phenotype of the worker (RG or MG). I tested 87 workers with a head width from 0.5 to 1.69 mm. I divided the tested workers into 5 different groups according to their size and added the information about the existence of a MG (Fig. 7). All five groups covered a range of 0.26 mm head width. The first group consisted of the 14 smallest workers with a head width from 0.50 to 0.76 mm. In the second group, there are 23 workers with a head width from 0.77 to 1.02 mm. In both groups, the RG-phenotype was found in the AL. The third group contained 17 workers with a head with from 0.99 to 1.25 mm, the fourth group 25 workers from 1.26 to 1.51 mm head width and the last group 8 workers with a head width from 1.52 to 1.69 mm. In these three groups, all workers showed the MG-phenotype. There is no significant difference in trail-following performance between both RG-groups and there is also no significant difference between the three MG-groups. We found a highly significant difference in the trail-following performance of workers between the RG- and the MG-groups (Kruskal-Wallis-test, $p < 0.001$). Workers with an MG could walk a much larger part (in average about 11-12 sections compared to 2-3 sections) of the decreasing trail. The existence of the MG enables workers to follow the releaser component of the trail-pheromone in smaller concentrations.

2.4. Discussion

I described the trail-following performance of different sized workers of *A. vollenweideri.* The trail-following performance is dependent on the concentration of the trail-pheromone. The trail-following performance of the polymorphic workers is also depended on the existence of the MG in the AL. The behavioral paradigms used were suitable for analyzing the trail-following performance. I used only a single component of the trail-pheromone for testing but since this substance (M4MP2C) is the releaser component of the trail-pheromone of *A. vollenweideri*, this substance was sufficient to trigger the trail-following behavior.

2.4.1. Small workers on the foraging trail

When a foraging trail of leaf-cutting Atta is observed, also workers with a head width smaller than 1 mm can be found on the trail, although the medium size of workers, a suitable size to cut leaves, is the most dominant. But up to 10% of the workers found on the trails belong to the smallest size of workers [Hughes and Goulson, 2001]. For the existence of the smallest workers on the trail – not able to help by the leaf-cutting and transporting task - several possible explanations can be found in the literature. One discussed behavior of the smallest workers on the trail is the "hitchhiking" of those workers on leaf fragments carried by larger workers [Eibl-Eibesfeld and Eibl-Eibesfeld, 1967]. Several hypotheses for this hitchhiking behavior have been proposed, from which the theory that small workers defend the larger workers against parasitoids like phorid flies (Diptera, Phoridae) is the most accepted one [Eibl-Eibesfeld and Eibl-Eibesfeld, 1967; Feener and Moss, 1990]. Other explanations are, that the smallest workers reduce their transport costs on the trail [Stradling, 1978], or that they prepare the leaf fragments "on the way" for the fungus and collect plant fluids [Linksvayer et al., 2002; Yackulic and Lewis, 2007]. Although the hitchhiking behavior is described for seven *Atta* species, there is only a small percentage of hitchhiking workers. The majority of the smallest workers, which can be found on the foraging trail walks by their own. One possible explanation for their attendance on the foraging trail is a key functional role in maintaining the pheromone trail. It is shown that those smallest workers have a higher pheromone-laying frequency than larger workers [Evison et al., 2008]. But until now, the function of the smallest workers on the foraging trail within the social organization of the colony is not fully understood. My result of different trail-following ability in different sized workers adds to this complexity of the foraging and trail-using process.

2.4.2. Different trail-following performance in polymorph workers

The trail-following ability differs in different sized workers in *Atta sexdens*. For this species it was shown, that smaller workers (head width 1.2 - 1.95 mm) and extremely large workers (head width 3.0 – 3.95 mm) have a better trail-fidelity , which means trail-following performance that large workers (head width 2.0 – 2.95 mm) [Morgan et al., 2006]. In my study on *A. vollenweideri* workers, I added more information about the trail-following performance: the behavior of the group of smallest workers with a head width smaller than

1.2 mm and the comparison with the neuroanatomy of the workers. My study reveals that workers which show the MG-phenotype are better in discriminating the releaser component of the trail-pheromone than workers with the RG-phenotype. Similar neuroanatomical differences can be assumed in *A. sexdens* workers, where an MG is also known in large workers [Kleineidam et al., 2005; Kelber et al., 2009]. Not the size of the worker allone has the major influence on its trail-following ability, but also the size of the brain-area which is processing the pheromonal information and the possible higher number of ORNs.

2.4.3. Behavioral plasticity in the polymorph worker caste

The polymorph workers of the leaf-cutting ant *A. vollenweideri* show a pronounced plasticity in trail-following behavior. Behavioral plasticity leads to distinct task allocation which results in adaptive and flexible colony responses [Beshers and Fewell, 2001]. I show here that the basis for the behavioral plasticity is the underlying neuronal plasticity. There are other examples for correlations between neuronal plasticity and behavior, e.g. differences in the volume of different neuropiles between reproductive and non-reproductive workers in *Harpegnathos saltator* [Gronenberg and Liebig, 1999]. Another example is the mushroom body volume in the paper wasp *Polistes instablis*, which is correlating with the social aggression behavior [Molina and O'Donnell, 2007].

One of the main tasks of large workers, according to their larger size and larger mandibles, suited for the cutting of leafs, is the search for food resources and their exploitation. Therefore, the detection and usage of pheromonal trails is highly advantageous for them. For the smallest workers, which are specialized in taking care of the brood and the fungus, this ability is not so necessary. The evolution of a better detection and processing system for the trail-pheromone in large workers is the result of the polymorphism and division of labor in the worker caste.

3. Phenotypic plasticity in number of glomeruli and sensory innervation of the antennal lobe in leaf-cutting ant workers (*A. vollenweideri*)

3.1. Introduction

The enormous ecological success of social insects is based mainly on division of labor with workers specialized to various degrees for foraging, nest defense and other tasks, and often only a single queen monopolizes reproduction. In some ant species, the worker caste is highly polymorph, and this is associated with an elaborated social organization of the colony. Worker polymorphism mainly results from differences in environmental conditions during postembryonic development, and is influenced only to a little extent by genetic differences between workers [West-Eberhard, 2005; Hughes and Boomsma, 2007]. Size-variation and variations in morphological characters of workers may constrain their behavioral repertoire. However, neuronal plasticity during development probably is the predominant mechanism underlying distinct differences in behavioral repertoires of polymorph ant workers [Kleineidam et al., 2005; Gronenberg, 2008; Kuebler et al., 2009].

Leaf-cutting ant workers of *Atta vollenweideri* exhibit an enormous size polymorphism (~200-fold variation in body mass) [Weber, 1972; Wilson, 1980a], and worker size is related to behavior and task allocation (alloethism). Small workers take care of brood and fungus cultivation, and large workers are specialized in leaf-cutting and transport. The sense of smell is the most prominent sensory modality in leaf-cutting ants, and many behaviors of the workers, like foraging, are olfactory guided [Weber, 1972; Hölldobler and Wilson, 1990; Hölldobler and Wilson, 2009]. Several behavioral studies show how sensitive and fine-tuned the odor responses in leaf-cutting ants are [Tumlinson et al., 1972; Andryszak et al., 1990; Kleineidam et al., 2005; Morgan et al., 2006; Kleineidam et al., 2007]. It is therefore not surprising, that the olfactory system of leaf-cutting ants is well developed and occupies a large proportion of the entire brain [Kuebler et al., 2009]. Odors are detected by numerous hair sensilla on the antennae. The majority of olfactory sensilla are long, thin and elbow-bend hairs (Sensilla trichodea curvata) and a smaller number of thick and short hairs with socket (Sensilla basiconica) also serve olfactory function [Kleineidam and Rössler, 2009; Nakanishi et al., 2009]. Olfactory sensilla in Hymenoptera are equipped with a high number of olfactory receptor neurons (ORNs) [Schneider and Steinbrecht, 1968; Dumpert, 1972;

Esslen and Kaissling, 1976; Stepper et al., 1983; Butterfield and Anderson, 1994; Ochieng et al., 2000; Isidoro et al., 2001; Kelber et al., 2006]. Their axons project to the first olfactory neuropil - the antennal lobe (AL) – where they are sorted into several sensory tracts and terminate in the functional units of the AL, the glomeruli. This organization results in a spatial representation of odors with distinct glomerular activation pattern as shown e.g. with functional imaging techniques in the honeybee, and more recently also in the leaf-cutting ant (*Atta vollenweideri*) [Joerges et al., 1997; Galizia et al., 1999b; Sachse et al., 1999; Kuebler et al., 2009]. The AL of hymenopteran species (ants, wasps and bees) contain a high number of glomeruli [Goll, 1967; Arnold et al., 1985; Flanagan and Mercer, 1989; Galizia et al., 1999a; Smid et al., 2003; Hoyer et al., 2005; Nishikawa et al., 2008; Zube et al., 2008; Kelber et al., 2009], whereas the AL of other insects (except orthopteran species, which have a different organization of their ALs) have less than 100 glomeruli and differ in AL tract morphologies [Stocker, 1994; Laissue et al., 1999; Huetteroth and Schachtner, 2005; Ignell et al., 2005; Schachtner et al., 2005; Galizia and Rössler, 2010].

Recently, two size-related AL-phenotypes have been described for workers of the leaf-cutting ant (*Atta vollenweideri*): An MG-phenotype (containing a macroglomerulus) and an RG-phenotype with all glomeruli of regular size. This neuroanatomical polyphenism is established during pupal development and separates the worker caste into two neuroanatomical sub-castes [Kleineidam et al., 2005; Kuebler et al., 2009]. Correlated with these two AL-phenotypes, workers differ in their behavioral response to the trail pheromone [Kleineidam et al., 2007]; Kelber, unpublished data). The releaser component of the trail pheromone is represented in the MG and that suggests alternative information processing dependent on the presence of the MG [Kuebler et al., 2009]. In the same study, a relatively large variance in the number of glomeruli, ranging from 396 to 442 has been described.

In the present study, we quantified the variance in glomerular number by investigating many *A. vollenweideri* workers of different size and subsequently we analyzed whether glomerular number is related to workers' body size. We found fewer glomeruli in small workers and analyzed whether the number of glomeruli correlates with the existence of an MG (MG-phenotype).

In order to describe the organization of the antennal lobe according to the sensory tracts from the antenna, we conducted mass-stainings of ORNs and analyzed which clusters contain less glomeruli in small workers. Using selective staining of single sensilla (S. basiconica and S. trichodea curvata) and their associated olfactory receptor neurons, we analyzed the sensilla specific innervation of glomerular clusters. Finally, we assessed the size related variance in number of S. basiconica on the antenna and compared this number to the volume of the corresponding glomeruli in the AL.

3.2. Methods

3.2.1. Animals

We used *Atta vollenweideri* workers from a laboratory colony. The founding queen was collected by M. Bollazzi in Formosa, Argentina in 2005. The colony was reared in an environmental chamber at 25°C and at a humidity of 50% in a 12h/12h photoperiod. The colony was fed mainly with dog rose (*Rosa canina*) and with privet leaves (*Ligustrum vulgaris*).

3.2.2. Preparation of the brains

Prior to neuroanatomical investigations, the body size of the investigated workers was measured. As a universal measure of body size we used the head width (H_w) of workers to allow comparisons of different neuroanatomical characters within the polymorphic worker caste. The head capsule was opened and the brain was dissected in saline solution (127 mM NaCl, 7 mM KCl, 1.5 mM $CaCl_2$, 0.8 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.8 mM TES, 3.2 mM Trehalose). After dissection, brains were immediately transferred to ice-cold Fix-Mix (2% paraformaldehyde/2% glutaraldehyde) in phosphate-buffered saline (PBS, pH 7.2) and stored for 3-5 days at 4°C. This fixation leads to increased autofluorescence which, compared to solely formaldehyde fixation, allowed us to better identify e.g. the outlines of glomeruli.

The brains were then rinsed in PBS (3 times 10 minutes) and dehydrated in an ascending series of ethanol (50%, 70%, 80%, 90%, 95% and 3 x 100%, 10 min. each) and finally transferred into methylsalicylic acid (M-2047, Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

3.2.3. Confocal microscopy and 3D-reconstruction

Confocal image stacks at a resolution of 1024 x 1024 pixel and optical sections of 1 µm depth were taken of the ALs in whole mount preparations with a laser-scanning confocal microscope (Leica TCS SP2 AOBS, 20 x 0.7 NA lens, Leica Microsystems AG, Wetzlar, Germany). 3D-analyses software (AMIRA 3.1.1, Mercury Computer Systems, Berlin, Germany) was used for 3D-reconstruction of individual glomeruli within the AL, the AL as a whole and the antennal nerve. Based on the measured volume of each glomerulus, we calculated the radius of a sphere with the same volume, and in the following we use this value (R_G) as a measure of glomerular size.

3.2.4. Number and size of glomeruli

For 28 workers with H_w ranging from 0.59 mm (small, fungus gardening worker) to up to 3.4 mm (large worker) each of all glomeruli within one AL were 3D-reconstructed. As we found that the number of glomeruli is related to H_W, similarly to the result in our previous study that the MG is related to H_W [Kuebler et al., 2009], we analyzed whether glomerular number is related to the presence or absence of an MG. Therefore, we calculated the radius value (R_{Vmax}) of the largest glomerulus (R_{Gmax}) the following way: $R_{Vmax} = (R_{Gmax} - R_{Gmean}) / SD$. R_{Vmax} describes how much bigger the largest glomerulus is compared to the mean size of a glomerulus (R_{Gmean}), and this with respect to the variance of glomerular volumes (SD). As criterion to define a glomerulus as a macroglomerulus, we set $R_V > 5$ [Kelber et al., 2009].

