Sensory basis of thermal orientation in leaf-cutting ants

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Sensorische Grundlagen der thermischen Orientierung bei Blattschneiderameisen

Doctoral thesis for a doctoral degree at the Graduate School
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Section: Integrative Biology

Submitted by

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I. Kleineidam CJ., Ruchty M., Casero-Montes ZA. and Roces F.

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II. Ruchty M., Romani R., Kuebler LS., Ruschioni S., Roces F., Isidoro N. and Kleineidam CJ.

The thermo-sensitive sensilla coeloconica of leaf-cutting ants (*Atta vollenweideri*)
Arthropod Structure and Development **38**: 195-205, **2009**

III. Ruchty M., Roces F. and Kleineidam CJ.

Detection of minute temperature transients by thermo-sensitive neurons in ants
Journal of Neurophysiology (Submitted)

IV. Ruchty M., Helmchen F., Wehner R. and Kleineidam CJ.

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I. Summary

Leaf-cutting ants have a highly developed thermal sense which the insects use to regulate the own body temperature and also to optimize brood and fungus development. Apart from the already described temperature guided behaviors inside the nest it is unknown to what extent the ants may use their thermal sense outside the nest. As part of the present thesis, the question was addressed whether leaf-cutting ants (*Atta vollenweideri*) are able to learn the position of a warm object as landmark for orientation during foraging. Using absolute conditioning, it was shown that ten training trials are sufficient to elicit the association between food reward and the temperature stimulus. In the test situation (without reward) a significantly higher amount of ants preferred the heated site compared to the unheated control. Importantly, thermal radiation alone was sufficient to establish the learned association and served as orientation cue during the test situation (**chapter IV**). Based on the experimental design used in the previous chapter, the localization of thermosensitive neurons, which detect the underlying thermal stimuli, is restricted to the head or the antennae of the ants. The antennal sensillum coeloconicum is a potential candidate to detect the thermal stimuli during the orientation behavior. In **chapter V** the sensillum coeloconicum of *Atta vollenweideri* was investigated concerning its gross morphology, fine-structure and the physiology of the associated thermosensitive neuron. The sensillum is predominantly located on the apical antennal segment (antennal tip) where around 12 sensilla are clustered, and it has a peg-in-pit morphology with a double walled, multiporous peg. The sensory peg is deeply embedded in a cuticular pit, connected to the environment only by a tiny aperture. The sensillum houses three receptor neurons of which one is thermosensitive whereas the sensory modality of the other two neurons remains to be shown. Upon stimulation with a drop in temperature, the thermosensitve neuron responds with a phasic-tonic increase in neuronal activity (cold-sensitive neuron) and shows rapid adaptation to prolonged stimulation. In addition, it is shown that thermal radiation is an effective stimulus for the thermosensitive neuron. This is the first evidence that sensilla coeloconica play an important role during the thermal orientation behavior described in chapter IV. During the test situation of the classical conditioning paradigm, the ants showed rapid antennal movements, indicating that they scan their environment in order to detect the heated object.

Rapid antennal movements will result in rapid discontinuities of thermal radiation that require thermosensitive neurons with outstanding sensitivity and high temporal resolution. In **Chapter VI** the question was addressed whether the thermosensitive neuron of the sensilla coeloconica fulfils these preconditions. Extracellular recordings revealed that the neuron is extremely sensitive to temperature transients and that, due to the response dynamics, an estimated stimulus frequency of up to 5 Hz can be resolved by the neuron. Already a temperature increase of only 0.005 °C leads to a pronounced response of the thermosensitive neuron. Through sensory adaptation, the sensitivity to temperature transients is maintained over a wide range of ambient temperatures. The discovered extreme sensitivity, the high temporal resolution and the pronounced adaptation abilities are further evidence supporting the idea that sensilla coeloconica receive information of the thermal environment, which the ants may use for orientation.

In order to understand how the ants use their thermal environment for orientation, it is necessary to know where and how thermal information is processed in their central nervous system. In **Chapter VII** the question is addressed where in the brain the thermal information, specifically received by the thermosensitive neuron of sensilla coeloconica, is represented. By selectively staining single sensilla coeloconica, the axons of the receptor neurons could be tracked into the antennal lobe of *Atta vollenweideri* workers. Each of the three axons terminated in a single functional unit (glomerulus) of the antennal lobe. Two of the innervated glomeruli were adjacent to each other and are located lateral, while the third one was clearly separate and located medial in the antennal lobe. Using two-photon Ca^{2+} imaging of antennal lobe projection neurons, the general representation of thermal information in the antennal lobe was studied. In 11 investigated antennal lobes up to six different glomeruli responded to temperature stimulation in a single specimen. Both, warm- and cold-sensitive glomeruli could be identified. All thermosensitive glomeruli were located in the medial half of the antennal lobe. Based on the correlative evidence of the general representation of thermal information and the results from the single sensilla stainings, it is assumed that thermal information received by sensilla coeloconica is processed in the medial of the three target glomeruli. This part of the thesis shows the important role of the antennal lobe in temperature processing and links one specific thermosensitive neuron to its target region (a single glomerulus).

In chapter V it was shown that the sensilla coeloconica are clustered at the antennal tip and have an extraordinary peg-in-pit morphology. In the last chapter of this thesis **(Chapter VIII)** the question is addressed whether the morphology of the sensilla coeloconica predicts the receptive field of the thermosensitive neuron during the detection of thermal radiation. The sensory pegs of all sensilla coeloconica in the apical cluster have a similar orientation, which was not constraint by the shape of the antennal tip where the cluster is located. This finding indicates that the sensilla coeloconica function as a single unit. Finally the hypothesis was tested whether a single sensillum could be direction sensitive to thermal radiation based on its eye-catching morphology. By stimulating the thermosensitive neuron from various angles around the sensillum this indeed could be shown. This is the last and most significant evidence that the sensilla coeloconica may be adapted to detect spatially distributed heated objects in the environment during the thermal landmark orientation of ants.

II. Zusammenfassung

Blattschneiderameisen besitzen einen hochgradig entwickelten Temperatursinn, den sie hauptsächlich zur Regulation ihrer Körpertemperatur, aber auch zur Optimierung der Brut- und Pilzentwicklung einsetzen. Abgesehen von temperaturgesteuerten Verhaltensweisen innerhalb des Nests ist nicht bekannt, ob die Tiere ihren Temperatursinn auch außerhalb des Nests einsetzen können. Im ersten Teil der vorliegenden Arbeit wird der Frage nachgegangen, ob Blattschneiderameisen (*Atta vollenweideri*) die Position eines warmen Objektes detektieren können und ob sie das Objekt anschließend als erlernte Landmarke zur Orientierung während des Furagierens nutzen können. Mit Hilfe eines absoluten Konditionierungsparadigmas konnte gezeigt werden, dass nach zehn Trainingsdurchläufen die Assoziation zwischen Futter und einem thermischem Stimulus von den Tieren gebildet wird. In der unbelohnten Testsituation entscheiden sich die signifikant höhere Anzahl der Tiere für die erwärmte Seite. Alleine die thermische Strahlung des erwärmten Körpers ist bereits ausreichend, um die Assoziation zu bilden und während des Tests als Orientierungssignal zu dienen **(Kapitel IV)**.

Durch die Art und Weise der Durchführung des Experiments im vorangegangenen Kapitel, konnte der Ort, an dem sich die nötigen thermosensitiven Neurone befinden, auf den Kopf bzw. die Antennen der Tiere beschränkt werden. Aufgrund ihrer Position auf den Antennen gelten die Sensilla coeloconica als potentielle Kandidaten für die Detektion der notwendigen Stimuli während des thermischen Orientierungsverhaltens. In **Kapitel V** dieser Arbeit wird das Sensillum coeloconicum in Bezug auf seine Morphologie, seine Ultrastruktur und die Physiologie des assoziierten thermosensitiven Neurons untersucht. Sensilla coeloconica befinden sich hauptsächlich auf dem letzen Antennensegment, der Antennenspitze, in einer Gruppe von bis zu 12 einzelnen Sensillen. Morphologisch kann das Sensillum als Grubensensillum klassifiziert werden und es enthält einem doppelwandigen Zapfen, der von zahlreichen Poren durchzogen ist. Der Zapfen ist tief in eine kutikuläre Grube eingelassen und nur über eine winzige Apertur mit der Umwelt verbunden. Das Sensillum beherbergt drei Rezeptorneurone, von denen eines thermosensitiv ist, während die sensorische Modalität der anderen beiden Neurone bis auf weiteres unklar ist. Als Antwort auf eine Abnahme in der Stimulustemperatur generiert das thermosensitive Neuron eine phasisch-tonische Erhöhung der neuronalen Aktivität (kältesensitives Neuron) und adaptiert sehr schnell an anhal-

tende Stimulationen. Zusätzlich kann gezeigt werden, dass thermische Strahlung ein wirksamer Stimulus für das thermosensitive Neuron ist. Die Ergebnisse dieser Untersuchungen sind ein erster Hinweis darauf, dass die Sensilla coeloconica eine wichtige Rolle während des thermischen Orientierungsverhaltens spielen.

Bei der klassischen Konditionierung wurden schnelle Antennenbewegungen bei den Ameisen festgestellt, die sich in der Testsituation zwischen dem warmen Objekt und dem Kontrollobjekt entscheiden mussten. Diese schnellen Bewegungen könnten bedeuten, dass die Tiere Ihre Umgebung nach dem konditionierten warmen Objekt absuchen. Solche schnellen Antennenbewegungen führen zu schnellen Temperaturänderungen und die Detektion dieser Stimuli setzt thermosensitive Neurone mit besonderer Sensitivität und erhöhtem zeitlichen Auflösungsvermögen voraus. In **Kapitel VI** wird untersucht, ob das thermosensitive Neuron der Sensilla coeloconica diese Voraussetzungen erfüllt. Extrazelluläre Ableitungen zeigen, dass das Neuron extrem sensitiv auf Temperaturänderungen reagiert und dass aufgrund der Antwortdynamik Stimulationsfrequenzen von bis zu fünf Hertz aufgelöst werden können. Schon eine Temperaturänderung von 0.005 °C führt zu einer ausgeprägten Antwort des thermosensitiven Neurons. Durch sensorische Adaption bleibt diese erhöhte Sensitivität über einen großen Umgebungstemperaturbereich erhalten. Die außergewöhnliche Sensitivität, die hohe zeitliche Auflösung sowie die Adaptionsfähigkeit des thermosensitiven Neurons sind weitere Hinweise darauf, dass die Sensilla coeloconica in der Lage sind Stimuli zu rezipieren, welche zur thermischen Orientierung genutzt werden könnten.