3.2.5. Clusters of glomeruli in the antennal lobe

The AL is subdivided into clusters of glomeruli and each cluster is innervated by one of several sensory tracts of receptor-neuron axons [Kirschner et al., 2006; Zube et al., 2008]. We counted the number of glomeruli in each cluster and compared small and large workers. To this aim, four workers (two large and two small, with H_w from 0.68 to 3.16 mm) were immobilized in a plexi-glass holder, and one antenna was cut off at the base of the scapus. A droplet of tetramethylrodamine-dextran (“micro-ruby”, D-7162, Molecular Probes, Eugene, USA), dissolved in distilled water was applied in a ring of petroleum jelly on the cut base and left in place for one hour. After staining, workers were allowed to move freely for 4 hours before the brains were dissected in saline solution followed by fixation and dehydration as

described above. Subsequently, confocal image stacks were taken and the antennal nerve, the sensory tracts and all glomeruli were reconstructed. The data on the number of glomeruli of these four workers are part of the 28 workers analyzed as described above. The reconstruction of the AL of one large worker was used as a template map to identify in which glomerular cluster each of the ORNs from selectively stained sensilla terminate.

3.2.6. Selective sensilla stainings and projection of their ORN axons in the AL

Stainings of the ORNs of single olfactory sensilla allowed us to investigate their axonal innervation patterns in the AL. Workers with H_w larger than 1.5 mm were immobilized in a plexi-glas holder with dental wax (surgident periphery wax, Heraeus Kulzer, Germany) and the right antenna was fixed with white-out correction fluid (Tipp-Ex, Bic, France). A glass electrode mounted on a Piezo-element that was connected to a function generator was used to cut the sensilla with the vibrating tip of the electrode. The tips of several hair-shaped sensilla were removed with this technique. Immediately after cutting off the sensilla, a second glass electrode filled with dextran-biotin (D-7135, Molecular Probes, Eugene, USA) dissolved in distilled water was slipped over the stump of one single sensillum. After 30 min, the staining electrode was removed and the ants were allowed to move freely for 8-10 hours. Then, the head was cut off and the brain was dissected in saline solution. After fixation, the brains were incubated for 48 hours in Alexa 488-conjugated streptavidin (S-11226, Molecular Probes, Eugene, USA) in PBS with 0.2% TritonX (1:125), and subsequently rinsed in PBS (3 x 10min.). In the following, the brains were treated and investigated as described above.

We reconstructed all glomeruli with stained arborizations of receptor neurons and assigned them to the different clusters according to the sensory tracts, or, in cases where this was not possible, we used the AL of the large worker (H_W = 3.16) in which we reconstructed all sensory tracts and identified all clusters of glomeruli as a template map. For visualization, the stained ORNs of one S. trichodeum curvatum and one S. basiconicum were reconstructed by using the skeleton-tool of AMIRA 3.1.1.

3.2.7. Allometry between number of sensilla and size of glomeruli

We assessed the relation between the number of one particular type of sensilla (S. basiconica) and the volume of the corresponding glomeruli in 12 workers with H_w ranging

from 0.76 mm to 3.48 mm. The S. basiconica of the segment next to the antennal tip (second distal segment) were used as a measure because they are easy to identify and to distinguish from all other hair-shaped sensilla based on their external morphology. The antennae were cut off, fixed with dental wax (surgident periphery wax, Heraeus Kulzer, Germany) on a plexi-glass holder and investigated by using epifluorescent illumination (568 nm) on a microscope (Olympus, BX51WI, XLUMP LD 20 x NA 0.95, immersion lens). Overview pictures of the second distal antennal segments were taken with a CCD-camera (model 8484-03G, Hamamatsu Photonics, Japan) that was mounted on the microscope. From these pictures, length and diameter of the segment was measured and the surface area calculated. Subsequently, two close-up pictures from each side of the segment were taken to identify and count the S. basiconica in an area of 18528 μm^2 in the smallest and 90497 μm^2 in the largest worker. Based on these measurements, the total numbers of the S. basiconica was estimated for the entire surface area of the second distal segment (N_{Sb}).

For 20 workers (H_w ranging from 0.59 to 3.40 mm) that were selected from the 28 workers in which the number of glomeruli have been analyzed, the volume of the 50 most posterior glomeruli of the T6-cluster was measured. As we will show later, the S. basiconica innervate exclusively glomeruli of the T6-cluster. We calculated the relation between H_w and the mean size of the measured glomeruli (R_{Gmean} of $T6_{50}$), and used this relation to estimate R_{Gmean} of $T6_{50}$ for the 12 workers in which we assessed the number of S. basiconica.

Statistical analyses were done using Statistica 7.1 (StatSoft, Tulsa, USA), which was also used to plot the data.

3.3. Results

3.3.1. Number and size of glomeruli

We investigated the number and size of glomeruli in the AL of 28 workers of different size. The total number ranged from 376 to 457 glomeruli, and the size of glomeruli (R_{Gmean}) showed a linear correlation with the body size (H_w) of the workers (Pearson Product Moment correlation; $R_{Gmean} = 3.15 * H_w + 12.04$, $R^2 = 0.88$; $p < 0.01$; $n = 28$; Fig. 8A). The smallest worker (H_w = 0.64 mm) had 401 glomeruli and a mean glomerular volume of only 802 μm^3 compared to the largest worker (H_w = 3.4 mm), which had 457 glomeruli and the mean glomerular volume was with 4824 μm^3 six times larger than in the smallest worker.

The MG-phenotype (R_{Vmax} >5) was found in workers with $H_w \geq 1.00$ mm, and these workers had 424 to 457 glomeruli (mean = 443; SD = 10.18; n = 17). In all of these workers, the number of glomeruli was not related to H_w ($R^2 = 0.08$; $p = 0.27$; n = 17; Fig. 8B). Four RG-phenotype workers (H_w = 0.88 to 1.02 mm) had a similar number of glomeruli (432 to 444), which is well in the range of glomerular numbers found in MG-phenotype workers (Fig. 8C). The remaining workers (all RG-phenotype) had 376 to 392 glomeruli (mean = 383; SD = 5.38; n = 7), which is a significantly lower number than found in the two groups described above (Student's t-test Type 3; $t = -5.09$; $p < 0.01$), and within this group, H_w is positively correlated with the number of glomeruli (number of glomeruli = $28.42 * H_w + 360$; $R^2 = 0.28$; $p < 0.05$). Based on the neuroanatomical trait MG/RG and the distinct number of glomeruli, three AL-phenotypes can be distinguished: MG-HN-phenotype and RG-HN-phenotype with around 443 glomeruli (HN: high number of glomeruli), and an RG-LN-phenotype with fewer glomeruli (LN: low number of glomeruli).

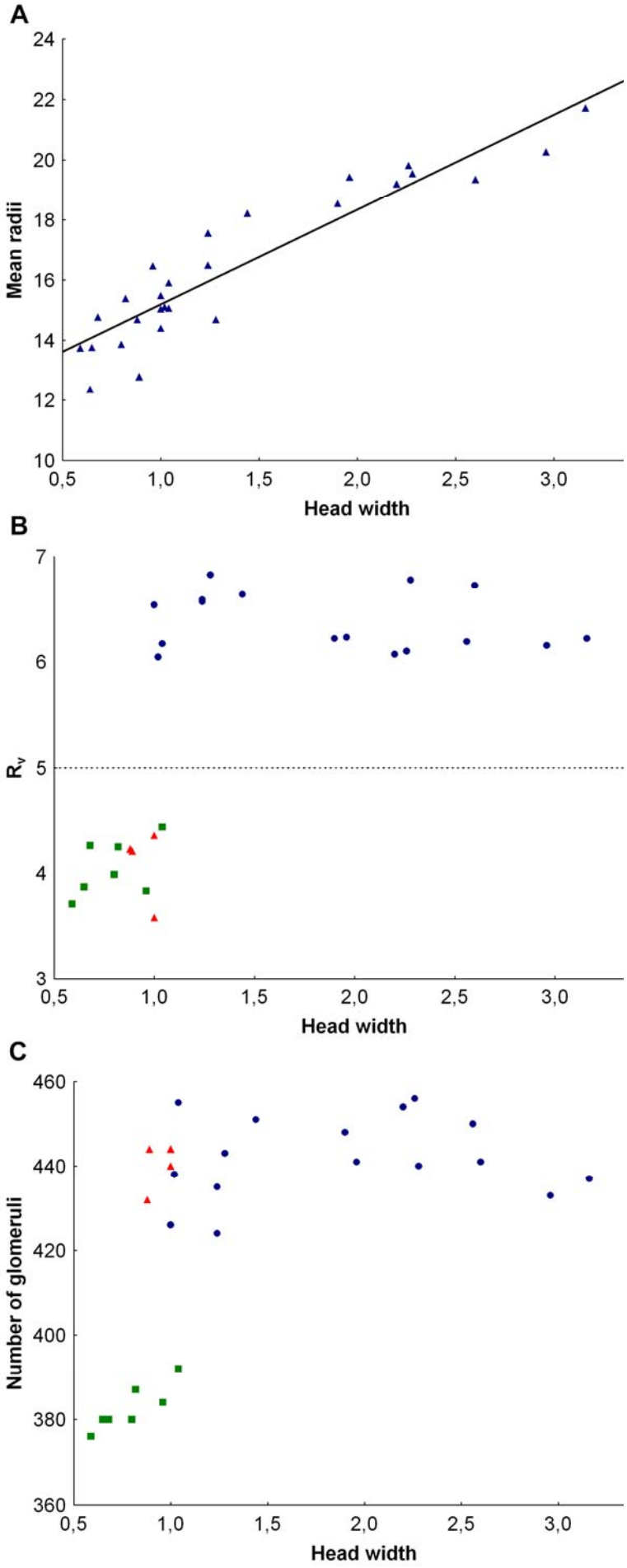
A
Mean radii
24
22
20
18
16
14
12
10
0,5
1,0
1,5
2,0
2,5
3,0
Head width
B
R_v
7
6
5
4
3
0,5
1,0
1,5
2,0
2,5
3,0
Head width
C
Number of glomeruli
460
440
420
400
380
360
0,5
1,0
1,5
2,0
2,5
3,0
Head width

Figure 8: Comparison of the mean radii of the glomeruli (**A**), size of the largest glomerulus (**B**) and the number of glomeruli (**C**) in 28 differently sized workers of *Atta vollenweideri*. **A**: A linear regression describes the relation between head width (H_w) of the workers and the mean size of glomeruli (R_{Gmean} = 3.15* H_w + 12.04, R^2 = 0.88; p < 0.01; n = 28). **B**: For the largest glomerulus within the antennal lobe, the radius value R_V is given as $R_V = (R_L\text{-}R_M)/SD$. A R_V-value of five (dotted line) was used to classify macroglomeruli (MG). All MG-phenotype workers are marked with blue circles and are separated from RG-phenotype workers (green squares and red triangles). **C**: The number of glomeruli is related to body size (H_w). One group with RG-phenotype (green-squares) has fewer glomeruli and thereby separates from a group with or without MG (MG-phenotype: blue-circles; RG-phenotype: red triangles).

3.3.2. Clusters of glomeruli in the antennal lobe

The sensory tracts separate the AL into several clusters, and each of the clusters contains a particular number of glomeruli. We investigated how the clusters differ in number of glomeruli in LN- and HN-phenotype workers. To this aim, we mass-stained receptor neurons anterogradely (Fig. 9A, B) and reconstructed the sensory-tract specific innervation of glomeruli in two LN-phenotype workers and two HN-phenotype workers (Table 1). The antennal nerve divides into six different sensory tracts termed T1-T6. The T1-glomeruli are located in the rostral part of the AL, T2- and T3-glomeruli cover the anterior part of the AL with the T2-glomeruli located more laterally (Fig. 9C-F). T4-, T5- and T6-glomeruli make up the posterior part of the AL. T5- is a small group of glomeruli, and is, like the T4-glomeruli, located more laterally. The T6-cluster is divided from the other posterior glomeruli by the dorsal tract that contains e.g. mechanosensory axons projecting to the dorsal lobe. The T6-glomeruli are relatively small in size, and the T6-cluster contains the highest number of glomeruli (Fig. 9D, F). In five of the six clusters (T1, T2, T3, T5 and T6), we counted only minor differences in the number of glomeruli (2-6 glomeruli, Table 1). In the T4-cluster of the LN-phenotype workers we counted ~50 glomeruli less than in the HN-phenotype workers, which is exactly the same difference we found in our analysis of the total number of glomeruli.

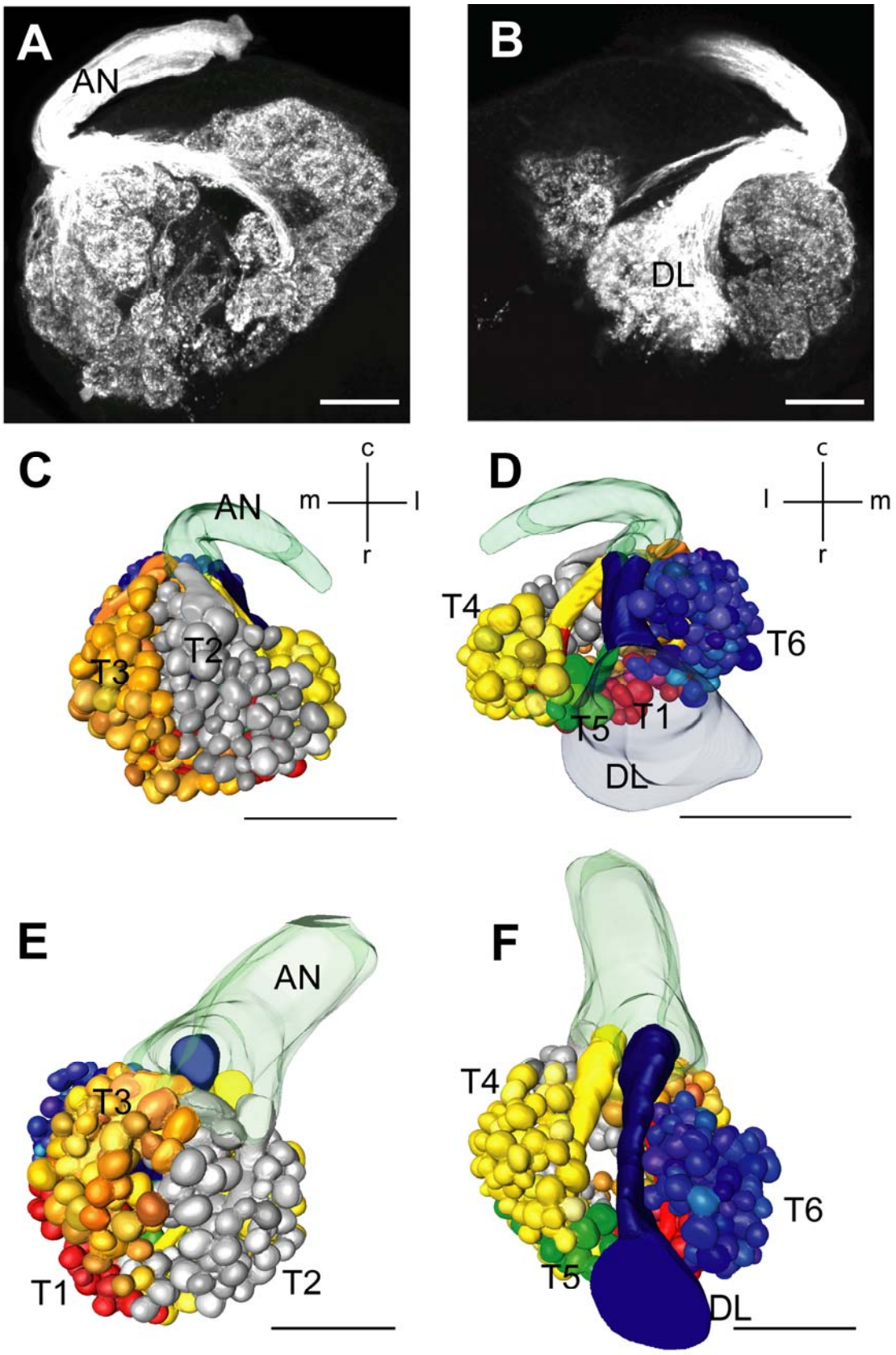

Figure 9: Mass stainings of antennal receptor neurons were used to assign each glomerulus of the antennal lobe to a distinct cluster of glomeruli according to their innervation. **A** and **B**: Two examples of confocal images showing stained ORNs in the AL of a small worker at

different focal depths. The innervated glomeruli, different sensory tracts and the antennal nerve (AN) are clearly visible. Based on such stainings, 3D-reconstructions of all glomeruli were made for a small (**C, D**) and a large worker (**E, F**). The antennal nerve is shown in transparent blue. In order to facilitate visual orientation, the dorsal tract and the dorsal lobe are shown in dark blue. The different clusters of glomeruli associated with the six sensory tracts are indicated in different colors: red = T1, grey = T2, orange = T3, yellow = T4, green = T5, blue = T6, AN = antennal nerve, DL = dorsal lobe, m = medial, l = lateral, c = caudal, r = rostral, Scale = 50 µm.

Table 1: Number of glomeruli in the six glomerular clusters and innervation patterns of different olfactory sensilla

Specimen	T1	T2	T3	T4	T5	T6	Total	Tracts
	Mass-stained sensilla							
Small 1	42	77	87	41	10	122	379	6
Small 2	43	86	85	41	14	115	384	6
Rel. %	**11.15**	**21.36**	**22.54**	**10.74**	**3.15**	**31.06**	**100**	
Large 1	44	81	81	91	13	129	430	6
Large 2	51	84	79	89	17	123	443	6
Rel. %	**10.88**	**18.00**	**18.32**	**20.61**	**3.43**	**28.86**	**100**	
	Sensilla trichodea curvata							
1	6	15	20	5	0	2	48	5
2	2	2	2	2	0	2	10	5
3	2	2	3	0	0	0	7	3
4	1	5	3	1	0	0	10	4
5	0	2	2	1	1	1	7	4
6	0	1	1	2	0	0	4	3
Total	**11**	**27**	**31**	**11**	**1**	**5**	**86**	
Rel. %	**12.79**	**31.39**	**36.05**	**12.79**	**1.17**	**5.81**	**100**	
	Sensilla basiconica							
1	0	0	0	0	0	11	11	1
2	0	0	0	0	0	44	44	1
3	0	0	0	0	0	3	3	1
4	0	0	0	0	0	39	39	1
5	0	0	0	0	0	53	53	1
Rel. %	**0**	**0**	**0**	**0**	**0**	**100**	**100**	
Total	**0**	**0**	**0**	**0**	**0**	**150**	**150**	

Specimen: ORNs of individual workers were either mass-stained, or individual sensilla, S. basiconica or S. trichodea curvata were stained selectively; **Small:** small *A. vollenweideri* worker (HW: 0.68 mm and 0.96 mm, respectively); **Large:** large *A. vollenweideri* worker (HW: 3.16 mm and 1.28 mm, respectively); **Total:** total number of glomeruli with stained arborizations; **Rel. %:** Percentage of glomeruli with stained arborizations; **T1 to T6:** number of innervated glomeruli belonging to the glomerular clusters T1 to T6; **Tracts:** Total number of sensory tracts containing axons with projections in glomeruli found in each single sensillum staining.

3.3.3. Selective sensilla stainings and projections of their ORN axons in the AL

The multiple ORNs of both olfactory sensilla (S. trichodea curvata and S. basiconica) project their axons to glomeruli in the AL. We investigated how the innervations of ORN axons are distributed across the glomerular clusters of the AL and further analyzed whether each ORN innervates only a single glomerulus.

In six successful stainings of single S. trichodea curvata, we found between 4 and 48 innervated glomeruli in the AL, and the total of 86 glomeruli with arborizations of ORNs were distributed across the entire AL (Table 1, Fig. 10A, C). ORNs from each single sensillum innervated at least three of the six different clusters. The innervation patterns indicate that the glomeruli of the clusters T1-T4 are innervated more often than the clusters T5 and T6 (Table 1: Rel. %). Only about 5 percent of the arborizations were found in the T6-cluster, although it contains about 30 percent of all glomeruli. The MG of large workers belongs to the T2-cluster and is also innervated by ORNs associated with the S. trichodea curvata (Fig. 10A).

In five successful stainings of single S. basiconica, we found between 11 and 53 innervated glomeruli in the AL, and the total of 150 glomeruli with arborizations of ORNs were located exclusively in the T6-cluster (Table 1, Fig. 10B, D and F). From the 11 selectively stained sensilla (S. trichodea curvata and S. basiconica) we found 246 distinct arborizations of ORN axons in glomeruli. In ~100 cases, the axons of single ORNs could be traced from the arborisation in the glomeruli retrogradely to the sensory tract and sometimes even back to the antennal nerve. In all these cases, the axons of ORNs terminated only in a single glomerulus. In few cases we found axons branching before entering the glomerulus,

however all branches of an axon finally arborized in the same glomerulus. This result supports the idea that the axon of each ORN terminates in only one glomerulus.

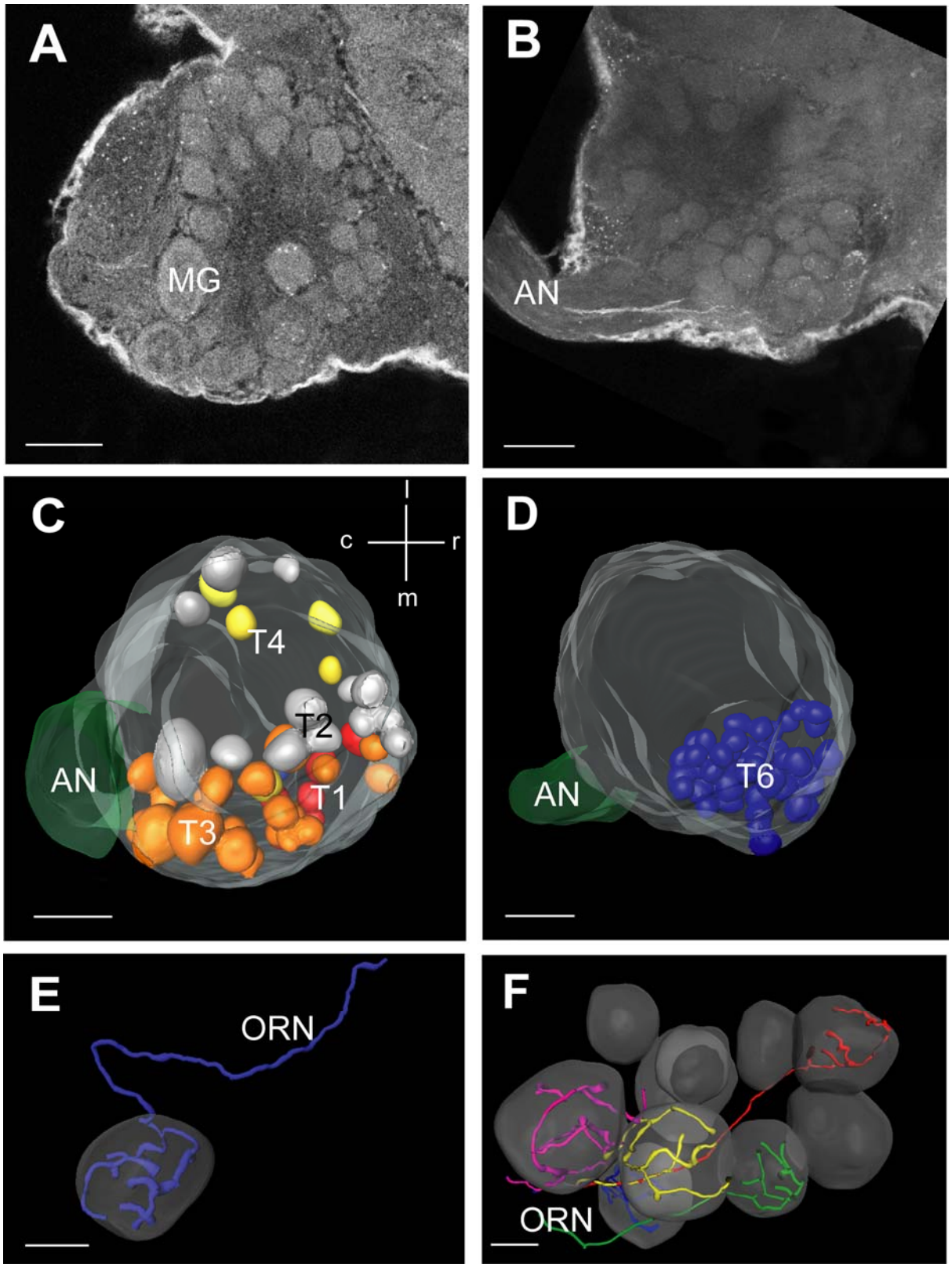

Figure 10: Two examples showing the innervation patterns of two different types of olfactory sensilla. **A** and **B**: Confocal images of single sensillar stainings of Sensilla trichodea curvata (**A**) and Sensilla basiconica (**B**). **C**: The ORNs of one S. trichodeum curvatum innervate several (3D-reconstructed) glomeruli through all six sensory tracts. **D**: The ORNs of one S. basiconicum innervate exclusively (3D-reconstructed) glomeruli of the T6-cluster. **E** and **F**: 3D-reconstructions of individual ORNs innervating single glomeruli, shown for one ORN associated with a S. trichodeum curvatum (**E**) and for several ORNs of a S. basiconicum innervating single glomeruli of the T6-cluster (**F**). Red = T1, grey = T2, orange = T3, yellow = T4, green = T5, blue = T6, AN = antennal nerve, MG = macroglomerulus, ORN = olfactory receptor neuron, m = medial, l = lateral, c = caudal, r = rostral. Scale A-D = 50 µm, scale in E and F = 10 µm.

3.3.4. Allometry between number of sensilla and the size of glomeruli

We found a similar number of T6-glomeruli in both HN- and LN-phenotype workers, and most if not all of these glomeruli were innervated by ORNs from the S. basiconica. We assessed the number of S. basiconica and related this measure to the size of the corresponding T6-glomeruli. First, we counted the number of S. basiconica (N_{Sb}) in two selected areas on the second distal segment for 12 workers. The density of S. basiconica ranged between 5.47 and 11.74 sensilla per 10^4 μm^2 (n = 12). We found no correlation between body size (H_w) of workers and density of S. basiconica (Pearson Product Moment correlation, p = 0.86; n = 12; data not shown). The total number of S. basiconica (N_{Sb}) on the second distal segment was about ten times larger in the largest investigated worker (H_w = 3.48 mm) compared to the smallest investigated worker (H_w = 0.76 mm). To our surprise, we found a linear relation between body size (H_w) and number of S. basiconica (Pearson Product Moment correlation; $N_{Sb} = 49.05 * H_w - 10.65$; $R^2 = 0.95$; $p < 0{,}001$; n = 12; Fig. 11A). This means that the surface area of the second distal segment scales isometrically with H_w, and not to the square as we expected. Indeed, the length (L) of the second distal segment scales with an exponent of 0.73 ($L = 142.05 * H_w^{0.73}$; $R^2 = 0.95$; $p < 0.01$; Fig. 11B), whereas the width (W) scales with an exponent of 0.57 ($W = 103.66 * H_w^{0.57}$; $R^2 = 0.79$; $p < 0.01$). Thus, the second distal segment scales not isometric, and compared to large workers, it is relatively long and broad in small workers. This results in a relatively large surface area of the second distal segment in small workers.