Um zu verstehen, wie sich die Tiere anhand ihrer thermischen Umwelt orientieren, ist es nötig zu wissen, wo im Zentralnervensystem die thermische Information prozessiert wird. In **Kapitel VII** wird analysiert, in welchem Bereich des Gehirns die thermische Information der Sensilla coeloconica repräsentiert ist. Mittels selektiver Färbung einzelner Sensilla coeloconica können die Axone der Rezeptorneurone im Gehirn verfolgt werden. Jedes der drei Axone endet in jeweils einer funktionellen Einheit (Glomerulus) im Antennallobus. Zwei der innervierten Glomeruli sind direkt benachbart und liegen im lateralen Teil des Antennallobus während der dritte Glomerulus im medialen Bereich zu finden ist. Mit Hilfe von zwei-Photonen Ca^{2+} Imaging der Projektionsneurone wurde die Repräsentation von thermischer Information im Antennallobus untersucht. In 11 untersuchten Antennalloben antworten bis zu sechs einzelne Glomeruli auf die Temperaturstimulation. Sowohl warm- als auch kalt-sensitive Glomeruli konnten identifiziert werden. Alle thermosensitiven Glomeruli befanden sich in der medialen Hälfte des Antennallobus. Basierend auf diesen Ergebnissen sowie auf den Ergebnissen der Einzelsensillenfärbungen ist davon auszugehen, dass thermische Information, die von den Sensilla coeloconica rezipiert wird, im medialen der drei innervierten Glomeruli prozessiert wird. Dieser Teil der Doktorarbeit zeigt, dass der Antennallobus in der Temperaturverarbeitung eine wichtige Rolle spielt und zeigt die Verbindungeines spezifischen thermosensitiven Neurons mit seiner Zielregion, einem einzelnen Glomerulus.

In Kapitel V wurde gezeigt, dass die Sensilla coeloconica gruppiert an der Antennenspitze vorkommen und eine außergewöhnliche Morphologie besitzen. Im letzten Kapitel dieser Doktorarbeit **(Kapitel VIII)** wird untersucht, ob anhand der Morphologie der Sensillen Voraussagen über das rezeptive Feld bezüglich thermischer Strahlung getroffen werden können. Die sensorischen Zapfen aller Sensilla coeloconica sind ähnlich in ihrer Grube ausgerichtet wobei diese Ausrichtung nicht durch die Form der Antennenspitze bedingt ist. Diese Erkenntnis ist ein Hinweis darauf, dass alle Sensilla coeloconica eine funktionelle Einheit bilden. Zum Schluss dieser Arbeit wird die Hypothese untersucht, ob ein einzelnes Sensillum aufgrund seiner Morphologie richtungssensitiv gegenüber thermischer Strahlung ist. Durch Stimulation des thermosensitiven Neurons mit thermischer Strahlung aus verschiedenen Winkeln kann dies tatsächlich nachgewiesen werden. Dieses Resultat ist ein deutlicher Hinweis darauf, dass die Sensilla coeloconica an die Detektion räumlicher verteilter, strahlender Objekte angepasst sein könnten und damit das thermische Orientierungsverhalten ermöglichen.

III. General Introduction

The ability to detect temperature is fundamental for all species and often the sensory system provides the organisms with detailed information on their thermal environment. The most important purposes of the thermal sense are 1) keeping the body at an optimal working temperature and 2) the detection of potentially harmful or advantageous temperatures. The impact of the environmental temperature on the daily life of an animal is not equal and largely depends on how the respective species regulates its body temperature. Endothermic animals like mammals generate metabolic heat and maintain a constant body temperature, usually higher than the environmental temperature. Thus the impact of the environmental temperature is rather low. In contrast, ectothermic animals as for instance insects regulate their body temperature via exchanging heat with their surroundings and are thus prone to hypo- or hyperthermia which can rapidly cause death. The following paragraphs will provide some basic information on the general principles of thermosensation across species and will introduce social insects as model organisms.

Principles of thermosensation across animal species

Principles of thermosensation are highly conserved throughout the animal kingdom. Irrespective of the purpose of temperature detection across animal clades, thermal stimuli are detected by thermosensitive neurons which are located in specific body parts like e.g. the skin in mammals or the antennae in insects. Based on their physiological characteristics, the thermosensitive neurons can basically be divided into warm- and cold-sensitive neurons, respectively. Across species, thermosensitive neurons share similarities, but also differ in terms of e.g. their physiological characteristics or the morphology of their accessory structures. In mammals, thermal stimuli are received by thermosensitive neurons which are located with their free nerve endings in the dermal or epidermal skin layers (Weddell et al. 1955). Both cold-and warm-sensitive neurons occur in similar numbers. The neurons are spontaneously active (2-5 imps/s) and typically respond with a phasic-tonic response characteristic to the respective temperature stimulus (warm or cold) (Hensel 1973). The perception of temperature might change dependent on the stimulus amplitude e.g. from cool to pain for cold stimuli and from warmth to pain in warm stimuli (Schepers and Ringkamp 2009).

Insects are ectothermic animals and thus the detection of environmental temperatures inevitably has a higher priority compared to most mammals. In order to keep their body at a stable working temperature, insects establish precise thermal preferences. *Drosophila,* for example, resides at ≈ 24°C when placed upon a thermal gradient (Sayeed and Benzer 1996), and this temperature guided behavior seems to be innate (Liu et al. 2003). The thermal preference is very stable in this insect and is not influenced by preimaginal or adult experience (McKemy 2007). The thermal preference is established by means of two antagonistic thermosensitive neurons. One is located in the brain of the animal and sets the upper threshold (Hamada et al. 2008) whereas the second one is localized on the antennae and sets the lower threshold (Sayeed and Benzer 1996; Zars 2001). Once these thresholds are exceeded, the neurons fire and trigger avoidance behavior (Hamada et al. 2008; Sayeed and Benzer 1996; Zars 2001). As in *Drosophila,* most of the so far described thermosensitve neurons in insects are located on the antennae in cuticular structures termed sensilla. In contrast to the mammalian system, the majority of the thermosensitive neurons in the sensilla are classified as cold-sensitive whereas warm-sensitive neurons have been rarely described (Davis and Sokolove 1975; Loftus and Corbière-Tichané 1981). Similar to mammalian neurons, insect cold-sensitive neurons are spontaneously active and generate a phasic-tonic increase in neuronal activity to a decrease in temperature (Altner and Loftus 1985; Ameismeier and Loftus 1988; Gödde and Haug 1990; Lacher 1964; Loftus 1968; Merivee et al. 2003; Must et al. 2006; Nishikawa et al. 1992; Tichy 1979; Tichy and Loftus 1987; Waldow 1970).

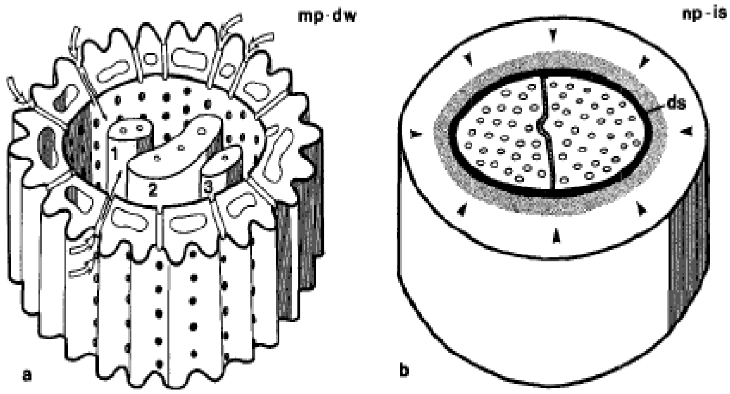


Figure 1: Schematic drawing of the two morphological types

a) The multiporous double walled type houses three receptor neurons. One responds to temperature, the other two neurons are usually chemosensitive. **b)** The second type of thermosensitive sensilla has a single walled nonporous peg which houses the combination of one thermosensitive and antagonistic hygrosensitive neurons. **DS:** dendritic sheath. Adapted from (Altner and Loftus 1985)

The sensilla which house thermosensitve neurons usually have a characteristic peg-in-pit morphology. Based on their fine-structure, the thermosensitive peg-in-pit sensilla can be separated in two morphological classes. The first type houses a sensory peg which is double walled (DW) and establishes many pores or spoke channels which connect the environment to the sensory structures (Altner et al. 1977). Usually between two and four receptor neurons are associated with this morphological type (Altner and Loftus 1985), of which one neuron is thermosensitive, and the others serve a chemosensory function (Altner et al. 1981; Altner et al. 1977; Altner et al. 1983; Davis and Sokolove 1975; Hunger and Steinbrecht 1998) (Fig. 1a). The second sensillum type can be easily distinguished from the MP-DW sensilla by the single walled, nonporous peg. Similar to the MP-DW sensilla, three dendrites are associated with the nonporous type. One receptor neuron is thermosensitive, whereas the other two are antagonistic hygro-receptors (Altner and Loftus 1985; Altner et al. 1981; Tichy 1979; Waldow 1970; Yokohari 1983) (Fig. 1b).

Social insects as model organism

Effects of temperature on the development

Temperature has a strong influence on the daily life of social insects, which establish huge colonies with thousands of individuals. In their specific case, temperature not only affects the survival of an individual insect but, in addition, has a strong impact on the development of the brood and thus determines the success of a whole colony. For example in the fire ant *Solenopsis invicta,* the brood development is significantly shortened with an increased rearing temperature (Porter 1988). The developmental rate first rises linearly as a function of temperature, and reaches its optimum at 32°C. Above this temperature optimum the developmental rate suddenly drops (Calabi and Porter 1989; Porter 1988; Roces and Núñez 1989). The rearing temperature does not only affect the developmental period and the mortality rate of the brood in social insects, but can also can lead to anatomical abnormities (Koeniger 1978). In honeybees it has been shown that the development of the central nervous system is influenced (Groh et al. 2004), thereby negatively affecting the behavioral performance and communication abilities of adult bees (Tautz et al. 2003).

Temperature regulation in honeybees

To prevent such negative temperature induced effects but rather to optimize the brood development and thereby to increase the colony fitness, social insects either relocate the brood, as it is the case in ants, or actively regulate the temperature inside their nest. Honeybees keep the temperature in their nest hive in a range between 33° and 36°C (Seeley 1985) with only minor fluctuations around these values (Heinrich 1993; Kleinhenz et al. 2003). Elevated temperatures inside the nest are counteracted by e.g. wing fanning to ventilate the hive or water intake for evaporative cooling (Lindauer 1954). At low ambient temperatures e.g. during winter, the honeybees crowd in their brood area (Harrison 1987; Kronenberg and Heller 1982) and produce metabolic heat via activating their wing muscles (Esch et al. 1991; Heinrich 1993; Heinrich and Esch 1994).

Temperature regulation in ants

In contrast to winged Hymenoptera like bumblebees, wasps or honeybees, ants are unable to produce metabolic heat through muscle activation for heating the nest. In these insect species primarily the nest architecture buffers fluctuations in climate parameters (Frouz 1996; Horstmann and Schmid 1986; Kleineidam et al. 2001). In addition to this architecture based climate control, ants actively regulate the temperature of their brood. As soon as there is a deviation from preferred temperature conditions, worker ants relocate the pupae to areas in the nest with favorable thermal conditions for their development (Brian 1973; Porter 1988; Roces and Núñez 1989; Weidenmüller et al. 2009). This brood tending behavior follows a circadian rhythm (Roces and Núñez 1995), and is extremely plastic as it can be influenced by preimaginal or adult experience (Weidenmüller et al. 2009). In leaf-cutting ants, nest temperature has an even higher impact on the colony fitness compared to the other social insect species. These ants forage fresh leaf material as a substrate for a symbiotic fungus, which is the sole food source for the developing brood (Quinlan and Cherrett 1979). The optimal growth rate of the fungus is reached at an ambient temperature between 25° and 30°C (Powell and Stradling 1986; Quinlan and Cherrett 1979) and it has been shown that leaf-cutting ant workers, similar to the brood tending behavior, relocate their fungus from unsuitable towards suitable temperatures (Bollazzi and Roces 2002).