Second, we assessed the relation between body size (H_w) and the mean volume of 50 glomeruli of the T6-cluster (R_{Gmean} of $T6_{50}$) in 20 workers. The isometric relation we found is described by the linear regression: R_{Gmean} of $T6_{50}$ = 3.10 * H_w + 9.86 (R^2 = 0.90; $p < 0.01$; n = 20). Using this equation and the equation above that describes the body size (H_w) related number of S. basiconica (N_{Sb}), we calculated the expected R_{Gmean} of $T6_{50}$ for the 12 workers in which we assessed the number of S. basiconica. Finally, we could calculate the relation between the number of S. basiconica (N_{Sb}) and their expected mean volume of the T6-glomeruli (V_{Gmean} of $T6_{50}$). We found that the mean glomerular volume scales with the power of 2.54 to the number of S. basionica (V_{Gmean} of $T6_{50} = 0.09*N_{Sb}^{2.54} + 892.22$; R^2 = 0.98; N = 12; Fig. 12).

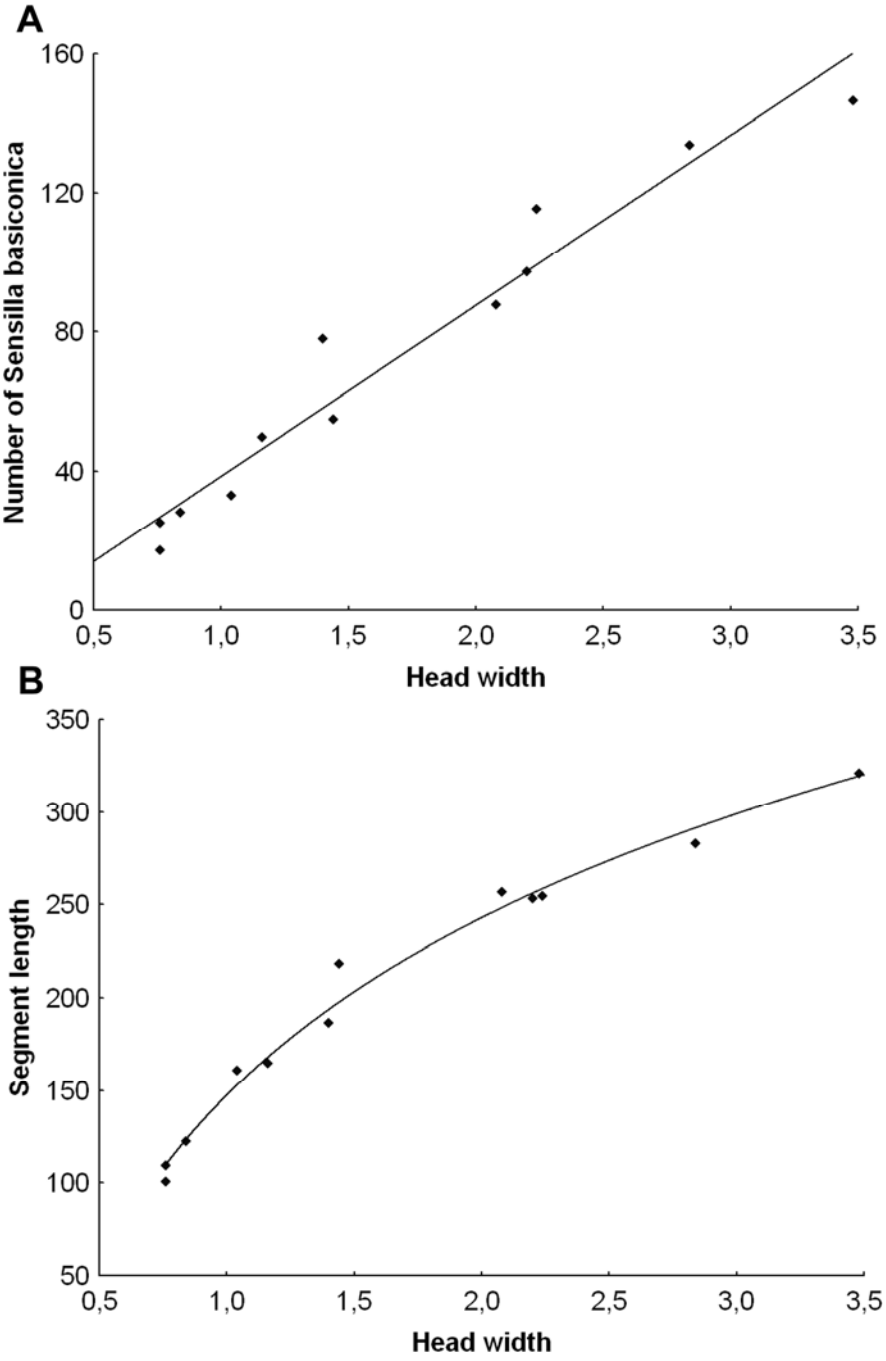

Figure 11: Number of Sensilla basiconica and segment length of the second distal antennal segment relates to body size (H_w). **A**: Large workers have more S. basiconica, and the number of sensilla scales isometric with head width (N_{Sb} = 49.05 * H_w – 10.65; R^2 = 0.95; p < 0,001; n = 12). **B**: The length of the second distal antennal segment scales logarithmic with head width (L = 142.05 * $H_w^{0.73}$; R^2 = 0.95; p < 0.01). The scaling factor < 1 results in a relatively large surface of the antennae of small workers and the surface areas are equipped with S. basiconica at a similar density (see text).

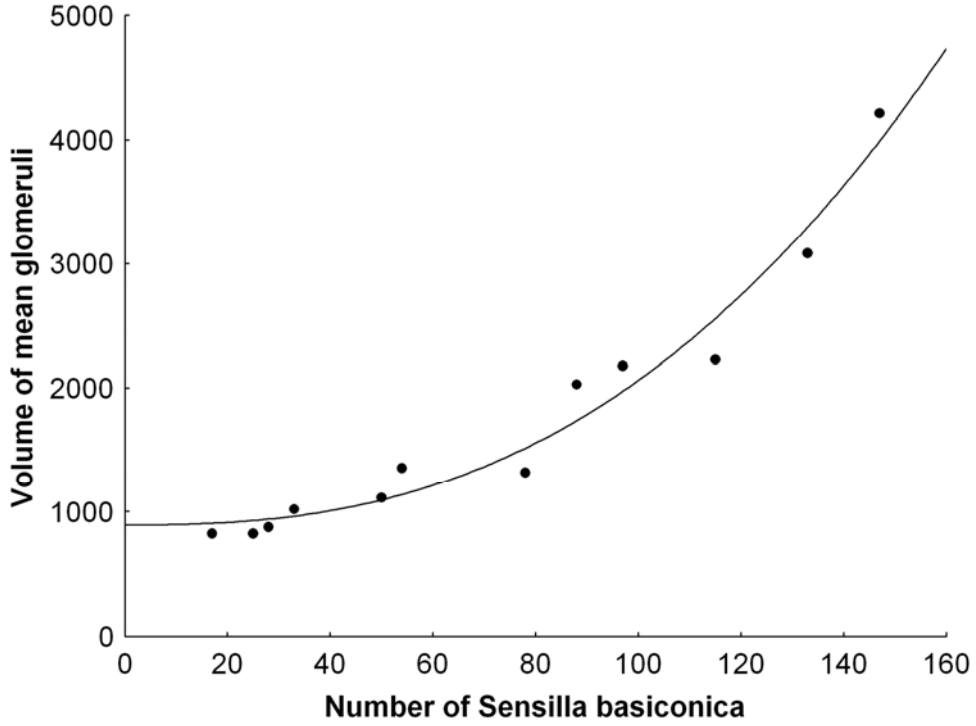

Figure 12: Correlation between the calculated mean volume of 50 glomeruli of the T6-cluster in the antennal lobe and the number of Sensilla basiconica on the second antennal segment (V_{Gmean} of $T6_{50}$ = 0.09*$N_{Sb}^{2.5356}$ + 892.22; R^2 = 0.9816; N = 12).

3.4. Discussion

Our study revealed that, in addition to the two previously described AL-phenotype workers (MG- and RG-phenotype), two other distinct AL-phenotypes (LN- and HN-phenotype) do exist. All workers with a low number of glomeruli have only regular sized glomeruli in their AL (RG-LN-phenotype), few workers were found with a high number but also only regular sized glomeruli (RG-HN-phenotype) and all workers with an MG have a high number of glomeruli (MG-HN-phenotype). Small leaf-cutting ant workers have a reduced olfactory system not only in their number of olfactory sensilla but also in the number and volume of glomeruli. The differences in the organization of the AL are the result of distinct developmental patterns and divide the worker caste of leaf-cutting ants into distinct neuroanatomical sub-castes. These sub-castes presumably differ in their olfactory-guided behavior, and this may support different behavioral phenotypes resulting in an elaborated social organization among workers of the colony.

3.4.1. Number of glomeruli

Compared to other hymenopteran species [Galizia et al., 1999a; Smid et al., 2003], the ALs of ants have a high number of glomeruli [Hoyer et al., 2005; Nishikawa et al., 2008; Zube et al., 2008; Kelber et al., 2009; Kuebler et al., 2009]. The fungus growing ants (Attini), to which the leaf-cutting ants of this study belong to, contain species with up to 630 glomeruli [Kelber et al., 2009]. Across ant species, *A. vollenweideri* workers with 376-457 glomeruli are in the upper range, and high glomerular numbers are considered to reflect outstanding abilities in odor discrimination [Kleineidam and Rössler, 2009]. As functional units for odor information processing, the number of glomeruli are one measure of the ALs' complexity.
Intraspecific variation in number of glomeruli is well documented for many insect species, and in Hymenoptera males usually possess fewer glomeruli than females [Brockmann and Bruckner, 2001; Hoyer et al., 2005; Schachtner et al., 2005; Groh and Rössler, 2008; Zube and Rössler, 2008; Kuebler et al., 2009]. Within the female castes we found in our previous study on *A. vollenweideri* that queens have less glomeruli than workers [Kuebler et al., 2009]. We now documented that even within the worker caste the number of glomeruli varies systematically. Related to body size, two AL-phenotypes with different number of glomeruli exist within workers of *A. vollenweideri*. A worker either has around 383 glomeruli (LN-phenotype) or it has around 443 glomeruli (HN-phenotype). The underlying mechanisms leading to either of the two alternative developmental patterns (polyphenism) is unknown. We hypothesize, however, that this may be linked to the regulation of OR-gene expression [Nijhout, 1999; Ray et al., 2008; Suzuki and Nijhout, 2008]. Genomic studies indicate that the number of functional OR-genes is a reasonable estimate for the number of glomeruli found in the AL, and vice versa [Clyne et al., 1999; Vosshall et al., 1999; Robertson and Wanner, 2006]. In *Drosophila melanogaster*, but also in humans, it has been shown that only a subpopulation of the OR-gene repertoire is expressed [Hasin et al., 2008; Laissue and Vosshall, 2008]. The number of OR-genes in *A. vollenweideri* is yet unknown and we speculate that only a fractional number of OR-genes is expressed in LN-phenotype workers. In MG-phenotype workers, we propose that in more ORNs, tuned to the releaser component of the trail pheromone, the corresponding OR-genes are expressed [Kuebler et al., 2009]. Surprisingly, we found not only RG-LN phenotype and MG-HN-phenotype workers, but also some RG-HN-workers at intermediate body size (H_W: ~1 mm). This indicates that the developmental patterns leading to RG- or MG- and LN- or HN- phenotype are not strictly

coupled. However, the developmental pattern leading to the HN-phenotype seems to be necessary for the potential development of the MG-phenotype.
In addition to differences in the ALs, further neuroanatomical phenotypes and accordingly more sub-castes may exist due to differences in higher integration centers like the mushroom bodies. It has been shown that e.g. volume differences of the mushroom body may be correlated with aggression behavior in the paperwasp *Polistes instabilis* [Molina and O'Donnell, 2007]. Because of the potential plasticity of the mushroom body, we expect that also across sub-castes of *A. vollenweideri* workers differences in mushroom body volume may exist, which remains to be investigated.

3.4.2. Cluster of glomeruli

The general AL morphology of *A. vollenweideri* is comparable to other social Hymenoptera, like the honeybee (*Apis mellifera*) or the carpenter ant (*Camponotus floridanus*) [Kirschner et al., 2006; Zube et al., 2008]. In all of these species, the antennal nerve divides into several sensory tracts. While four sensory tracts innervate the glomeruli in the honeybee [Arnold et al., 1985; Flanagan and Mercer, 1989; Galizia et al., 1999a; Kirschner et al., 2006], seven sensory tracts are described for *C. floridanus* [Zube et al., 2008]. In contrast to *C. floridanus*, six sensory tracts innervate the AL in *A. vollenweideri*. The ORNs of the most abundant olfactory sensillum on the antenna (S. trichodea curvata) arborized predominantly in glomeruli of clusters T1 to T4. In the T4-cluster of an LN-phenotype worker we found 50 glomeruli less than in an HN-phenotype worker. Visual inspection of several ALs with and without labeled ORNs confirmed that the T4-cluster, which is located at the dorsal part of the antennal lobe, consists of considerably less glomeruli in the LN-phenotype workers. It seems that only the number of T4-glomeruli is reduced and that the number of glomeruli in the other five clusters is similar in both, LN- and HN- phenotypes. The T5-glomeruli in *A. vollenweideri* show structural similarities to the T4-glomeruli in the honeybee and the T7-glomeruli in *C. floridanus*. All these clusters consist of few glomeruli that are larger in size and their sensory innervation pattern is special, with a sensory innervation throughout the whole glomerulus [Galizia et al., 1999a; Kirschner et al., 2006; Zube et al., 2008]. The T4-glomeruli in the honeybee are innervated only by sensory neurons from the ventral antennal nerve, which led to the speculation that non-olfactory but e.g. temperature-sensitive or CO_2-sensitive sensilla innervate these glomeruli [Nishikawa et al., 1995; Sachse et al., 2007;

Nishino et al., 2009]. Our investigation of selectively stained olfactory sensilla (S. basiconica and S. trichodea curvata) revealed that of all the 236 glomeruli in which we found axonal arborizations of ORNs, only one single glomerulus was located in the T5-cluster (that is the T4 corresponding cluster of the honeybee). This supports the idea that non-olfactory information is processed in T5-glomeruli.

The T6-glomeruli in *A. vollenweideri* and in *C. floridanus* are comparable, in both species this cluster consists of a large group of relatively small glomeruli, separated from the other glomeruli by the dorsal lobe tract [Nishikawa et al., 2008; Zube and Rössler, 2008]. Our study revealed that the S. basiconica are innervating exclusively glomeruli of the T6-cluster. Interestingly, some of the ORNs stained in single S. trichodea curvata also arborized in glomeruli of the T6-cluster. Possibly both types of sensilla may house the same type of ORN, however, this remains to be investigated in greater detail.

3.4.3. Multiple ORNs of olfactory sensilla

We selectively stained single sensilla and their associated ORNs. Based on the number of innervated glomeruli we conclude that both types of olfactory sensilla, the S. trichodea curvata and the S. basiconica, house multiple ORNs. Our finding is consistent with the finding of multiple ORNs of basiconic and trichoid sensilla described for the carpenter ant *Camponotus japonicus* [Nakanishi et al., 2009], and it seems to be a common character of Hymenoptera [Schneider and Steinbrecht, 1968; Esslen and Kaissling, 1976; Isidoro et al., 2001]. However, the functional significance of this organization is still unknown [Butterfield and Anderson, 1994; Ochieng et al., 2000; Kelber et al., 2006]. The total number of ORNs in one antenna is therefore a multiple of the number of sensilla. We did not quantify or estimate the total number of olfactory sensilla on the antenna but we assessed the total number of ORNs for several workers (H_W: 1.96 – 3.48 mm) using transmission electron microscopy of cross-sectioned antenna. We found between 32.000 and 40.000 ORNs, characterized by their small diameter in cross-section (data not shown). This number is comparable to e.g. the number of ORNs in the antenna of the honeybee that was calculated from the number of sensilla and their ORNs [Esslen and Kaissling, 1976].

In *A. vollenweideri*, each ORN innervates only a single glomerulus. We followed many of the stained ORN axons from the two main olfactory sensilla (S. basiconica and S. trichodea curvata) and we never observed that one ORN innervates more than one single glomerulus.

Instead, several ORNs could be followed from the sensory tract to their arborisation in a single glomerulus, supporting the "one ORN-type – one glomerulus" theory. Although we are convinced that all ORNs terminate in a single glomerulus, we cannot rule out that the same ORN-type may alternatively arborize in different glomeruli.

In some insect species like moths, pheromonal ORNs are housed in sensilla specialized for pheromone detection [Kaissling and Kasang, 1978; Berg et al., 1995]. For the *A. vollenweideri* workers, we found that the MG-ORNs (ORNs terminating in the MG and sensitive for the releaser component of the trail-pheromone [Kuebler et al., 2009]) are associated together with other types of ORNs within the S. trichodea curvata. Based on our neuroanatomical data, we could not identify any kind of sensilla that are specialized solely for the detection of the trail-pheromone.

3.4.4. AL miniaturization

Compared to large workers (H_W: >1mm), the brains and ALs of small workers are tiny. Possibly, the ALs in LN-phenotype workers are just too small to contain over 400 glomeruli while maintaining their functionality. Indeed, the smallest glomeruli we found in small *A. vollenweideri* workers (mean: 299 μm^3; LN-phenotype workers) are just slightly smaller than the smallest glomeruli in the large workers (mean: 330 μm^3; in HN-phenotype workers). However, other Attini species, e.g. *Apterostigma cf. mayri* have small, monomorph workers with many more glomeruli (630) in the AL than *A. vollenweideri*. All their glomeruli are very small (200-250 μm^3), and the smallest glomeruli are only about 100 μm^3 [Kelber et al., 2009]. In case the organization of glomeruli is similar in all Attine species, glomerular size is not a limiting parameter that may explain the LN-phenotype. Another example of small glomeruli have been described for the AL of male mosquitoes [Ghaninia et al., 2007], and the small glomeruli of *Drosophila* are also only 100-200 μm^3 in volume [Laissue et al., 1999].

We rather propose that the lower complexity of the AL in LN-phenotype workers may allow less complex odor information processing with less energy consumption. How this reduction in AL-complexity affects e.g. odor discrimination is not yet known.

3.4.5. Allometry between sensilla number and glomerular volume

The variation in body size allowed us to investigate the relation between the number of sensilla and the volume of the innervated glomeruli. We selected the S. basiconica because

on the antennae they are easy to identify and innervate a single cluster of glomeruli in the AL. We assume that, irrespectively of body size, the number of ORNs in each S. basiconicum is similar since the density of this sensillar type is the same in large and small workers. This finding stands in contrast to a study on poreplate sensilla of the bumblebee *Bombus terrestris*, where both an increase of sensillar number and sensillar density was found in larger workers [Spaethe et al., 2007].

With more sensilla and associated ORNs in large leaf-cutting ant workers their glomeruli are larger. The volume of glomeruli scales with an exponent of 2.54 to the number of sensilla, and a large proportion of the glomerular volume is occupied by the arborisations of ORNs. The large scaling exponent indicates that in large workers each ORN occupies a larger volume of the glomerulus than in small workers. We propose that the ORNs' innervation of glomeruli differs in large and small workers with large workers having more, or longer branching patterns of ORN axons. Since it was not aim of the present study to quantify this proposed difference, it remains to be investigated in greater detail in future studies.

3.4.6. Neuroanatomical sub-castes and social organization

We found three different AL-phenotypes in the worker caste according to glomerular number and the occurrence of an MG. The different phenotypes correlate with the size of the workers, and to their behavior (alloethism). We propose that the differences in the organization of the AL are adaptations leading to distinct odor-guided behaviors and specialization to particular tasks within the colony. For the MG/RG-phenotypes, we have good evidence that the MG-phenotype workers are more sensitive to the releaser component of the trail pheromone, whereas the RG-phenotype workers are better in discriminating conspecific from heterospecific trail-pheromones [Kleineidam et al., 2007; Kuebler et al., 2009]. For the LN/HN-phenotypes newly described in this study, we can only speculate about the functional significance for behavior. A higher number of glomeruli (in T4) in large workers possibly is advantageous for detection of host plant odors and the processing of information necessary to select suited plant material for fungus cultivation. Finding new and digestible plant substrate is the main task for large workers, while small workers do not select the plants that are cut and therefore may have lower requirements in leaf assessment. In order to investigate this hypothesis, physiological data on glomeruli in the T4-cluster are needed.

The polyphenisms of the olfactory system within the worker caste of leaf-cutting ants we described in this and previous studies exemplify how distinct developmental patterns result in various sub-castes of workers. How odor information is processed depends on the organization of the ALs, and we now face the challenge to understand the adaptive value and the constraints of the different AL-phenotypes in detail as well as the underlying genetic control that results in distinct phenotypes. Ultimately, this will lead to a better understanding of the evolution of task specialization in an insect species that has extremely large colonies and one of the most complex social organizations.

4. The antennal lobes of fungus-growing ants (Attini): neuroanatomical traits and evolutionary trends

4.1. Introduction

The tribe Attini is a monophyletic taxon with 13 genera and over 230 species restricted to the new world. Based on studies of behavioral ecology [Weber, 1956; Wilson, 1971; Hölldobler and Wilson, 1990], larval morphology [Schultz and Meier, 1995], and mDNA sequence analyses [Wetterer et al., 1998] three major groups are distinguished. The first group comprises the lower (formerly basal) Attini with nine different genera and small colonies housing only several hundred individuals. The second group, the higher (formerly intermediate) Attini contains the two genera *Sericomyrmex* and *Trachymyrmex* with colony sizes restricted to several thousands of individuals [Weber, 1972; Hölldobler and Wilson, 1990]. The last group, the leaf-cutting Attini, comprises two genera, *Atta* and *Acromyrmex,* with colonies composed of up to millions of individuals. A recent phylogenetical study allows a more detailed separation of species groups based on their agricultural systems [Schultz and Brady, 2008], but the basic division in three major groups remains unchanged.

All Attine species are characterized by an obligate cultivation of symbiotic fungi; an association which originated 45-60 million years ago in the early Tertiary. Since then the success of fungus gardening improved by coevolution of both partners – the ants and the fungus - and includes derived fungus-care procedures by the ants like fertilization and the use of antibiotic substances [Currie et al., 1999; Mueller et al., 2001]. The complexity of

resource selection and fungus cultivation are expected to pose high demands on the Attini olfactory system. Furthermore, chemical communication along the foraging trails and the evolution of complex foraging systems add to the multitude of relevant odors in the life of Attini ants.

The three Attini groups differ in both the food resources and the foraging strategies they use. The fungus of lower and higher Attini colonies is reared mainly with insect carcasses, feces, dead plant parts, and to a lesser extent with living plant tissue [Weber, 1972; Hölldobler and Wilson, 1990; Mueller and Wcislo, 1998; Mackay et al., 2004]. The small body size and the small mandibles of lower and of most higher Attini workers are unsuited to cut fresh leaves [Wilson, 1980b; Wilson, 1980a]. In the leaf-cutting Attini, the worker caste shows a pronounced size-polymorphism, and division of labor is largely dependent on worker size (alloethism). The large workers are equipped with powerful mandibles suited to cut and transport fresh leaves and grasses [Weber, 1972; Roces and Lighton, 1995; Röschard and Roces, 2002]. Therefore, a huge and renewable food source stands open for the leaf-cutting Attini. Important for the utilization of this resource is an efficient mass recruitment system. Indeed, the majority of leaf-cutting Attini forage on well established pheromone trails that are even physically maintained, and division of labor can be observed along transport chains on the trail [Röschard and Roces, 2003]. In contrast, lower Attini workers forage alone or in small groups [Weber, 1972; Leal and Oliveira, 2000]. We expect that all fungus-growing Attini have a highly developed olfactory system, necessary to meet the demands of complex olfactory-guided tasks like pheromone communication or substrate selection. Indeed, several behavioral studies show how sensitive and fine-tuned the odor responses in leaf-cutting Attini are [Tumlinson et al., 1972; Andryszak et al., 1990; Kleineidam et al., 2005; Morgan et al., 2006; Kleineidam et al., 2007]. We expect that the demands on the olfactory system of fungus growing Attini differ among the different groups according to differences in olfactory-guided behaviors like foraging strategies and the use of chemical mass recruitment.

In ants - like in other insects - axons of antennal olfactory receptor neurons (ORNs) project to the first olfactory neuropil, the antennal lobe (AL) in the brain and terminate in the functional units of the AL, the glomeruli. For both vertebrate and invertebrate olfactory systems, it is assumed that axons from ORNs that express the same odorant receptor gene

converge onto the same glomerulus [Rodrigues, 1988; Vassar et al., 1994; Mombaerts et al., 1996]. This is supported by a good match in the number of functional odorant receptor genes and the number of glomeruli found in the AL, as shown for example in the honeybee *Apis mellifera* or in *Drosophila* [Vosshall et al., 2000; Robertson and Wanner, 2006]. This organization results in a spatial representation of odors in the AL glomeruli, which was documented by functional calcium-imaging studies, especially in the honeybee, and more recently in the carpenter ant *Camponotus floridanus* [Joerges et al., 1997; Galizia et al., 1999b; Sachse et al., 1999; Zube et al., 2008].

The olfactory system in social Hymenoptera shows some interesting peculiarities compared to the olfactory system in other insects [Kleineidam and Rössler, 2009]. Individual olfactory sensilla on the antennae are generally equipped with a high number of ORNs [Schneider and Steinbrecht, 1968; Esslen and Kaissling, 1976; Kelber et al., 2006] compared to e.g. moths or flies with only one to three receptor neurons in a single sensillum. The advantages of multiple ORNs are not yet clear. Another difference is the high number of glomeruli in the AL. Glomerular numbers in most insects studied so far do not exceed 100, for example ~43 glomeruli in *Drosophila melanogaster*, ~50 glomeruli in the mosquito *Aedes aegypti,* and ~65 in *Manduca sexta* [Stocker, 1994; Laissue et al., 1999; Huetteroth and Schachtner, 2005; Ignell et al., 2005], whereas the number of glomeruli in social Hymenoptera ranges from ~164 in the honeybee *Apis mellifera* to up to ~460 in the Carpenter ant *Camponotus floridanus* [Arnold et al., 1985; Flanagan and Mercer, 1989; Galizia et al., 1999a; Zube et al., 2008]. It seems that the high number of glomeruli in social insects is related to the diverse demands on the olfactory system and possibly reflects the high level of social organization based on olfactory communication using pheromones and chemical recognition cues. Regarding the size of the glomeruli in the AL, striking differences can be found. Substantially enlarged glomeruli (macroglomeruli, MG) have been found in moths, but also in males of social Hymenoptera [Arnold et al., 1985; Hansson and Anton, 2000] and these MG were shown to process information about sex-pheromones [Arnold et al., 1985; Sandoz, 2006]. As the size of a glomerulus is mainly determined by the number of ORN axons terminating in it, individual AL glomeruli can be considered as a trait, and an enlarged glomerulus or MG is likely to represent an over-representation of a particular trait. Enlarged glomeruli may also reflect the importance of an odor, which was shown to be the case for sex-pheromone

specific MG. Recently, a MG was described in the AL of large leaf-cutting ant workers of *Atta vollenweideri* and *Atta sexdens* [Kleineidam et al., 2005]. Preliminary physiological data show that olfactory information about the trail-pheromone is represented in this MG [Kleineidam, unpublished results].

In this study we investigate the neuroanatomy of the AL in many species across all three major Attini groups to reveal potential differences in the glomerular organization. The selected species differ in life history, behavioral ecology and olfactory-guided behavior, and we compare several aspects of the AL: the number of glomeruli, their size and their position. This study is aimed to gain new insights into the evolution of an important brain compartment by a comparison across many species within a monophyletic taxon.

4.2. Methods

We investigated workers of 25 Attini species, both from laboratory colonies as well as from field colonies in Central and South America. The species investigated, their origin and the major AL parameters analyzed are summarized in table 2.