These examples emphasize the importance of the thermal sense in social Hymenoptera and describe amazing behavioral adaptations of these insects to cope with temperature fluctuations inside the nest. In addition, a few studies revealed that the thermal sense has a function outside the nest in the context of foraging. In honeybees, it has been shown that individual foragers can associate a temperature stimulus with a food reward (Hammer et al. 2009; Heran 1952; Menzel et al. 2001) and in bumblebees the temperature of flowers influences the foraging decision of these pollinators (Dyer et al. 2006). It is assumed that the thermal sense strongly contributes to the success of Hymenoptera across latitudes and altitudes. Social insects are therefore important model organisms to study the principles of thermosensation. It is of great interest to understand the behavioral physiology of the various temperature guided behaviors and their underlying neuronal mechanisms.

Thesis Outline

The following aspects will be addressed in the chapters IV-VIII of the present thesis:

1. ***Thermal orientation outside the nest***

   Leaf-cutting ants mainly utilize their thermal sense to thermo-regulate or to detect the most suitable conditions for their brood and fungus within the nest. In the present chapter, the question is addressed whether the ants can use their thermal sense for orientation outside the nest (thermal orientation). The most prominent example for such thermal orientation in insects is known in fire beetles, which detect thermal radiation emitted from forest fires to orient towards the fire sites in order to meet mates and to reproduce (Evans 1964). In haematophagus bugs, thermal radiation alone is sufficient to guide these insects to their endothermic hosts where these animals receive a blood meal (Lazzari and Núñez 1989; Schmitz et al. 2000). Using a classical conditioning paradigm, the ability of leaf-cutting ants to associate a thermal stimulus (convective, conductive and radiant heat) with a food reward is investigated. Subsequently it is tested whether the ants can use the thermal stimuli as learned landmark for orientation.

2. *The thermosensitive sensillum coeloconicum*

The experimental design of the previous study (chapter IV) revealed that the involved thermosensitive neurons are located in the antennae of the leaf-cutting ants. Thermosensitive neurons on the antenna of insects are commonly housed in peg-in-pit sensilla (Altner and Loftus 1985). In *Atta vollenweideri*, the thermosensitive sensillum coeloconicum is a potential candidate for the detection of thermal stimuli during the thermal orientation behavior. In chapter (V) the morphology, fine-structure and physiology of the sensillum coeloconicum and its associated receptor neurons is investigated in detail, using extracellular recordings and electron microscopy.

3. *Physiological characteristics of the thermosensitive neuron*

In chapter IV rapid antennal movements have been observed during the thermal orientation and it has been shown for a closely related species (*Atta sexdens*) that the antennae can reach up to 3.6°/ms (Ehmer and Gronenberg 1997). In the context of temperature detection such movements may generate rapid and minor temperature transients at thermosensitive neurons. Thus, to successfully detect a warm object during the thermal orientation behavior, thermosensitive neurons with specific physiological characteristics are required. Using electrophysiological recordings the thermosensitive neuron associated with the sensilla coeloconica is investigated in chapter VI and the question is addressed whether it fulfills the preconditions of the detection of rapid and minor temperature transients.

4. *Representation of thermal information in the antennal lobe of ants*

In order to understand how insects orient in their thermal environment, it is necessary to know where thermal information is processed in the central nervous system. Until now, the central projections of thermosensitive neurons have been investigated only in few studies and all of them revealed the antennal lobe as the first integration centre (Nakanishi et al. 2010; Nishikawa et al. 1995; Nishino et al. 2009). In Chapter VII the question is addressed, where in the brain thermal information specifically received by the thermosensitive neuron of sensilla coeloconica is represented. The central projections of single sensilla coeloconica are investigated, and the antennal lobe is screened for thermosensitive projection neurons using two-photon Ca^{2+} imaging.

5. *Direction sensitivity of sensilla coeloconica to thermal radiation*

For the thermal orientation behavior, leaf-cutting ants have to assess the position of the radiant source and morphological adaptations of the thermosensitive sensilla are expected. The most prominent example of a morphological adaptation to detect thermal radiation is known in snakes. Boids and pit vipers possess a pair of pit organs (Newman and Hartline 1982) which these animals use to detect thermal radiation emitted by their endothermic prey (Buning et al. 1981a; b). Based on the morphology of the pit organ, the thermal environment is imaged topographically onto the pit membrane and each thermosensitive neuron is sensitive to a temperature stimulus from a certain direction (Bakken and Krochmal 2007). Because the sensilla coeloconica resemble the pit organs in miniature, a direction sensitivity to thermal radiation is expected as well. This hypothesis is tested using thermal radiation as stimulus, emitted from various angles around the sensillum.

IV. Thermal orientation in the leaf-cutting ant *Atta vollenweideri*

Abstract

We explored the ability of leaf-cutting ants (*Atta vollenweideri*) to learn the location of a food reward by using thermal information as an orientation cue. During training of single workers, the conditioned stimulus was a distant thermal source placed frontally, 15 mm away from a platform having a leaf fragment as reward. After training, single workers were confronted with the choice between two sides, one being coupled, in a pseudo-randomized design, with a thermal stimulus heated 5°C above environmental temperature. After 10 learning trials, workers significantly chose the side with the thermal stimulus. This showed that workers can use thermal information for spatial orientation in the context of foraging, which may help them to locate, for instance, highly attractive sun-exposed leaves. Thermal radiation alone as orientation cue was sufficient to allow learning, since preclusion of thermal convection during training and test did not impair workers' response. Shielding of both thorax and gaster from the thermal source did not weaken learning, suggesting the sole participation of head and antennae in temperature reception. A thermal stimulus heated 1°C above environmental temperature could not be used as a learned orientation cue, even when foragers were allowed to directly contact the thermal source.

Introduction

Environmental temperature plays a decisive role in the metabolism and energy balance of ant societies, since ants are essentially ectothermic (Heinrich 1993). Temperature primarily affects both worker activity by inhibiting or reducing foraging, as well as the speed of brood development, parameters that directly determine the growth rate of a colony (Porter 1988; Porter and Tschinkel 1993). Excessive temperatures negatively affect colony growth by reducing brood survival and worker longevity (Calabi and Porter 1989; Roces and Núñez 1989). Since temperature can have a massive effect on ant colony fitness, behavioral adaptations to cope with seasonal variations in environmental temperature, as well as with the naturally occurring daily temperature fluctuations, are expected to occur (Roces 1995; Roces and Núñez 1996; Steiner 1929). Long-term thermal regulatory responses of ants have been described at the individual and colony level. They involve, for instance, the selection of a suitable place to locate the nest by founding queens (Brian 1952), and the construction of nest mounds that to some extent compensate the natural fluctuations of climatic parameters (Frouz 1996; Kleineidam et al. 2001; Scherba 1962). Short-term responses for temperature control in ants involve, for instance, behavioral tracking of preferred temperatures inside the nest, and transporting the brood to the selected values (Brian 1973; Ceusters 1977; Roces and Núñez 1989).

Leaf-cutting ants of the genus *Atta* construct the largest ant nests, which house up to five million individuals and may contain up to 7000 underground chambers, mainly for fungus cultivation on collected leaf material (Autuori 1941; Moreira et al. 2004a; Moreira et al. 2004b). The fungus gardens represent the sole food source for the developing larvae, and they have strict demands of high humidity and temperatures between 25 and 30°C for proper growth (Powell and Stradling 1986; Quinlan and Cherrett 1978). As a consequence, workers are expected to maximize the growth of their symbiotic fungus not only by selecting suitable vegetable substrate, but also by an active manipulation of climatic conditions and proper location for fungus cultivation inside the nest. For instance, workers of a laboratory colony of *Atta sexdens rubropilosa* were observed to relocate the fungus garden along a humidity gradient, choosing the environment with the highest humidity (Roces and Kleineidam 2000).

Regarding temperature control, workers of the leaf-cutting ant *Acromyrmex heyeri* relocate the fungus and select temperatures that closely match the thermal range in which maximal growth of the symbiotic fungus occurs (Bollazzi and Roces 2002). A prerequisite for the control of nest climate, and ultimately colony growth, is the ability of workers to precisely sense the relevant nest climate parameters, such as humidity, carbon dioxide and temperature. Leaf-cutting ant workers have been shown to be equipped with thermo- and CO_2-sensitive antennal sensilla (Kleineidam et al. 2000; Kleineidam and Tautz 1996). The responses to CO_2 concentrations were shown to be relevant in the context of nest ventilation (Kleineidam et al. 2001; Kleineidam and Roces 2000). Relocation of brood and fungus as a thermoregulatory response in leaf-cutting ants (Bollazzi and Roces 2002) implies the use of temperature as an orientation cue. Temperature information may also serve outside the nest as an additional orientation cue, together with chemical, visual, gravitational and magnetic information (Vilela et al. 1987), when foraging workers explore the area around their nest. And it may also help foraging workers, upon arrival at a food plant, to find leaves warmed by the sun, which have been shown to be highly palatable (Folgarait et al. 1996; Nichols-Orians 1991).

In the present study we explored the ability of workers of the leaf-cutting ant *Atta vollenweideri*, in a foraging context, to learn the location of food by using thermal information for orientation. Further, we investigated whether the temperature response of the ants is based on the reception of convective heat or thermal radiation. In addition, we assessed the temperature difference necessary to allow learning of a heat source, both, in far range (by reception of thermal radiation or convective heat) and in contact range (by reception of conductive heat).

Materials and Methods

Animals

The colony of *Atta vollenweideri* (Forel) used in the experiments was collected in 2002 at the Reserva Ecológica El Bagual, Formosa, Argentina (Bollazzi M. and Geissler O.). It was reared at the Biozentrum, University of Würzburg, in an environmental chamber at 26°C and 50% relative humidity in a 12 h/12 h photoperiod, and fed mainly with privet leaves (*Ligustrum vulgaris*) and dog rose (*Rosa canina*).

At time of the experiments the fungus garden occupied a volume of about 12 l in 9 plastic boxes (19x19x9 cm each), interconnected with plastic tubes. One of the plastic boxes was used as central feeding box (CF box) at times when no experiments were done. During the experiments, the CF box was connected via a wooden bridge of 1 m with a perpendicular bifurcation (0.45 m each) to two additional feeding boxes. The colony had free access to the two feeding boxes. Foragers were allowed to carry leaf fragments from the feeding boxes and to develop two foraging columns (established trails) merging at the bifurcation (Fig. 1A).