4.2.1. Fixation and preparation

We used specimens fixed and stored under different conditions. Some of the specimens were fixed as whole animals in an alcoholic Bouin solution (0.9% Picric acid, 40% formaldehyde, 4.8% glacial acetic acid in 70% ethyl alcohol) and then stored in 70% ethanol. For other specimen, the perforated heads were fixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde (fix-mix) or in a solution containing 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2). For all specimens, the head width was measured between the outer edges of the eyes. Afterwards, heads were cut open and the brains were dissected in saline solution (127 mM NaCl, 7 mM KCl, 1.5 mM $CaCl_2$, 0.8 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.8 mM TES, 3.2 mM Trehalose). For specimens collected from laboratory colonies, the head was cut off immediately after removing these workers from the colony and the brains were also dissected in saline solution.

After dissection, brains were immediately transferred to ice-cold fix-mix and then stored for 5-7 days at 4°C. Brains were then rinsed in PBS (3 times 10 minutes), dehydrated in an

ascending series of ethanol (50, 70, 80, 90, and 95% and 2 times 100%, 10 minutes each) and finally transferred to methylsalicylic acid (M-2047, Sigma-Aldrich, Steinheim, Germany). Glutaraldehyde fixation intensifies the autofluorescence of the brain and allows confocal analyses and 3D-reconstructiions of neuropiles without any additional staining. The brains were examined with a laser-scanning confocal microscope (20x 0.7 lens, Leica TCS SP2 AOBS, Leica Microsystems AG, Wetzlar, Germany).

4.2.2. Antennal lobe anatomy across 25 species

The number and the volume of all glomeruli in a single AL were determined for all investigated species by 3D-reconstruction of all glomeruli with the help of the 3D-software AMIRA 3.1.1 (Mercury Computer Systems, Berlin, Germany). Prior to the 3D-reconstructions, we inspected the confocal image stacks of the ALs visually to ensure adequate image quality. For most species, confocal stacks of 2-4 specimens were selected for detailed investigation and one was chosen for reconstruction. The other stacks have also been examined for the existence of e.g. an enlarged glomerulus, but no 3D-reconstruction was made (see table 2). Each glomerulus in the AL was labeled individually in all three planes (xy, xz, yz) and subsequently reconstructed by using the wrapping function of AMIRA 3.1.1. The antennal nerve was reconstructed as a landmark for the comparison of the position of glomeruli in different species. Subsequently, we measured the volume of each glomerulus and the total number of glomeruli in the AL.

4.2.3. Classification of a macroglomerulus

Across all investigated species and also across workers within polymorphic species, the size of the AL and its glomeruli varied considerably. In order to compare the size of glomeruli between different ALs, we used a relative measure based on the variance found in all (or a large subset of) glomeruli. First, the volume of glomeruli obtained from the 3D-reconstructions was used to calculate the radius of a sphere having the same volume as the glomerulus. Second, the mean radius (R_M) of these spheres and the standard deviation (SD) were calculated. Third, for the largest and the second largest radius (R_L and R_S), the radius value (R_V) was calculated using: $R_V = (R_L\text{-}R_M)/SD$. We used the calculated radii of the glomerular volumes because the size distribution based on volumes is skewed, whereas the size distribution based on the radii is normally distributed. Our measure of R_V describes how

much bigger the largest (second largest) glomerulus is compared to the mean size of a glomerulus and with respect to the variance of glomerular volumes. We defined a glomerulus as macroglomerulus if its radius is 5xSD larger than the mean radius of glomeruli of the AL (MG: R_V>5).

4.2.4. A macroglomerulus in leaf-cutting Attini

We found an MG only in leaf-cutting Attini, which all have a polymorphic worker caste. The position of the MG in the AL was described by using the antennal nerve as a landmark. We compared the positions to the two already known positions of the MG in *A. sexdens* and *A. vollenweideri* [Kleineidam et al., 2005]. Additionally, we investigated the ALs of small leaf-cutting Attini workers (head width < 1mm) in search of a MG. Therefore a subset of 100 glomeruli near the antennal nerve entrance was reconstructed in the AL of small workers of four *Atta* species (*A. vollenweideri, A. colombica, A. laevigata* and *A. cephalotes*) and five *Acromyrmex* species (*Ac. striatus, Ac. ambiguus, Ac. fracticornis, Ac. heyeri* and *Ac. lundi*).

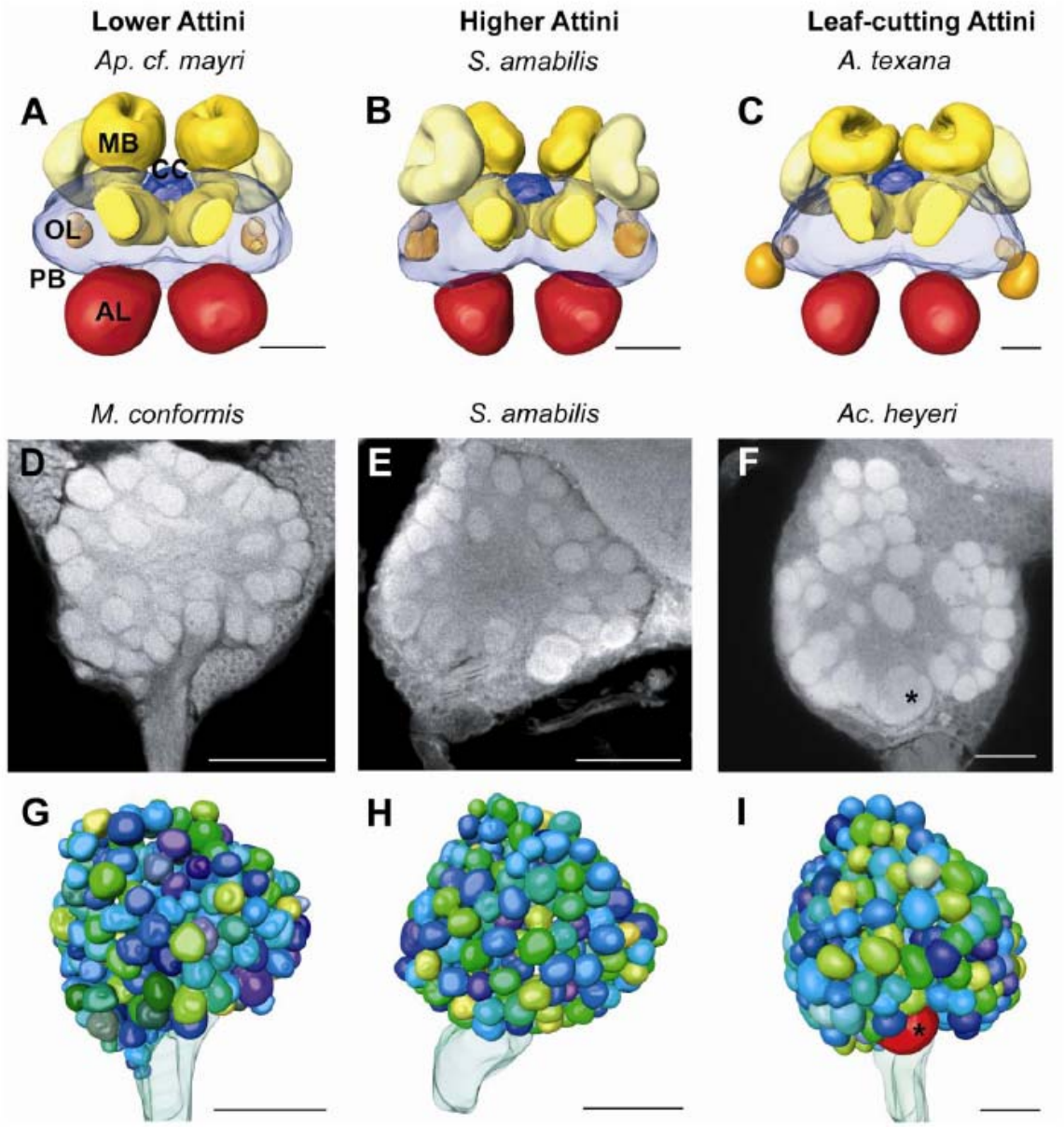

Fig 13: Antennal lobes of lower, higher and leaf-cutting Attini

3-D reconstructions of the brains (A, B, C), single confocal slices of the antennal lobes (D, E, F), and 3-D reconstructions of the antennal lobes (G, H, I) of different Attini species. For the lower Attini, the brain of *Apterostigma cf. mayri* is shown (A); the second brain belongs to *Sericomyrmex amabilis* as an example of the higher Attini (B); the third brain belongs to *Atta texana*, a leaf-cutting Attini (C). The confocal images show the ALs of *Mycetophylax conformis* (D), *Sericomyrmex amabilis* (E) and *Acromyrmex heyeri* (F) at a position close to the antennal nerve entrance. G, H and I show complete 3D-reconstructions based on confocal image stacks of the antennal lobes shown above. In both, the lower and higher

Attini, no MG was found. In the leaf-cutting Attini *Acromyrmex heyeri* the MG is clearly visible in the confocal section (*) and in the 3D-reconstruction. AL = Antennal lobe, CC = central complex, MB = mushroom bodies, OL = optic lobe, PB = protocerebrum. Scales: 100 µm in A-C, 50 µm in D-I.

4.3. Results

We measured the number, the size and the position of all glomeruli in the AL of 25 Attini species. For most species the fixation with either Bouin or fix-mix resulted in confocal scans of good quality and the glomeruli in the ALs could be distinguished easily for 3D-reconstruction (Fig. 13 D-I). Even specimens stored in Bouin for several years allowed confocal scans of reasonable quality (Fig. 13 D). We found a large variability in the number of glomeruli within the Attini. A MG was found only in leaf-cutting Attini and in all cases it was positioned close to the antennal nerve entrance.

4.3.1. Number of glomeruli

One outstanding character of the ALs in all Attini is the high number of glomeruli. In the five investigated genera of the lower Attini we found between 257 glomeruli in *C. costatus* and 630 glomeruli in *Ap. cf. mayri* (table 2). The large range in the number of glomeruli illustrates the high variation of this character within the Attini. For the higher and leaf-cutting Attini, both groups consist of only two genera and in all four genera we found a similar number. For the higher Attini, we found from 344 glomeruli in *S. amabilis* to 362 glomeruli in *T. septentrionalis,* and 363 in *T. cornetzi*. For the large workers of leaf-cutting Attini, we found between 369 glomeruli (*Ac. striatus*) and 477 glomeruli (*Ac. lundi*) in *Acromyrmex*, and a similar range - between 336 glomeruli (*A. texana*) and 452 glomeruli (*A. laevigata*) in *Atta*. In all species, the basic organization of the AL seems to be similar, although there are large differences in the glomerular number. The antennal nerve enters the AL and divides into several sensory tracts, in which the axons of the receptor neurons are bundled until they terminate in their target glomerulus. In most species, no glomeruli are located in the core of the AL. The somata of interneurons and projection neurons are located in several clusters on the outer part of the AL.

4.3.2. Size of glomeruli

The size of the glomeruli ranged from 97 to 5009 μm^3 in the lower Attini, from 81 to 6846 μm^3 in the higher Attini, and from 165 to 47645 μm^3 in the large workers of leaf-cutting Attini (table 2). A single and very large glomerulus was found in large workers of leaf-cutting Attini (head width between 1.03 and 2.96 mm) and it extended the range of glomerular volumes considerably. This glomerulus is about 10-14 times larger than the volume of average sized glomeruli. Within the ALs of lower and higher Attini, no glomerulus with such an extreme volume was detected. As a size measure for the largest glomeruli of an AL, we calculated the radius value (R_v) which refers to the variance of all glomerular volumes found in the AL. In all investigated Attini species, the second largest glomeruli have a R_v between 2 and 5 and therefore are not considered as extremely large glomeruli. The largest glomeruli in the AL of lower and higher Attini have an only marginally larger R_v, whereas the largest glomeruli in all leaf-cutting Attini have a R_v larger than 5 (ranging from 5.8 to 7.7; Fig. 14A). The difference in the R_v of the largest and second largest glomerulus in leaf-cutting Attini illustrates that only the largest glomerulus has an extreme volume and henceforth is denominated as macroglomerulus (MG). The least prominent MGs were found in *Ac. striatus* and *Ac. balzani*, and none of the investigated lower and higher Attini posses a MG. The prominent size and position of the MGs enabled us to identify them by visual inspection of confocal stacks which were used to confirm our results.