Training procedure

For the experiments, single outgoing foragers were gently branched off from the wooden bridge, onto a wooden toothpick. While on the toothpick for the first time, the foragers were marked on one leg or thorax with a small dot of water soluble white out fluid (Tipp-Ex, Clichy Cedex, France) for individual identification during training and test. The toothpick was then briefly connected for transfer of the forager to a wooden training stick (TS) on which training took place (see Fig. 1B, left). Once on TS, the forager encountered a leaf fragment, picked it up in most cases, and turned back to return to the nest. The toothpick was used again for transfer, to allow the marked forager to return to the bridge and to enter the foraging column. Typically, a successful forager dropped the carried fragment on the trail after having walked 10 or 20 cm, turned back and tried to return to the discovered “foraging site” within a few minutes. Under natural conditions, this is a common behavior that allows the rapid branching and establishment of a secondary trail when an alternative food source is discovered (Hubbell and Wiemer 1983; Röschard and Roces 2003a; b). It is therefore possible to maintain an experienced worker foraging solitarily along such a side-trail, repeating this cycle of picking a fragment, dropping it upon arrival at the foraging column, and returning to the side-trail. Occasionally, some foragers were observed to return to the foraging column without collecting the leaf fragment they encountered on the platform. After a brief interaction with nestmates on the bridge, they returned to the spot from where they were transferred to the training stick (TS), and looked for the TS to reach again the platform with the leaf fragment. Such foragers, although unloaded, typically enforce recruitment of nestmates by laying pheromone trails (Jaffe and Howse, 1979; Geissler O., pers. comm.), so that such foraging cycles were also considered successful training trials.

Because of the training of freely-foraging workers in an appetitive context, the intervals between successive training trials varied between 1 and about 10 minutes. Nevertheless, this procedure was preferred, instead of forcing workers to make decisions at regular time intervals.

Testing procedure

Single foragers were challenged to learn the location of a leaf fragment as reward by using a heated object as orientation stimulus. Foragers of the control groups did not experience a heated object during training, and experienced the same conditions in the non-rewarded test situation as the experimental groups. In the test situation, the majority of foragers decided quickly for one of both sides (< 5s). Only a few turned back and left the decision point, which lead to the removal of these foragers from the analysis. The foragers' learning abilities were tested in a non-rewarded choice situation by comparing the behavior of foragers of the experimental groups versus the control groups, so as to expose any learned thermal preference. Tested foragers and those that did not complete training were removed from the colony to avoid repeated measurements.

Stimulus device

Peltier plates of 15x15 mm were used as warm and cold objects, respectively. The temperature of the Peltier plates was adjusted manually with a power supply and the surface temperature was measured with a contact-free thermometer (Quicktemp 850-T1, Testo, Germany). Adjusted temperatures were either 5°C above, 5°C below or 1°C above ambient temperature, that is 31°C, 21°C or 27°C, respectively. One surface (front face) of the Peltier plates was covered by a foil with an emissivity of 0.93; the other surface was mounted on a rod for heat conduction. Changes of temperature after switching the heat/cool current were fast and almost complete after one minute (Fig. 2). In steady-state use, the front face temperature was stable over a measured period of 30 min (data not shown).

E1: Learning experiment

In the first experiment we asked whether foragers are able to learn the location of a leaf fragment by using a heated object as a remote orientation stimulus, without the need of touching it with the antennae. We employed a paradigm of absolute conditioning. During training, foragers on TS encountered a leaf fragment (reward) placed at the center of the platform (ø 20 mm) at the end of TS (Fig. 1B left). The Peltier plate was mounted in front of the approaching forager at a distance of 15 mm from the platform offering the reward. Thus, single foragers were about 25 mm away from the Peltier plate when collecting the leaf fragment. The front face of the Peltier plate was heated to 5°C above ambient temperature for two groups of foragers; for the control group the Peltier plate had ambient temperature. After either 5 or 10 training trials (group: *5 trials* and group: *10 trials*, respectively), individual foragers were tested once at a modified setting. Two Peltier plates were mounted on both sides of the platform at an equidistance of 15 mm (Fig. 1B right). One was heated 5°C above and the other cooled 5°C below ambient temperature. The forager to be tested entered the platform without reward, and normally continued walking onto one of the two choice sticks (CPs) offered at the distal end of the platform. Only foragers that continued walking on one CP were included into the data analysis. After that, the forager was removed and its choice recorded. Sides were changed pseudo randomly in the subsequent tests with other foragers.

E2: Radiation vs. Convection

In the previous experiment (E1), learning may be based on the perception of thermal radiation, convective heat, or both. In this experiment (E2), we asked whether convective heat or thermal radiation is the relevant stimulus that allows learning. Due to the close proximity of the Peltier plate to the forager’s head, convective heat might be perceived by the forager. If convective heat acts as a relevant stimulus, removal of thermal convection should impair learning of the heated object as an orientation cue. In order to reduce the possible influence of thermal convection on temperature reception, air was blown across the platform, the forager and the Peltier plate via an air tube (diameter of 50 mm) mounted below the platform (Fig. 1C). A continuous flow of air at a velocity of about 13 mm*s^{-1} was used to drag warm air from the front face of the Peltier plates and from the platform.

Inside the air tube, four layers of copper mesh were mounted to induce a laminar air flow directed upwards.

Besides reaching the antennae, thermal radiation may in addition warm the substrate the forager stands on (TS) while experiencing the thermal stimulus. Thus, temperature differences across the substrate might influence workers' response. In order to test whether the known temperature-sensitive sensilla on the antenna (Kleineidam and Tautz 1996; Lacher 1964) are sufficient for reception of the thermal stimulus in the learning paradigm, the radiation of the heated Peltier plate was restricted to the forager's head by a shield during testing. During training, single foragers had to pass an aperture in the shield to reach the platform with the reward (Fig. 1C left). The front face of the Peltier plate, positioned ahead of the forager as in E1, was heated to 5°C above ambient temperature (group: *5°C shield*); for the control group the Peltier plate had ambient temperature. After 10 training trials, foragers were tested at a modified setting. The platform was removed and single foragers were not allowed to pass the aperture. Instead, the end of TS was positioned in the center of the aperture and two further sticks (CPs) were arranged as a Y-maze, as in E1 (placed horizontally and 90° to each other; Fig. 1C right). Two Peltier plates were mounted horizontally along the shield on both sides of the aperture, and at an equidistance of 15 mm from the center of the aperture. One was heated to 5°C above ambient temperature; the other was at ambient temperature. Sides were switched pseudo randomly between successive tests. Due to the shield, solely the head of the foragers was exposed to thermal radiation during test, but not the legs or thorax. Only foragers entering one of the two CPs were included into data analysis.

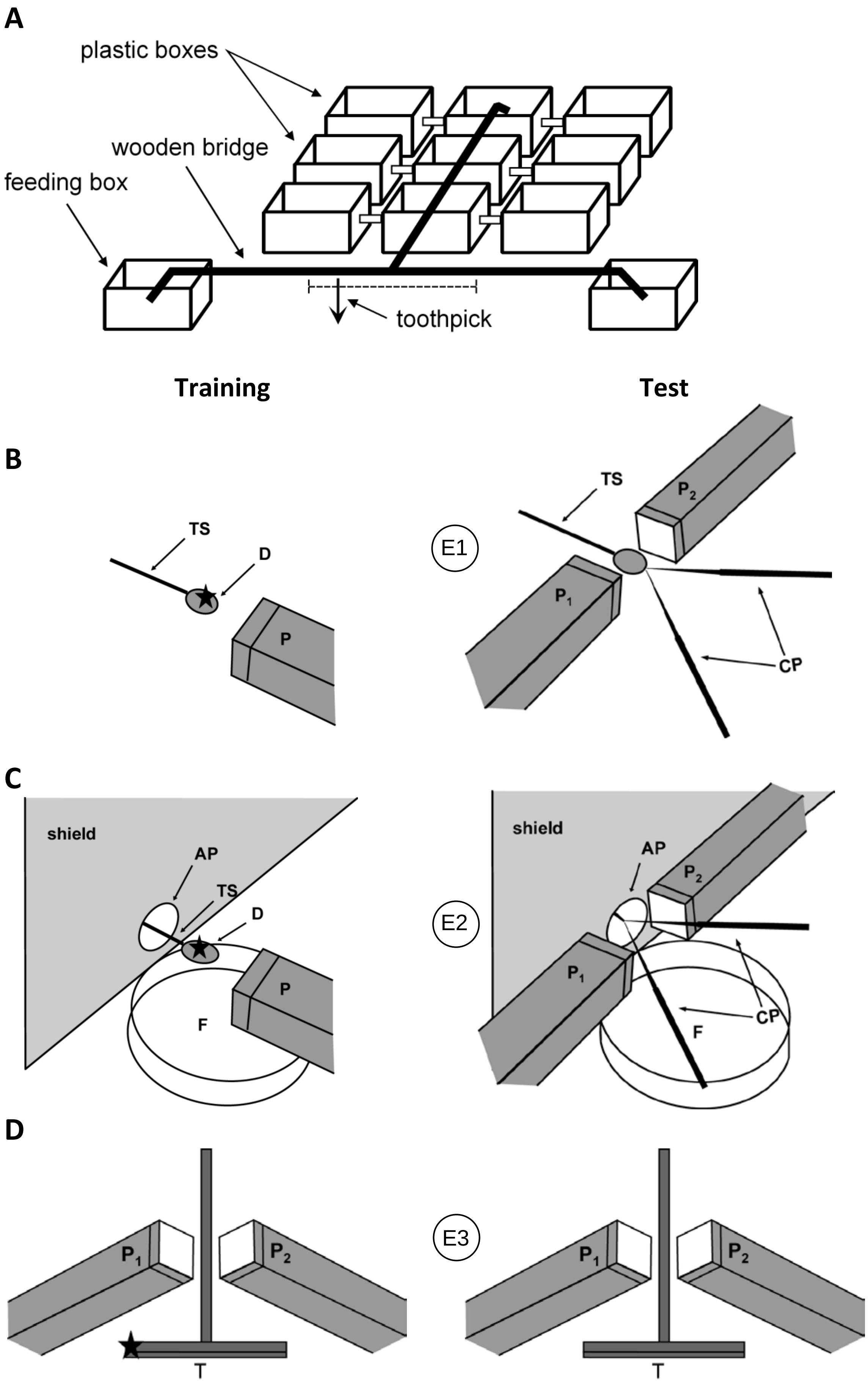
E3: Radiation vs. Conduction experiment

The sensitivity of foragers to thermal radiation might be low compared to direct assessment of surface temperature by making contact with the object. In this experiment (E3), foragers were trained to learn the location of a leaf fragment by using a heated object as orientation stimulus, as previously described, and then tested whether they are also able to orient when the same object was heated to a temperature lower than that experienced during training (training at 5°C and non-rewarded tests at either 5°C or 1°C above ambient temperature). In contrast to E1 and E2, foragers were able to touch two Peltier plates on their way to the reward (Fig. 1D left).

During training, the foragers encountered a T-maze and entered the T at the base. Before reaching the T-bifurcation, the single foragers passed the two Peltier plates. One of them was heated above ambient temperature, with the side chosen pseudo randomly during training trials, while the other plate remained at ambient temperature. The reward was offered on the same side of the approaching forager as the thermal stimulus, and the worker's decision at the bifurcation was used as measure for thermal preference. Workers were tested after 10 learning trials. During testing, i.e., during the 11th visit, single workers were confronted with the same spatial arrangement, and their choice for one of the sides (both unrewarded) was recorded (Fig. 1D, right). Thus, in experiment E3, a differential conditioning paradigm rather than an absolute conditioning paradigm, as in E1 and E2 was used. Two experimental series were performed using the same training schedule, but differing in the test situation. Foragers were tested either with one Peltier plate heated 5°C above ambient temperature (group: *5°C @test*), or with one Peltier plate heated 1°C above ambient temperature (group: *1°C @test*). Foragers that were trained and tested once with one Peltier plate heated 5°C above ambient temperature (group *5°C @test*) were tested again in second test trial. In this case, the first test trial possibly acted as an extinction trial, since no reward was offered. For the control group, the Peltier plates were at ambient temperature during the first 10 trials, and in the 11th trial, one was heated to 5°C above ambient temperature (as in group *5°C @test*), allowing to control for possible spontaneous preference for thermal stimuli. Only foragers completing the 10 training trials and the test trial were included into data analysis.

Statistical analysis

All statistical analyses were performed using the statistic software STATISTICA 7.1 (StatSoft, Oklahoma). Differences between groups were evaluated with the Chi^2-test and Fishers exact p one-tailed at a significance level of 0.05.


A
plastic boxes
wooden bridge
feeding box
toothpick
Training
Test
B
TS
D
P
E1
TS
P2
P1
CP
C
shield
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TS
D
P
F
shield
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Figure 1: Foraging-, training- and test-setups for the experiments E1, E2 and E3
A: Arrangement of nest boxes and foraging setup with wooden bridge, bifurcation and two feeding boxes. The colony established foraging columns along the wooden bridge to the two feeding boxes. Single foragers were then branched off with a toothpick in an area indicated by the dashed line, and gently transferred to one of the experimental setups shown below. **B:** Training- (left) and test-setup (right) for the learning experiment (E1). During training (on the left), single foragers approached the reward (leaf fragment, indicated by a star) on the platform (D) via the training stick (TS), and experienced the thermal stimulus (P: Peltier plate) ahead. In the test situation (on the right), two Peltier plates (P_1 and P_2) were mounted on both sides of the platform and two choice sticks (CP) were offered at the distal end of the platform. **C:** Training- (left) and test-setup (right) for the radiation-convection experiment (E2). During training (on the left), the approaching foragers had to pass an aperture (AP) in a shield before reaching the platform (D) with the reward (star). A constant and laminar air flow from a tube (F) was used to remove thermal convection from the platform and the forager. In the test situation (on the right), two Peltier plates (P_1 and P_2) were mounted on both sides of the shield and two choice sticks (CP) were offered at the distal end of the training stick (TS). **D:** Training- (left) and test-setup (right) for the radiation-conduction experiment (E3). During training (on the left), the approaching foragers passed two Peltier plates (P_1 and P_2), both of which could be touched with the antennae. One of the plates was heated. The reward (star) was offered at the same side as the heated Peltier plate on one end of the bifurcation (T). In the test situation (on the right), the arrangement was similar but no reward was offered.

Results

Side preference

In all experiments, the side where the thermal stimulus (heated Peltier plate) was positioned in the test situation was alternated pseudo randomly. This was done in order to equalize a possible bias as a result of a stimulus-independent side preference. The experimental arrangement of the colony with the T-shaped wooden bridge and two feeding boxes was used to branch off foragers inline of their foraging column (Fig. 1A). Foragers showed a slight preference for one side. Pooling the worker responses (n=190) recorded across all experiments revealed a side preference of 60 % of foragers to the right side in the test situation ($Chi^2=3.85$, df=1, $p<0.05$). However, in none of the single experiments was the stimulus-independent side preference statistically significant.

E1: Learning Experiment

In experiment E1, foragers experienced the heated object during approach and collection of the reward. In the test situation, one Peltier plate was heated and the other cooled (Fig. 1B). After 10 training trials (group *10 trials*, n=20), foragers showed a marked preference for the side with the heated object (learned warm preference) (Fig. 3A; Chi²=8.64, df=1, p<0.01). Five training trials (group *5 trials*, n=20) failed to cause any thermal preference compared to the control group (n=20) (Fig. 3A; Chi²=0.10, df=1, p=0.50). In the control experiment, foragers that experienced a heated Peltier plate only during the test showed a slight tendency, although not statistically significant, to avoid the side with the thermal stimulus, when compared with an expected 1:1 distribution of responses (Chi²=0.40, df=1, p=0.38).

E2: Radiation vs. Convection

As in E1, foragers experienced the heated object during approach and collection of the reward. In the test situation, only one Peltier plate was heated and the other remained at room temperature. The experimental arrangement excluded the influence of thermal convection, thus allowing assessing the role of thermal radiation as stimulus for learning. Furthermore, in the training and test situation any heating of the forager's body surface except at the head was prevented, because radiation was shielded from all body parts other than the head (Fig. 1C). Foragers experiencing a heated Peltier plate during the 10 training trials (group *5°C shield*, n=20) showed a learned thermal preference in the non-rewarded test trial (Fig. 3B; Chi²=6.40, df=1, p<0.05). In contrast, trained foragers of the control group (n=20) slightly,

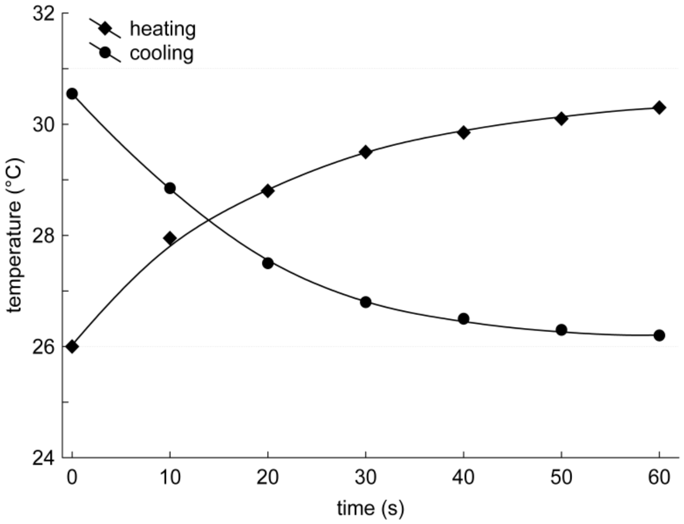

Figure 2: Temperature change at the surface of the Peltier plate after switching on (heating; closed circles) or switching off (passive cooling; open circles) the power source. After switch-on, the temperature at the Peltier plate surface rises quickly up to the maximum of 5°C above ambient temperature after 60 s. After switch-off, the temperature at the Peltier plate surface drops rapidly to almost ambient temperature of 26°C after 60 s. A similar temperature dynamic was measured by cooling the Peltier plate to 5°C below ambient temperature and passive warm up (data not shown).

although not statistically significant, tended to avoid the side with the thermal stimulus (Chi^2=1.67, df=1, p=0.17).

E3: Radiation vs. Conduction

The ability of foragers to sense thermal radiation does not exclude the possibility that conductive heat can also be sensed and used as a potent temperature stimulus for learning. In order to investigate how sensitive foragers are to conductive heat, we modified the whole experimental setup and used a paradigm of differential conditioning. After the 10 training trials experiencing a Peltier Plate heated 5°C above ambient temperature, workers belonging to different groups experienced in the 11th trial (first test trial) either a Peltier plate heated 5°C above ambient temperature, as during training (group *5°C @test*), or a plate heated 1°C above ambient temperature (group *1°C @test*).

Only foragers of the group *5°C @test* (n=24) showed a significant learned thermal preference in the first test trial (Fig. 3C; Chi^2=4.46, df=1, p<0.05). Foragers of the group *1°C @test* (n=42) showed no preference in the first test trial, when one Peltier plate was heated only 1°C above ambient temperature (Fig. 3C; Chi^2=0.14, df=1, p=0.45). Differential conditioning allowed the analysis of the dynamic of the learned thermal preference during training of all foragers (n=66). In the first 8 training trials, foragers showed no learned thermal reference (Fig. 4; Chi^2≤2 .50, df=1, p>0.05). This indicates that during the initial training trials, foragers find the reward on the T-bar based on exploration of both sides rather than based on e.g. odor cues present at the bifurcation. In the last two training trials, foragers showed a learned thermal preference (9th trial: Chi^2=4.51, df=1, p<0.05; 10th trial: Chi^2=9.38, df=1, p<0.01). Analysis of the group of foragers that was tested with one Peltier plate heated 1°C above ambient temperature (group *1°C @test*, n=42) revealed a learned thermal preference during the last training trial (10th trial: Chi^2=4.04, df=1, p<0.05), yet they failed to show a preference during test, as indicated above.

All foragers that were tested with one Peltier plate heated 5°C above ambient temperature (group *5°C @test*, n=24) could be tested again (second test trial), although the first test trial was not rewarded and thus possibly acted as an extinction trial. The learned thermal preference was slightly reduced, although statistically not significantly, compared to the first test trial (Fig. 4; $Chi^2=0.44$, df=1, $p>0.05$).