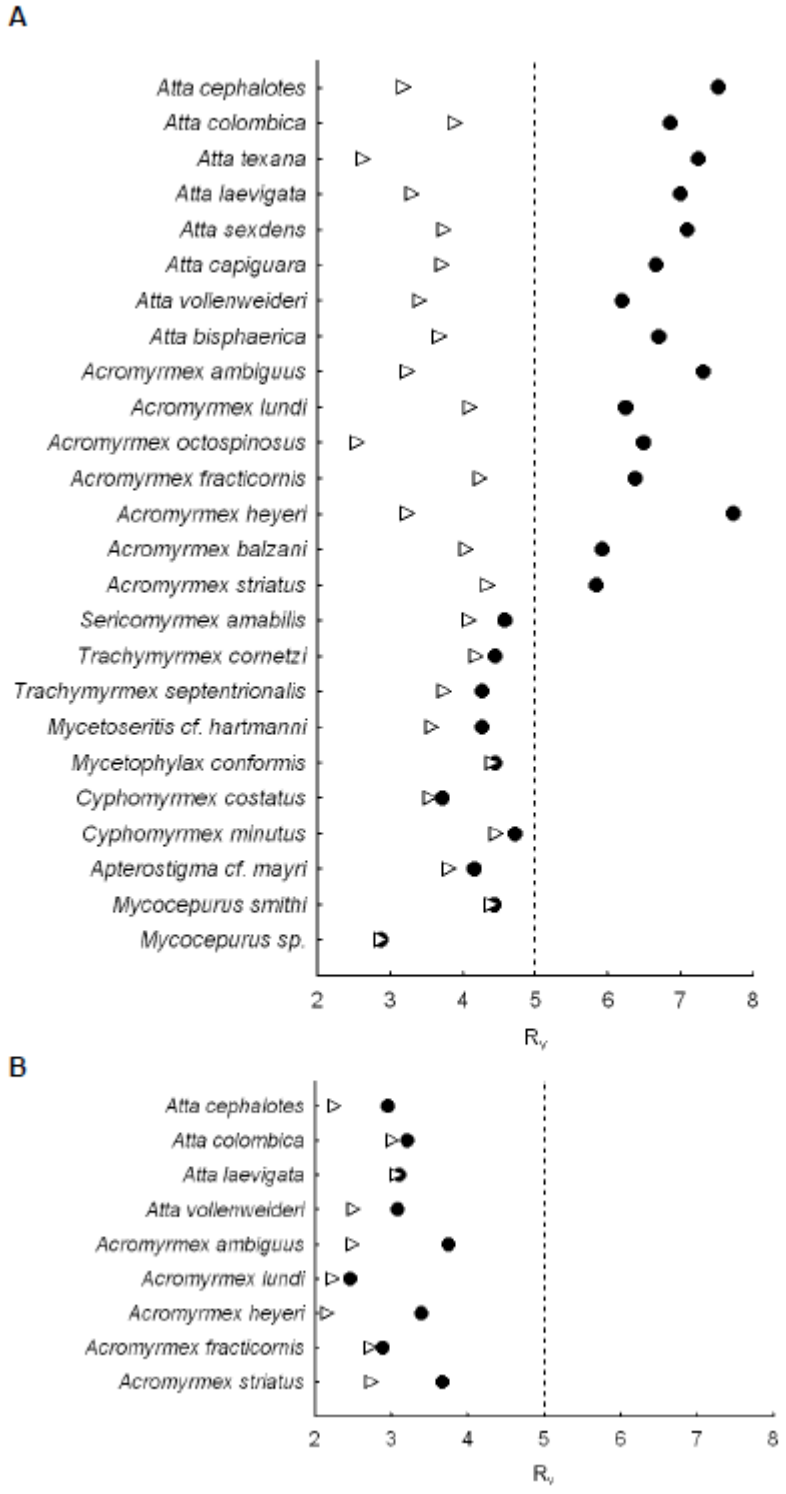

Figure 14: Relative size of the largest and second largest glomerulus

Comparison of the two largest glomeruli in the antennal lobes of workers across 25 different Attini species **(A)** and among the small workers of nine different leaf-cutting Attini species **(B)**. In order to compare the size of glomeruli among different ALs, we used a relative measure based on the variance found across all (or a large subset of) glomeruli: $R_V = (R_L - R_M)/SD$. The dots show the R_V of the largest and the triangles the R_V of the second largest

glomeruli for each species. A R_V-value of five (dotted line) was used to classify macroglomeruli (MG). Large workers of all leaf-cutting species possess a MG and in none of the antennal lobes of lower or higher Attini a MG was found (A). A MG was also absent in the ALs of small workers of four *Atta* and five *Acromyrmex* species (B).

4.3.3. Position of the macroglomerulus

Kleineidam et al [2005] showed that the MG in *A. vollenweideri* and *A. sexdens* are located in species-specific configuration close to the antennal nerve entrance. In *A. vollenweideri*, the MG was found adjacent to the antennal nerve, while in *A. sexdens*, it was positioned more medial with one normally sized glomerulus in between the nerve and the MG. We analyzed the MG-position in other *Atta* and *Acromyrmex* species. By visual inspection we found that the MG in different *Atta* and *Acromyrmex* species were adjacent to the antennal nerve (similar to the position found in *A. vollenweideri*): in *A. laevigata, A. texana, A. capiguara* and *A. bisphaerica* and furthermore in *Ac. ambiguus, Ac. heyeri* (Fig. 13 F, I)*, Ac. lundi, Ac. octospinosus* and *Ac. striatus*. The MG-position with one regular glomerulus in between (similar to the position in *A. sexdens*) was found in *A. cephalotes, A. colombica*, and *Ac. balzani*.

4.3.4. A macroglomerulus in large and in small workers?

Small workers (head width < 1 mm) in *A. vollenweideri* and *A. sexdens* do not possess a MG [Kleineidam et al., 2005]. We analyzed whether this size-based difference is unique to *Atta* species, or whether it also exists in the less polymorphic *Acromyrmex* species. We therefore compared the R_V of the largest and second largest glomeruli to a subset of 100 glomeruli at the comparable part of the AL in nine different *Atta* and *Acromyrmex* species with head widths ranging from 0.68 to 0.88 mm (Fig. 14B). In addition to the specimens in which glomeruli were reconstructed, we inspected several confocal stacks visually. Both, visual inspection and volume analyses clearly showed that small workers of all investigated *Atta* and *Acromyrmex* species do not possess a MG.

4.4. Discussion

We investigated the ALs of more than 10 percent of the about 230 extant Attini from all three groups (lower, higher and leaf-cutting Attini). Our aim was to study different species

within the attine phylogeny to cover a range of species with different olfactory-guided behaviors. Compared to other insect species the olfactory system of the Attini comprises large ALs with a high number of glomeruli. We found a large variability in neuroanatomical traits of the AL e.g. in the number of glomeruli, and we expect that future studies will identify correlations between the number of glomeruli and distinct olfactory-guided behaviors across species. Extremely enlarged glomeruli (MG) were found exclusively in one group of the Attini, the leaf-cutting Attini. This indicates that the MG is a derived overexpression of a trait in polymorphic leaf-cutting species. For a given species, phenotypic plasticity of this trait (MG in large workers and its absence in small workers) among castes is likely to promote division of labor in the leaf-cutting Attini [Kleineidam et al., 2007].

4.4.1. High number of glomeruli and its potential origin

The ALs of the Attini are characterized by a large variance in the number of glomeruli which may reflect adaptations to species-specific demands on the olfactory system. We found the largest variance of the glomerular number in the five investigated genera of the lower Attini. In the higher and leaf-cutting Attini (a total of four genera), the variance is much lower. The lower Attini *Ap. cf. mayri* have more than twice the number of glomeruli compared to *C. costatus* which belongs to the same group. The 630 glomeruli found in the AL of *Ap. cf. mayri*, so far, is the highest number of glomeruli described for all insects with a similar organization of the olfactory pathway (except for the locust, in which AL glomeruli have been termed microglomeruli because of their different functional organization [Hansson and Anton, 2000]). A historical and often cited number of ~1000 glomeruli in the hornet *Vespa crabro* [Hanström, 1928] is incorrect, since our closer inspection using confocal techniques revealed only about 250 glomeruli [Kelber, unpublished data]. Although we find a high number of glomeruli in all Attini, and also in other ant species like *Camponotus* [Nishikawa et al., 2008; Zube et al., 2008], we can not make predictions about the grade of social organization on the basis of glomerular number. Instead we can assume that the high number of glomeruli e.g. in *Ap. cf. mayri* indicates that their olfactory system is more differentiated compared to other species. This idea is also supported by the volume ratio between the ALs and the optic lobes (OLs). In most ant species, the visual processing is less prominent than the olfactory and pheromone processing [Gronenberg, 2008]. The AL volume in *Ap. cf. mayri* is 17 times larger than the OL volume whereas in other lower and

higher Attini this ratio is much smaller (~6-9; see table 2). In the leaf-cutting Attini we also find a wide range for the AL/OL ratio (~4-16). While the AL/OL ratio may relate to the relative importance of the olfactory sense, the high number of glomeruli possibly is another indication for its relevance.

We asked the question why there is such a large variability in the number of glomeruli across related species. It is assumed that the number of glomeruli corresponds to the number of ORN-types and thus is related to the expression of different olfactory receptor genes. In *Drosophila melanogaster*, but also in humans, it was shown that only a subpopulation of an existing repertoire of OR-genes is expressed [Hasin et al., 2008; Laissue and Vosshall, 2008]. The number of OR-genes in the Attini is yet unknown. We found the two species with the highest and the lowest number of glomeruli at a phylogenetically basal position within the Attini. This may indicate that the common ancestor of the Attini possessed a high number of OR genes, and in the presently existing Attini - within all three groups - only a fractional number of them is expressed. We speculate that a high number of OR-genes may have favored possible adaptations for the numerous olfactory-guided tasks that need to be successfully performed for fungus cultivation.

4.4.2. The macroglomerulus

The size of a glomerulus is largely defined by the number of ORNs terminating in the glomerulus [Hansson et al., 1995; Berg et al., 1998]. A high number of ORNs probably leads to a higher absolute sensitivity for a particular odor (threshold sensitivity) and/or result in an improved signal to noise ratio. A high number of ORNs form terminal arborizations in the MG in *A. vollenweideri* and *A. sexdens*, and preliminary results show that the releaser component of the trail pheromone is represented in the MG [Kleineidam et al., 2005]; Kleineidam, unpublished data]. Probably the MG plays a prominent role in the perception of the trail pheromone. The releaser component of the trail pheromone mixture is sufficient to elicit trail following behavior, and several leaf-cutting Attini use the same releaser component [Tumlinson et al., 1971; Attygalle and Morgan, 1985; Kleineidam et al., 2007; Morgan, 2009]. The trail pheromones of the lower and higher Attini are not yet identified.

In the group of lower Attini, we investigated 7 species belonging to 5 out of 9 existing genera. In none of them we found a MG. We also investigated three species of the higher Attini, two *Trachymyrmex* and one *Sericomyrmex* species, which did not possess a MG. All these species do not use an elaborate trail system or chemical mass recruitment, and the worker caste is monomorphic. A slight worker-size variation was found for *T. septentrionalis* [Beshers and Traniello, 1996], but far less pronounced than in the leaf-cutting Attini. Although we did not investigate all lower and higher Attini species, our analyses of several genera within the lower and higher Attini make us confident that the described MG is restricted to the polymorphic leaf-cutting Attini. The genus *Acromyrmex* comprises ~24 species and is subdivided into the subgenera *Acromyrmex* s. Str. and *Acromyrmex* Moellerius [Fowler, 1988]. The genus *Atta* contains 15 highly polymorphic species, actually subdivided into the four following monophyletic groups, *Atta s. str., Archeatta, Neoatta* and *Epiatta*, a subdivision that slightly differs from a previous taxonomical division [Borgmeier, 1959; Bacci et al., 2009]. Our study covered species of all subgenera, and in all of the 15 investigated species we found a MG. This indicates that the existence of the MG is a derived overexpression of a trait and is restricted to the leaf-cutting Attini.

The existence of a MG is common in males of several insect species like moths, cockroach and the honeybee [Boeckh et al., 1977; Arnold et al., 1985; Brockmann and Bruckner, 2001; Wanner et al., 2007]. The leaf-cutting Attini were the first and so far the only species in which a MG was found in the sterile worker caste [Kleineidam et al., 2005]. Until now, studies on the olfactory system in other species of ants did not reveal a MG in the female worker caste [Goll, 1967; Gronenberg and Hölldobler, 1999; Nishikawa et al., 2008]. It was shown by physiological and neuroanatomical data in the carpenter ant *Camponotus floridanus* that workers process information about the trail-pheromone in regularly sized glomeruli [Zube et al., 2008]. But it can not be excluded that other ant species with a highly-developed trail-pheromone communication system also possess a MG. Eventually, the existence of size polymorphism is the precondition for the over-expression of a glomerulus for trail-pheromone components. It would be very interesting to investigate other ant species which show both, a highly developed trail-pheromone communication as well as a pronounced size polymorphism, like army ants or members of the *Pheidole* genus.

4.4.3. Position of the macroglomerulus

In moth species of the genus *Heliothis* it was found that the position of a glomerulus is conserved across closely-related species [Vickers et al., 1998; Vickers and Christensen, 2003]. The same pheromone components are represented at the same position in an array of glomeruli (macroglomerular complex). This is possibly also the case for the MGs in the leaf-cutting Attini, where two different releaser components were identified for different species. The releaser component methyl-4-methylpyrrole-2-carboxylate (M4MP2C) was found in *A. cephalotes*, *A. texana* and *A. vollenweideri*, and in *Ac. octospinosus* and *Ac. subteraneus* [Tumlinson et al., 1971; Nascimento et al., 1994; Tanaka et al., 2004; Morgan, 2009]. A different releaser component, 2-ethyl-3,6-dimethylpyrazine (2E3,6DMP), was found in *A. sexdens* [Cross et al., 1979]. *A. texana, A. vollenweideri and Ac. octospinosus* have the same releaser component, and we found a similar MG-position. *A. cephalotes* has a MG at a similar position as *A. sexdens*, but both use different releaser components. From this finding we conclude that the position of the MG in the different *Atta* and *Acromyrmex* species allows no clear prediction about the releaser component used by the species. However, the position close to the entrance of the antennal nerve is conserved across all species that have a MG.

4.4.4. A macroglomerulus in large workers only

Small workers can be found on trails of different leaf-cutting Attini [Stradling, 1978; Wilson, 1980b; Whitehouse and Jaffe, 1996; Hughes and Goulson, 2001], and it was shown that they can perceive the trail pheromone components, although they do not possess a MG. However, there are differences in the quality of perception of the trail pheromone, as demonstrated in behavioral experiments [Kleineidam et al., 2007]. We assume that in small workers the MG-corresponding glomerulus (responsible for the detection of the trail pheromonal releaser component) is located at a similar position in the AL as in large workers.