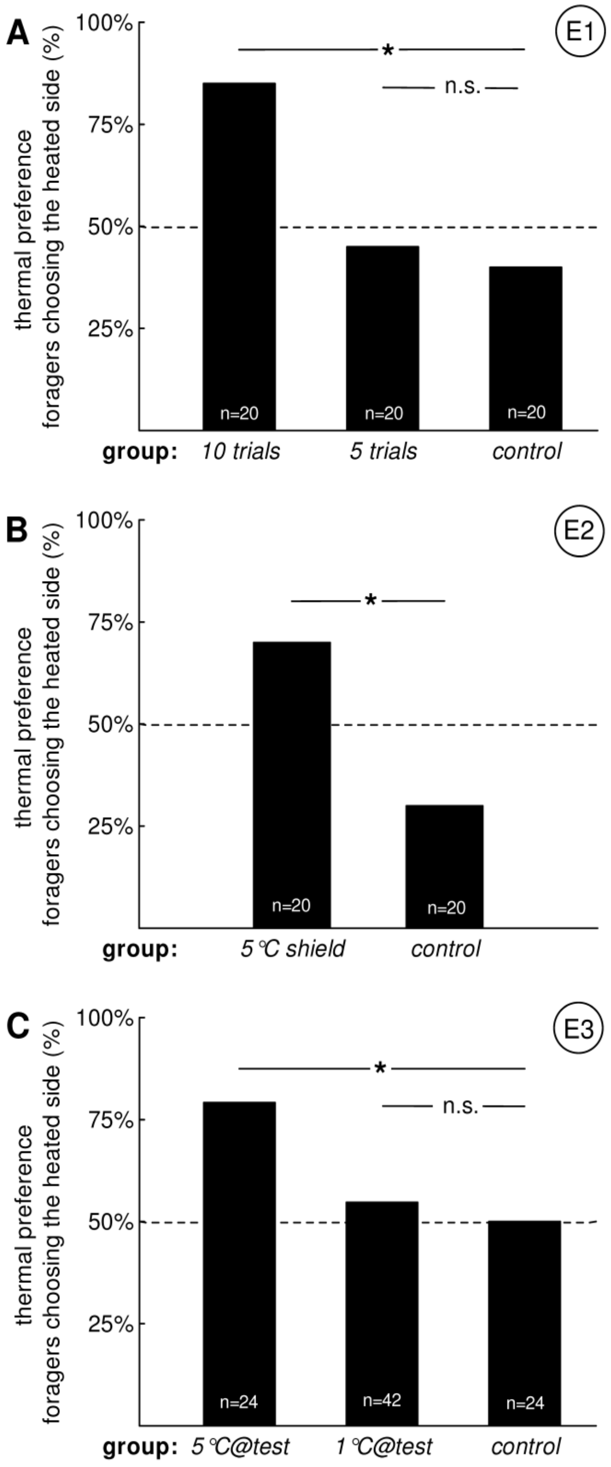

Figure 3: Thermal preference of foragers plotted as the percentage of workers choosing the heated side during the non-rewarded test situation. **A**: Learning experiment (E1). After 10 training trials (group *10 trials*) foragers showed a significant and learned thermal preference compared to the control group ($Chi^2=8.64$, df=1, $p<0.01$). After 5 training trials (group *5 trials*) no learned thermal preference was found ($Chi^2=0.10$, df=1, $p=0.50$). In the test situation, one Peltier plate was heated while the other was cooled ($\Delta T=10°C$). **B**: Radiation-convection experiment (E2). A laminar upward flow of ambient air had no influence on the learned thermal preference after 10 training trials (group *5°C shield*) compared to the control group ($Chi^2=6.40$, df=1, $p<0.05$). In the test situation, one Peltier plate was heated while the other had ambient temperature ($\Delta T=5°C$). Foragers of the control group slightly, although not statistically significant, tended to avoid the side where the Peltier plate was at 5°C above ambient temperature in the test situation ($Chi^2=1.67$, df=1, $p=0.17$). **C**: Radiation-conduction experiment (E3). A significant and learned thermal preference of foragers was measured when the Peltier plate was at 5°C above ambient temperature (group *5°C@test*) in the test situation ($Chi^2=4.46$, df=1, $p<0.05$). No learned thermal preference was found when the Peltier plate was only 1°C above ambient temperature (group *1°C@test*) in the test situation ($Chi^2=0.14$, df=1, $p=0.45$). Note that foragers of the control group partitioned exactly 1:1 at the bifurcation of the T. Differences between groups were evaluated with the Chi-square test and Fishers exact p, one-tailed, asterisks denote significant differences at the 5% level.

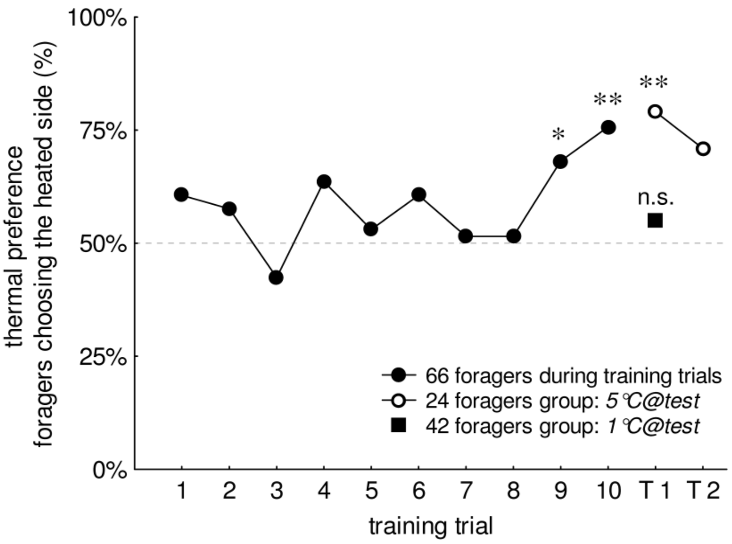

Figure 4: Thermal preference of 66 foragers on 10 successive training trials (closed circles) in the radiation-conduction experiment (E3). In the first 8 training trials, foragers showed no learned thermal preference (Chi^2 ≤ 2.50, df=1, p>0.05). In the 9th and 10th training trial the foragers showed a significant and learned thermal preference compared to an expected 1:1 partitioning (9th trial: Chi^2=4.51, df=1, p<0.05; 10th trial: Chi^2=9.38, df=1, p<0.01). In the test situation (11th trial), the foragers had a thermal preference as indicated with open circles (group *5°C@test*) or a square (group *1°C@test*), as shown in Fig. 3C. The 50% level indicates no thermal preference and a 1:1 distribution of foragers' responses to either side. Foragers of the group *5°C@test* were tested twice and the thermal preference did not change significantly between the first and the second non-rewarded test trial (T1 vs. T2; Chi^2=0.44, df=1, p>0.05).

Discussion

In insects, the thermal environment is decisive in influencing many different physiological processes (Beck 1983). Regarding metabolic control, insects function optimally within a more-or-less limited range of temperatures (Lighton 1989). Besides its effects on developmental speed and colony growth rates in social insects (Porter 1988), temperature is also used by insects during host-finding or to locate appropriate places for reproduction (Bradshaw 1980; Davis and Sokolove 1975; Lazzari and Núñez 1989; Schmitz and Bleckmann 1998). Thermal cycles, in addition, are powerful entrainment agents for circadian and circannual rhythms in insects (Dreisig 1976; North 1993), and social insects in particular proved to be especially sensitive to thermal changes (Roces and Núñez 1996; 1995).

In the present study, we show that workers of the leaf-cutting ant *Atta vollenweideri* are able to learn the location of a leaf fragment by using a heated object as orientation stimulus in a foraging context. Thermal radiation was the adequate stimulus modality, and a Peltier plate served as orientation stimulus when presented at a distance of 25 mm and heated 5°C above ambient temperature.

For leaf-cutting ants, behavioral responses to thermal cues have previously been described in the context of nest climate control (Bollazzi and Roces 2002; Kleineidam et al. 2001). Inside the subterranean nest, thermal radiation (e.g. originating from the fungus garden), may be used for orientation. Workers were observed to respond to unsuitable temperatures for their symbiotic fungus and developing brood by relocating them (Bollazzi and Roces 2002).

The ability to use thermal radiation as a learned orientation cue possibly allows foragers to improve their foraging efficiency. Leaf-cutting ants travel between food resources and nest by using a system of chemically-marked foraging trails (Kost et al. 2005). Pheromonal and architectural manipulations of these otherwise directionless trails provide foragers with additional orientation cues (Guajara et al. 1990; Vilela et al. 1987), and there is recent evidence that foraging workers may exploit the earth's magnetic field to infer direction (Banks and Srygley 2003). Once a potential food source such as a palatable plant has been located, temperature discontinuities at a small scale may guide foraging workers to find leaves warmed by the sun, which have been shown to be highly palatable (Folgarait et al. 1996; Nichols-Orians 1991).

Foragers in our experiments were able to use a thermal stimulus for orientation, yet they needed more than five training trials for successful learning. In the context of foraging, learning of food odors by leaf-cutting ants was shown to be very powerful. In *Acromyrmex lundi*, a single reward sufficed to change the response of workers (Roces 1994; 1990). Recently, a study investigating odor discrimination of individual *Camponotus* ants by using differential conditioning showed that more than 20 trials are necessary for full acquisition (Dupuy et al. 2006). In the ant *Camponotus rufipes*, experiencing a predictable food source four times suffices to change workers' decision rules (Schilman and Roces 2003). When solving difficult discrimination tasks in the context of color learning and 'delayed matching-to-sample' paradigms, bees also require a large number of training trials (Dupuy et al. 2006; Dyer and Chittka 2004; Dyer and Neumeyer 2005; Giurfa 2004; Giurfa et al. 2001).

We are aware that the use of 10 training trials in our experiments may have not sufficed for the ants to achieve a full acquisition, since the thermal preference increased up to the last training trial (see Fig. 4). More training trials may have further increased the thermal preference, and possibly foragers would have also been able to learn temperature differences smaller than 5°C as orientation cues. This aspect remains to be investigated.

We used the learning abilities of ants as a tool to investigate what kind of thermal stimuli (thermal radiation or convective heat) can be learned by the foragers, without further consideration of the learning processes underlying the observed flexibility in behavior. However, our data on the behavioral response of leaf-cutting ants to reinforced thermal stimuli are consistent with an associative learning process. In order to classify the observed performance as associative learning, further experiments for instance to trace the acquisition and extinction curves are needed. In Experiment E3 we tested the trained foragers twice. The first non-rewarded test is expected to act as an extinction trial, and in line with this argument, a tendency towards a reduced thermal preference was observed. We decided not to perform further test (extinction) trials because the lack of a reward would have probably induced workers to give up and not to voluntarily return to the test situation.

The learning of a thermal stimulus in leaf-cutting ants is probably not a very difficult task requiring advanced cognitive abilities. Rather, a thermal stimulus is unlikely to be such as prominent as for instance odor stimuli, and it might be less represented in the perception of the foragers at the beginning of the training. Whether repeated thermal stimulation during training improves the attention to thermal stimuli in general, remains to be shown. The foragers in our experiments moved their antennae from side to side and raised their body (scanning behavior) at the point of decision, just before climbing on one of the two sticks offered, and this may indicate an active search for the learned thermal stimulus. Although this behavior was not quantified, it might be a measure for the forager’s expectancy in the choice situation.

Regarding learning responses to temperature, honeybees are able to learn the location of a feeder based on its temperature, being able to resolve a temperature difference beyond 2°C in a choice set-up (Heran 1952). More recently, bumble bees were shown to prefer warmer flowers and are able to associate the temperature of a feeder with its color (Dyer et al. 2006). Bumble bees’ preferences were significant when the temperature difference was 4°C or more. In Heran’s (1952) study, bees were unable to use thermal radiation for orientation, and it is still an open question whether bees and bumble bees use thermal radiation, convective, and/or conductive heat as orientation stimuli when landing on flowers.

Our experiments provide strong evidence that thermal radiation is indeed an adequate stimulus used by leaf-cutting ant foragers. A heated object emits energy to the surrounding air by conduction and thermal radiation (IR-radiation).

Whereas heated air is transported away from the surface by convection, thermal radiation is not affected by air currents. In our second experiment (E2, Radiation vs. Convection), we used an air current to drag warm air from both the surface of the Peltier plate and the forager's head. The experimental air flow might have induced turbulences around e.g. the platform where the reward was presented, and the possibility that some filaments of warm air might still have contacted the forager's sensory organs cannot be excluded. In the test situation, however, the shield protected the forager much better from warm air currents. It is important to mention that in the control group, foragers tended to avoid the warm side during the test. Although speculative, it is tempting to consider that during training, foragers might have experienced a cold stimulus due to evaporative cooling caused by the air current, and therefore tended to prefer the cold side during the test. This possibility remains to be explored in the future.

The results from experiment E1 and E2 showed that thermal radiation was an effective stimulus in the rage of a few centimeters. The same heated object was also used in experiment E3 where foragers were allowed to touch its surface.

The temperature difference necessary to allow a learned thermal preference of foragers in the experiment E3 was in the same range as the temperature difference in the experiments where foragers could not touch the Peltier plate (5°C above ambient temperature). In addition, a similar number of training trials was necessary to allow learning. We addressed the question whether temperature differences smaller than 5°C suffice as a learned cue for orientation. Since the thermal source was in the close range and could be directly contacted, it was expected that foragers should be able to learn and use heated objects with smaller temperature differences for orientation. Ants and bees are able to resolve temperature differences as small as 0.2 - 0.25°C when searching for suitable temperatures for themselves or their brood (Heran 1952; Roces and Núñez 1995). Surprisingly, we found that foragers trained and tested at a ΔT of 1°C between the two Peltier plates did not prefer the warm side (10 foragers tested, data not shown).

Foragers trained at a ΔT of 5°C that showed a learned thermal preference when tested at a ΔT of 5°C failed to show a preference when tested at a ΔT of 1°C. It is suggested that foragers might be able to discriminate an object heated only 1°C above ambient temperature, but they are unable to use it as a learned orientation cue. It is an open question whether more than 10 training trials would be necessary to allow learning.

Two lines of evidence indicate that thermal radiation is the major stimulus used by ant foragers for orientation. First, our results of experiment E2 in which convective heat was precluded by an air current showed that the foragers still could use the learned thermal stimulus for orientation. Second, our results of experiment E3 in which, although both conductive and convective heat transfer to the antenna were not prevented, the learning performance remained similar to that of experiment E2. Nevertheless, convective or conductive heat may also activate the receptor neurons responsible for sensing thermal radiation, and convective heat may carry information used by foragers in a different context than orientation, e.g., nest construction.

For a number of insect species, the sensilla housing thermo receptive neurons have been identified. In most cases the thermo receptive neuron is associated with two hygroreceptive neurons (triad) in one sensillum (Altner and Loftus 1985; Ehn and Tichy 1996; Steinbrecht 1998). An extreme adaptation for thermo reception was found in e.g. Buprestid beetles of the genus *Melanophila* with elaborated cuticular structures of the sensillum and a single IR receptive neuron (Schmitz and Bleckmann 1997; Schmitz et al. 1997).

In Hymenoptera, the thermo receptive neurons are located in sensilla coeloconica and/or sensilla ampullacea (Dumpert 1978; Kleineidam and Tautz 1996; Lacher 1964). The thermo receptive neurons of several insect species seem to be adapted for reception of convective heat (Gingl and Tichy 2001; Gingl et al. 2005). However, the ability to use thermal radiation was also shown for other insects beside the fire-beetles, e.g., for the haematophagous bug *Rhodnius prolixus* (Schmitz et al. 2000). Unfortunately, the physiology and morphology of thermo receptive neurons and sensillar structures reported so far for Hymenoptera do not allow any conclusive view whether convective heat or thermal radiation is the adequate stimulus modality. The question whether the sensilla responsible for the reception of thermal radiation are direction-sensitive is still open, and we are far from understanding how temperature cues are processed in the insect brain and how the thermal environment is perceived by insects.

Acknowledgements

The leaf-cutting ant colonies used during this study were collected at the Reserva Ecológica El Bagual (Alparamis SA - Aves Argentinas) in eastern Chaco, Province of Formosa, Argentina. We are very much indebted to the ornithologist Alejandro G. Di Giacomo, his field assistants, and especially the family Götz for providing facilities and much help at the Biological Station during our stays. Many thanks to Silvia Naranjo Blanco, Franz Gruber and Yi-chun Chen for help in several of the experiments reported here. Financial support was provided by the DFG, Germany (SFB 554, A6 and E1) and DAAD (Vigoni D/05/54047).

V. The sensillum coeloconicum in *Atta vollenweideri*

Abstract

Social insects show a variety of temperature-guided behaviors. Depending on whether heat reaches the sensillum via air movements (convective heat) or as radiant heat, specific adaptations of thermosensitive sensilla are expected. In the present study the morphology and the physiology of thermosensitive peg-in-pit sensilla (sensilla coeloconica) of the leaf-cutting ant *Atta vollenweideri* were investigated. Sensilla coeloconica are located predominantly in a single cluster on the apical antennomere, and connect to the outside through a small aperture. The sensory peg is double-walled, embedded in a chamber and innervated by three unbranched dendrites. Using tungsten electrodes, activity of the sensory neurons was measured. In most cases, the neuron with the largest spike amplitude responds to changes in air temperature (convective heat) as well as to radiant heat. In response to a drop in air temperature, the neuron shows a phasic-tonic response followed by a complete adaptation within one minute (cold-sensitive neuron). Based on their morphology and physiology, it is suggested that the sensilla coeloconica are involved in the recently described thermal orientation behavior of *A. vollenweideri* leaf-cutting ants.

Introduction

The antennae are the most prominent sensory organs of insects. They are equipped with a vast number of different sensilla. The majority of sensilla serve an odor-receptive function; others are used for mechano-, temperature- or humidity-reception. Sensilla are classified based on morphological characters, which are expected to reflect an adaptation to improve stimulus reception. For example, the long trichoid sensilla for pheromone reception in the male silk moth *Bombyx mori* have a large surface area, and are arranged like combs on the antennae such that pheromone molecules are effectively captured from an air current. Another amazing morphological adaptation has been described for thermosensitive sensilla of fire beetles (genus *Melanophila*), which can detect and approach forest fires from a distance of up to 12 km to encounter mates and lay eggs into freshly-burned wood (Evans 1964; Schmitz and Bleckmann 1998). The thermosensitive sensilla contain a specialized structure (cuticular sphere) which absorbs IR radiation and acts as an outer pressure vessel. Inside the sphere a microfluidic component generates an increase in internal pressure which then causes a compression of the dendritic tip of a mechanoreceptor (Müller et al. 2008; Schmitz et al. 2007; Schmitz and Bleckmann 1997; Schmitz et al. 1997; Vondran et al. 1995). Information about the thermal environment is crucial for the reproductive success of fire beetles, but it is also of paramount importance for social insects which have the ability to manipulate the thermal environment within the nests to improve brood development. A number of different temperature-guided behaviors emphasize the significance of thermoreception in the life of ants, wasps and bees (social Hymenoptera). However, surprisingly little is known about the underlying sensory mechanisms. In most insect species, thermosensitive neurons are located in peg-in-pit sensilla, often combined with two hygrosensitive neurons forming a sensory triad (Altner and Loftus 1985; Altner et al. 1981; Tichy 1979; Waldow 1970; Yokohari 1983). In social insects such an organization has been described for the sensilla coelocapitula of the honeybee *Apis mellifera*. Other thermosensitive neurons have been found in sensilla ampullacea (Sa) and sensilla coeloconica (Sc) (Dumpert 1978; Kleineidam and Tautz 1996; Lacher 1964; Yokohari 1983). In the mosquito (*Aedes aegypti*), two antagonistic thermosensitive neurons (cold-warm neurons) have been found within one sensillum (Davis and Sokolove 1975). Even the combination of thermo- and chemosensitive neurons occurs in several insect species (Altner et al. 1981; Altner et al. 1977; Davis 1977; Hansson et al. 1996).

However, the peg-in-pit morphology does not unambiguously predict the housing of a thermosensitive neuron. For instance, in search of a thermosensitive neuron in the Sc of the moths *Bombyx mori*, solely chemosensitive neurons have been found (Boeckh 1967; Pophof 1997; Pophof et al. 2005). And furthermore, thermosensitive neurons occur in sensilla without peg-in-pit morphology (Altner et al. 1977; Loftus 1966; 1968; Loftus and Corbière-Tichané 1981).

In order to elucidate a structure-function relationship, both fine structure and the gross morphology of the sensilla are equally important. While morphological data on thermosensitive sensilla are available across different insect orders, data on their fine structure are fragmentary. Fine structural analysis of thermosensitive sensilla in several insect species indicate that some cold-sensitive neurons have a highly lamellated outer dendritic segment (Altner and Loftus 1985; Corbière-Tichané 1977; Corbière.G 1974; Corbière.G and Bermond 1972; Steinbrecht 1989). In Hymenoptera, data on the fine structure of peg-in-pit sensilla are available only for the Sa but are lacking for both, Sc and sensilla coelocapitula (Kleineidam et al. 2000).

Leaf-cutting ants (genus *Atta* and *Acromyrmex*) are evolutionary derived eusocial insects with pronounced size polymorphism, highly organized colonies and complex nest architecture (Weber 1966). For instance, a colony of *Atta vollenweideri* controls its nest climate with the help of a passive nest ventilation mechanism, and the construction of structures such as ventilation tunnels and turrets (Kleineidam et al. 2001; Kleineidam and Roces 2000). Inside the nest, brood and fungus translocation is used as a short term measure to track favorable conditions (Bollazzi and Roces 2002; Roces and Kleineidam 2000). Outside the nest, leaf-cutting ants are able to learn the location of a food source using radiant heat as an orientation cue (Kleineidam et al. 2007). Due to the different physical properties of the thermal stimuli (e.g. convective heat and radiant heat) used for different temperature-guided behaviors, specific adaptations of the thermosensitive sensilla are expected.

In the present study, the thermosensitive Sc of the leaf-cutting ant *A. vollenweideri* were investigated in detail regarding their arrangement on the apical antennomere, as well as their morphology. The neuronal activity of one of the three associated sensory neurons of the Sc was examined electrophysiologically. Finally, the functional morphology is discussed as a possible adaptation to the reception of either convective or radiant heat.

Materials and methods

Animals

Workers of *A. vollenweideri* were obtained from a laboratory colony collected by Bollazzi M. and Geissler O. in 2002 at the Reserva Ecológica El Bagual, Formosa, Argentina. The colony was reared at the Biozentrum, University of Würzburg at 25°C and 50% relative humidity in a 12h/12h photoperiod, and fed mainly with leaves of privet (*Ligustrum vulgaris*) and dog rose (*Rosa canina*). For the experiments, only medium sized workers from the feeding site were collected, thus it is assumed that only foragers were investigated.

Morphology (SEM)

The cuticular structures of the flagellum and the Sc were investigated by scanning electron microscopy (SEM). The flagellum was either fixed overnight in phosphate-buffered 4% formaldehyde (PBS, pH 7.2), or the apical antennomere was sectioned obliquely with a razor blade followed by tissue removal with warm 3M KOH-solution and 10 min cleansing in an ultrasonic bath. All specimens were dehydrated in a graded ethanol series (50%, 70%, 80%, 90% 95% and 100%; 10 min each) and Aceton (100% for 10 min), and critical point-dried (CPD030, Balzers Union, Liechtenstein). The specimens were glued onto SEM-supports, gold-palladium-coated (MED010, Balzers Union, Liechtenstein) and examined with a scanning electron microscope (DSM962, Zeiss, Germany). From each investigated worker, only one flagellum was analyzed (independent measurements).

Looking at the surface of the flagellum, only small apertures are visible and these belong either to Sc or to Sa. The length and the width of each sensillum aperture were measured in 7 specimens and their area calculated. The distances of all apertures of the 11th antennomere to the antennal tip were measured for 2 specimens.