4.4.5. The macroglomerulus and social organization

We propose that the MG is a specialization for a particular olfactory-guided foraging task: the trail-following behavior. This is supported by the fact that the size of the MG differs in different leaf-cutting species, and appears to correlate with their foraging system and their

effectiveness in leaf-cutting. We find the least developed MG of all investigated species in *Ac. striatus* and *Ac. balzani*, both belonging to the subgenus Moellerius. While most *Acromyrmex* species use large trail systems to supply their fungi with fresh plant material, *Ac. striatus* workers forage on less predictable resources and do not construct or maintain physical trails, using more loosely chemical trails instead [Carbonell, 1943; Bucher and Montenegro, 1974; Farji-Brener and Protomastro, 1992; Marschner et al., 1993]. Similarly, workers of *Ac. balzani*, a grass-cutting species with a relative small colony size, do usually employ short trails and a less complex chemical recruitment system [Fowler et al., 1986b; Lopes et al., 2003; Lopes et al., 2004]. In contrast, we found the largest MG (nearly 14 times larger compared to the median of the volumes of all other glomeruli) in *A. capiguara*, which use extended underground and superficial trails to forage enormous amounts of grass (up to 196 kg/colony/year; [Robinson and Fowler, 1982]). The presence of a MG, therefore, appears to be an adaptation to trail pheromone guided behavior of leaf-cutting Attini, and has reached the most prominent enlargement in species with an elaborate trail system.

Leaf-cutting Attini either forage mainly on grass (*A. capiguara, A. bisphaerica, A. vollenweideri, Ac. balzani, Ac. fracticornis, Ac. striatus* and *Ac. heyeri*) or forage on dicots (*A. sexdens, A. cephalotes, A. colombica* and *Ac. lundi*) [Fowler et al., 1986a]. It is assumed that one of both foraging preferences is basal and the other derived. Which of both represents the origin of leaf-cutting Attini is unclear [Weber, 1972; Fowler, 1982; Mayhe-Nunes and Jaffé, 1998]. Our analysis on the size of the MGs (R_v measure) revealed no correlation between MG size and foraging preference. Rather we found similar variations in both groups with a R_v from 5.8 to 7.7 in grass-cutting Attini, and a R_v from 6.2 to 7.5 in dicot-cutting Attini. This suggests that the MG may have evolved prior to the diversification in substrate selection, and further emphasizes the strong plasticity of this overexpressed trait.

The larger the colony, the higher is its need for large amounts of substrate to supply the fungus. Fresh material like leaves or grasses are available in a much larger amount and provide more energy compared to dead plant material. For being able to cut and harvest fresh plant material efficiently, however, an ant worker has to have a critical size and strong mandibles. A single large worker is able to harvest more plant material than a whole group of small workers [Beshers and Traniello, 1996]. On the other hand, small workers are needed

inside the nest for fungus care and for the delicate handling of the fungus hyphae to feed the larvae. These distinct tasks that need to be fulfilled by leaf-cutting ant workers in a colony probably promoted the evolution of worker polymorphism. While flexible division of labor within the worker caste is common in most social insects, the polymorphism constrains the flexibility of e.g. task switching. As a consequence, workers become specialized for particular tasks, and specific adaptations might further support this specialization. The MG in large workers of *Atta* and *Acromyrmex* is such an adaptation with a likely great impact on both the processing of odor information, and the responsiveness of large workers to odor stimuli. Thus, the neuroanatomical differences between small and large workers support the alloethism in polymorphic species and add on to the complexity of their social organization.

In the past, behavioral, ecological, morphological and genetic characters have been used to categorize the tribe Attini into three major groups (lower, higher and leaf-cutting Attini). This is the first study that compared neuroanatomical traits within this monophyletic but behaviorally divers group of ants. The results show that one group, the leaf-cutting Attini, is separated based on a unique neuroanatomical trait, the MG. It is expected that future studies on the behavioral ecology of attine species will highlight the behavioral significance of other neuroanatomical characters described in this study.

Table 2: Investigated Attini species and the major antennal lobe parameters analyzed.

Species		Type	Origin	Date	Fix.	Head width	3D-R.	No.	Vol. min	Vol. max.	R_v	AL Vol.	AL/ OL
A. cephalotes	Leaf-cutting Attini	l	Panama	2003	fix-mix	2.29	1 (2)	349	922	36651	7.5	1262390	5.83
A. colombica		l	Panama	2003	fix-mix	2.40	1 (2)	411	777	40161	6.8	1707621	5.89
A. texana		f	USA	2007	fix-mix	1.34	1 (2)	336	1086	42173	7.2	1709900	4.80
A. laevigata		l	Brazil	2005	fix-mix	2.96	2 (1)	452	500	47019	7.0	1897535	--
A.sexdens		l	Brazil	2002	fix-mix	2.16	1 (2)	382	646	32520	7.0	1125748	5.13
A. capiguara		f	Brazil	2007	fix-mix	2.4	1 (1)	389	724	47645	6.6	1690539	5.21
A. vollenweideri		l	Argentina	2005	fix-mix	2.56	6 (10)	450	165	16575	6.1	707377	4.90
A. bisphaerica		f	Brazil	2007	fix-mix	2.64	1 (1)	406	412	27564	6.7	1020307	--
Ac. ambiguus		l	Uruguay	2003	fix-mix	1.89	1 (3)	409	418	22210	6.7	961455	7.36
Ac. lundi		l	Argentina	1997	fix-mix	1.03	1 (1)	477	451	30448	6.2	1579262	15.84
Ac. octospinosus		l	Costa Rica	1995	fix-mix	2.08	1 (1)	438	465	30655	6.4	1544938	8.70
Ac. fracticornis		f	Argentina	2008	fix-mix	2.4	1 (2)	437	575	24629	6.3	1260251	--
Ac. heyeri		l	Uruguay	2000	fix-mix	1.99	1 (1)	459	416	35411	7.7	1422295	12.99
Ac. balzani		f	Brazil	2007	fix-mix	2.16	1 (2)	400	667	33445	5.9	1512781	--
Ac. striatus		f	Argentina	2006	fix-mix	1.48	2 (2)	369	405	13205	5.8	628966	3.89
S. amabilis	Higher Attini	l	Panama	2007	fix-mix	1.08	1 (1)	344	424	5853	4.5	476033	9.34
T. cornetzi		l	Panama	2007	fix-mix	0.8	1 (1)	363	81	1490	4.4	121733	--
T. septentrionalis		f	USA	1993	Bouin	1.02	1 (2)	362	312	6846	4.2	549439	7.25
M. cf. hartmanni	Lower Attini	f	Costa Rica	1995	Bouin	0.68	1 (1)	309	146	3285	4.2	229412	--
My. conformis		f	Trinidad	1995	Bouin	0.59	1 (1)	288	234	5009	4.4	283656	8.15
C. costatus		l	Panama	2007	fix-mix	0.52	1 (1)	257	161	2281	3.7	142993	--
C. minutus		f	USA	2001	Bouin	0.61	1 (1)	364	245	3600	4.7	290100	--
A. cf. mayri		f	Panama	1995	Bouin	0.64	1 (2)	630	97	4888	4.1	469871	17.17
Myc. smithi		f	Costa Rica	1995	Bouin	0.62	1 (2)	461	108	2058	4.4	206685	6.97
Myc. sp.		f	Costa Rica	1995	Bouin	0.64	1 (1)	360	127	1074	2.8	144779	6.17

Species: investigated workers of Attini species; affiliation to one of the three Attini groups [Wetterer et al., 1998; Schultz and Brady, 2008] **Type:** laboratory (l) or field (f) colony; **Origin:** country, where colony or founding queen was collected; **Date:** year, when founding queen (laboratory colonies) or workers (field colonies) were collected; **Fix:** used fixation method, either fixation with Bouin solution or fix-mix; **Head-width:** measured head width from eye to eye of the reconstructed worker (in mm); **3D-R:** number of 3D-reconstructed antennal lobes and manually inspected antennal lobes (in brackets); **No.:** number of glomeruli found in the antennal lobe; **Vol. min:** volume of smallest glomerulus (in μm^3); **Vol. max:** volume of largest glomerulus (in μm^3); **R_v:** radius volume of largest glomerulus; **AL Vol.:** Volume of all glomeruli in the antennal lobe (in μm^3); **AL/OL:** ratio of antennal lobe volume and optic lobe volume.

5. Outlook

I investigated the trail-following performance of different sized *A. vollenweideri* workers, which is depended of the AL-phenotype (existence of the MG). Neuroanatomical studies revealed that in addition to the two previously described AL-phenotype (MG- and RG-phenotype), two other distinct AL-phenotypes (LN- and HN-phenotype) exist. The differences in the organization of the AL divide the worker caste of leaf-cutting ants into distinct neuroanatomical sub-castes. The sub-castes of the *Atta vollenweideri* workers differ in their olfactory-guided behavior like trail-following, and this may support different behavioral phenotypes resulting in an elaborated social organization of the colony. I also investigated the neuroanatomy of the antennal lobe of several Attini species to find the evolutionary origin of the MG. The MG is a derived overexpression of a trait only found in the polymorphic leaf-cutting species.

The findings described above gave only first insights into how the individual's nervous system influences the social organization of the whole colony. Next to the behavioral experiments, neuroanatomical techniques where used to investigate the olfactory system of the workers. These techniques have, like all techniques, boundaries. We now have a good overview of the antennal lobe structure, while functional aspects and the genetic background are still unknown. The next steps in investigating underlying mechanisms of social behavior and organization should include three main approaches providing continuative information. First, more detailed behavioral studies are necessary to understand the specialization of different sized workers on diverse tasks. In this study, only the trail-following behavior is analyzed, but other tasks like brood care, fungus care and reaction to alarm pheromones are also important for the ecological success of the colony. For the investigation of size-dependent differences in those aspects, adequate behavioral paradigms have to be developed.

Second, the physiology of the olfactory system has to be analyzed in order to understand the function of the different glomeruli in different sized workers. Therefore, imaging techniques like calcium-imaging seem to be an appropriate method to uncover the differences in the processing of olfactory information in the polymorph worker caste. Beside the general study of glomeruli, two clusters should be in the main focus of investigations. These are the T4-cluster, where about 50 glomeruli with yet unknown function are missing in small workers,

and the T6 cluster, where the ORNs of the Sensilla basiconica terminate, to find out if those sensilla have a special function in the antennal lobe. To answer both questions, a 2–photon calcium imaging setup is necessary, because both areas lie to deep in the brain to gain information with a normal CCD calcium imaging setup.

Third, the molecular basis of the olfactory sense should be investigated. The odorant receptor genes (OR-genes) and their translated receptor proteins are completely unknown. Differences in the expression of OR-genes in different sized workers can be expected. The identifying of the OR-genes in the worker caste is technically possible, but this approach will be a large and long-lasting project, because until know, there are no information about the OR-genes in *Atta* and no preliminary tests have been made.

6. Literature

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Publikationen

Kelber C, Rössler W, Kleineidam CJ. Phenotypic plasticity in number of glomeruli and sensory innervation of the antennal lobe in leaf-cutting ant workers (*A. vollenweideri*), Developmental Neurobiology, submitted.

Kuebler LS, **Kelber C**, Kleineidam CJ. Distinct antennal lobe phenotypes in the leaf- cutting ant (*Atta vollenweideri*), 2009, Journal of Comparative Neurology, in press.

Kelber C, Rössler W, Roces F, Kleineidam CJ. The antennal lobes of fungus growing ants (Attini): neuroanatomical traits and evolutionary trends, 2009, Brain, Behavior and Evolution, 73:273–284.

Kelber C, Rössler W, Kleineidam CJ. Multiple olfactory receptor neurons and their axonal projections in the antennal lobe of the honeybee *Apis mellifera*; 2006; Journal of Comparative Neurology, 496:395-405 (diploma thesis).

Vorträge

Kelber C, Kleineidam CJ. Neuroanatomical sub-castes in polymorphic ants; 2009; 102. Jahresversammlung der Deutschen Zoologischen Gesellschaft; Regensburg

Kelber C, Rössler W, Roces F, Kleineidam CJ. Correlating neuroanatomical traits and social organization in attine ants; 2008; 4. Europäisches Treffen der IUSSI; La Roche-en-Ardenne, Belgien.

Kelber C. Multiple olfactory receptor neurons in hymenoptera; 2006; 17. Neuro-biologischer Doktorandenworkshop; Berlin.

Posterpräsentationen

Kelber C, Roces F, Rössler W, Kleineidam CJ. Correlating social organization and neuroanatomical characters in leaf-cutting ants; 2009; 32th Göttingen Neurobiology Conference; Göttingen.

Kübler LS, **Kelber C**, Rössler W, Kleineidam CJ. Neuroanatomical sub-castes in leaf-cutting ants: Differences in antennal lobe design correlate with olfactory guided behavior; 2007; 31th Göttingen Neurobiology Conference; Göttingen.

Kelber C, Kübler LS, Rössler W, Kleineidam CJ. Antennal lobe design is caste-specific in leaf-cutting ants; 2006; Forum of european neuroscience, Wien

Kelber C, Rössler W, Kleineidam CJ. Functional organization of poreplate sensilla in the honeybee *Apis mellifera*; 2005; 30th Göttingen Neurobiology Conference, Göttingen.

Rybak J, **Kelber C**, Kübler L. Serotonin-like immunoreaktivity in the beewolf, *Philantus triangulum* F.; 2003a; Tagung der Deutschsprachigen Sektion der IUSSI, Regensburg.

Rybak J, **Kelber C**, Meyer C. Sexual dimorphism in the brain of the beewolf, *Philantus triangulum* F.; 2003b; Tagung der Deutschsprachigen Sektion der IUSSI; Regensburg.

Ehrenwörtliche Erklärung

gemäß §4 Abs. 3 Ziff. 3,5 und 8 der Promotionsordnung der Fakultät für Biologie der Bayrischen Julius-Maximilians-Universität Würzburg.

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Die Dissertation wurde bisher weder vollständig noch teilweise einer anderen Hochschule mit dem Ziel einen akademischen Grad zu erwerben vorgelegt.

Im Mai 2005 wurde mir von der Universität Würzburg der akademische Grad „Diplom-Biologin Univ." verliehen. Weitere akademische Grade habe ich weder erworben noch versucht zu erwerben.

....

Datum Christina Kelber

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