Fine structure (TEM)

For transmission electron microscopy (TEM) observations, 13 workers were anaesthetized in CO_2, immersed in ice-cold 2.5 % glutaraldehyde in 0.1 M cacodylate buffer + 5% glucose, pH 7.2-7.3. In order to facilitate fixative penetration, the apical antennomeres were detached immediately from the rest of the antennae and the specimens were fixed at 4 °C for about 2 hours.

After rinsing overnight in cacodylate buffer, the specimens were post-fixed in 1% osmium tetroxide at 4 °C for about 1 hour and rinsed in the same buffer. Dehydration in a graded ethanol series was followed by embedding in an Epon-Araldite mixture (Fluka, Sigma-Aldrich; Buchs, Switzerland), using propylene oxide as bridging solvent. Thin (90-120 nm) sections were taken with a Diatome® diamond knife on a ultramicrotome (L.K.B. Nova, Bromma, Sweden) and mounted on collodium-coated 50 mesh grids. Finally, after staining with uranyl acetate (20 min, room temperature) and lead citrate (5 min, room temperature), the sections were observed with a TEM (Philips EM 208, FEI Company, The Netherlands). Digital pictures (1376x1032 pixels, 8 bit grayscale, uncompressed Tiff files) were taken using a high resolution digital camera (Megaview III SIS, Olympus, Japan).

Electrophysiology

In search for thermosensitive neurons, the associated receptor cells of Sc were investigated by extracellular recordings of neuronal activity. Workers were mounted on a plastic holder with adhesive tape and the scapus was glued onto the holder with white-out correction fluid (Tipp-Ex, Bic, France). Under visual control and at a magnification of 390x (Leitz microscope equipped with NPL-Fluotar L25/0.35, Leitz, Germany), an electrolytically sharpened tungsten electrode was superficially inserted into the cuticle next to the chamber of a Sc using a manual micromanipulator (HS-6, Märzhäuser, Germany). In order to achieve a good signal to noise ratio, the reference electrode was inserted deep into the flagellum in close vicinity to the recording electrode. Recordings were band-pass filtered (60 Hz-3 kHz) and amplified 1000 x (Neuroprobe Amplifier 100, A-M-Systems, USA and VBF8, Kemo, Great Britain). The electrical noise was significantly reduced using an additional digital filter (Humbug, Quest Scientific, Canada). Data were digitized at a sampling rate of 12 kHz (IDAC-4-USB, Synthec, Netherlands) and stored for analysis (AutoSpike 32, Syntech, Netherlands) on a PC. During all physiological experiments, an ambient airflow (flow rate 1.5 l/min) was continuously channeled via a glass tube (2 cm in diameter, at a distance of 1 cm from the antenna) and blown over the preparation (continuous air flow). The responses of the thermosensitive neurons to convective stimuli were investigated by using a second (stimulus) air flow. A metal tube (1 cm in diameter, at a distance of 1 cm from the antenna) was positioned opposite to the tube providing the continuous air flow.

Air temperature in both, the continuous- and the stimulus air flow were measured continuously with thermocouples (NiCr-Ni, Typ K, Conrad Electronics, Germany), logged on a PC, and controlled by heat exchangers close to the outlet of the air flows. The heat exchangers could be heated or cooled via two silicon tubes which were connected with two water baths (DC1, Haake, Germany). This setup allowed us to generate convective stimuli with temperatures ranging from 12°C to 45°C. A stimulus controller (CS-01, Synthec, Netherlands) was used to switch on the stimulus air flow for either 10 s or 10 min, resulting in rapid changes in air temperature at the antenna.

To study the physiological adaptation of the thermosensitive neurons in response to prolonged stimulation, a step change from room temperature (24°C, continuous air flow) to the stimulus air flow with either 6°C above room temperature (30°C) or 6°C below room temperature (18°C) was used, while simultaneously recording the neuronal activity. The adaptation process was described by measuring the mean instant frequency (bin size 1 s) once every minute.

The responses of the thermosensitive neurons to radiant heat (infrared radiation) were investigated by using a broad-band IR-emitter (Wavelength 0.4-11.2 µm, SVF 360-8M, Laser components, Germany). The IR-emitter was mounted on a micromanipulator at a distance of 3 cm above the antenna. For IR-stimulation, the IR-emitter was powered with 2.6 W and pulsed with a frequency of 0.25 Hz using a frequency generator (PM 5133 function generator, Phillips, Netherlands). The calculated radiation power at the preparation was 133 mW/cm^2. In order to exclude the influence of convective heat, e.g. from the heated air around the emitter, a temperature controlled air flow (continuous air flow) was blown over the preparation throughout the experiments. Statistical analysis was performed using STATISTICA 7.1 (Statsoft, Tulsa, USA) and data were plotted with ORIGIN 8 (OriginLab, USA).

Results

Morphology (SEM)

The SEM investigations revealed that peg-in-pit sensilla are located predominantly on the apical (11th) antennomere. The sensory peg embedded in the pit connects to the outside through a small round or oval aperture (Fig. 1A). The apertures, located on the latero-ventral side of the flagellum, belong either to the sensilla coeloconica (Sc) or the sensilla ampullacea (Sa). In 7 investigated flagella, 9 to 16 peg-in-pit sensilla were found on the apical antennomere, compared to only 0 to 2 apertures on the 7th to 10th antennomeres. The size of the apertures on the apical antennomere varied considerably, ranging from 0.7 to 2.3 µm in length diameter (n = 102), whereas on the more proximal antennomeres their size ranged only from 1.3 to 2.3 µm (n = 24). For two additional apical antennomeres, the size of the aperture (length and width) and its distance to the antennal tip were measured. Two size-classes of apertures with larger apertures located more distally than small apertures were found (Fig. 2). The internal view of the apical antennomere revealed a cluster of Sc located distally of a cluster of Sa (Fig. 1C). This arrangement indicates that the large apertures (> 0.5 µm^2) belong to the Sc and the small apertures belong to the Sa. The aperture sizes found on the more proximal antennomeres indicate that these also belong to Sc rather than to Sa. Subsequently, the Sc could be discriminated from Sa solely by examining the antenna from the outside. The aperture of the Sc opens into a chamber containing the sensory peg inside. The chamber has a width of about 6 µm and a height of about 8 µm, the peg is 4 to 5 µm long (Fig. 1B). At the cup-shaped base, the peg is formed of 4 to 6 finger-like ridges that split into two before they fuse at the tip. The Sc are almost completely embedded in the thick antennal cuticle such that the cup-shaped base and the perpendicularly standing peg inside are inclined towards the antennal tip. The peg’s tip faces right to the aperture, and all Sc of the apical cluster seem to face into the same direction (Fig. 1D).

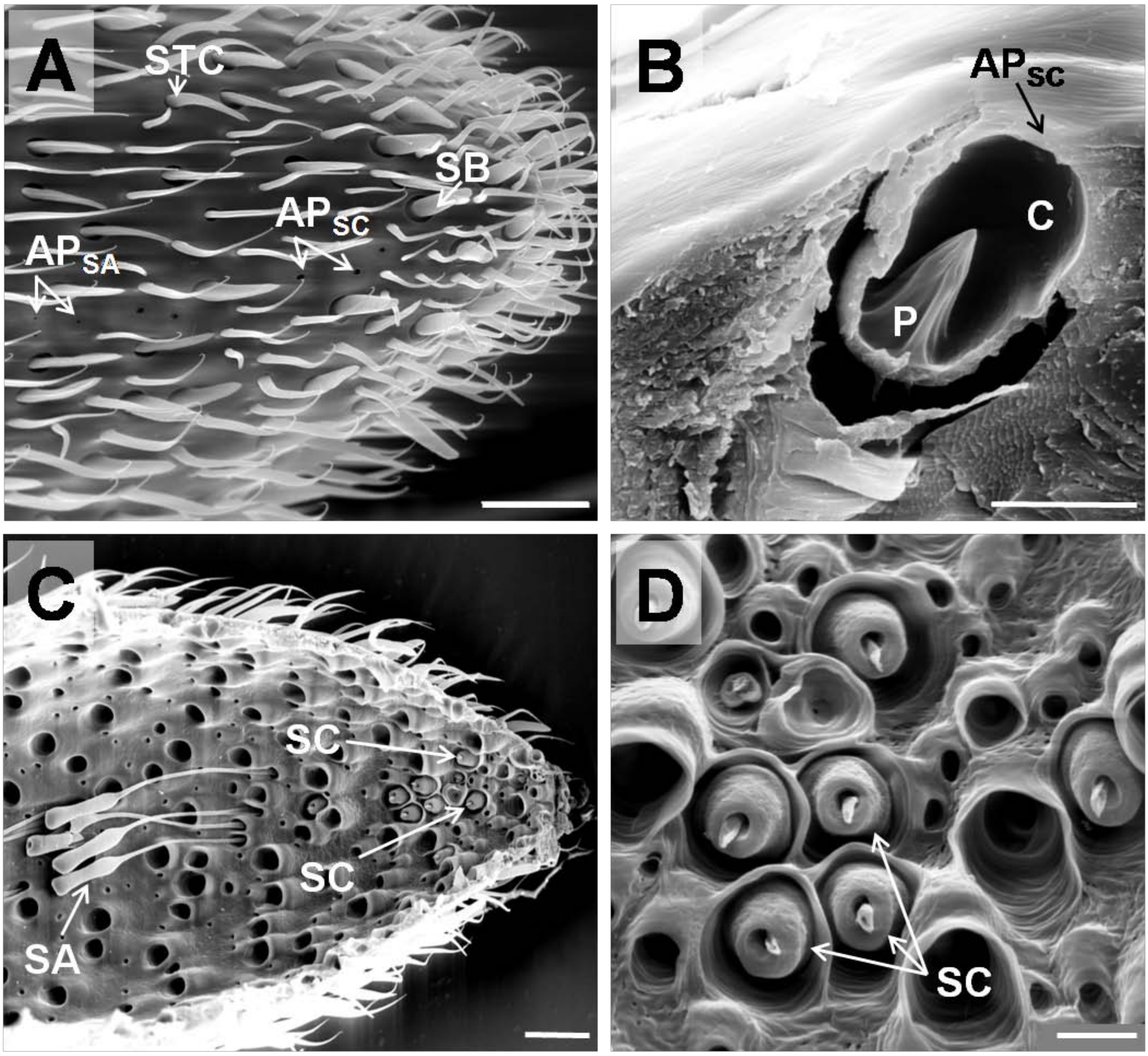

Figure 1: Location, morphology and orientation of the sensilla coeloconica

A: Overview of the apical antennomere with the apertures of the sensilla coeloconica at the distal tip and the more proximal apertures of the sensilla ampullacea. **B**: Longitudinal section of the chamber of a sensillum coeloconicum with the peg inside. The aperture is cut-open. **C**: Internal view of the last flagellar segment with sensilla coeloconica and sensilla ampullacea. **D**: Orientation of a group of sensilla coeloconica embedded into the antennal cuticle and inclinated towards the tip of the antenna (internal view). AP, aperture of the peg-in-pit sensilla ; C, chamber of a sensillum coeloconicum; SA, sensillum ampullaceum, SB, sensillum basiconicum; SC, sensillum coeloconicum; STC, sensillum trichodeum curvatum; P, peg of the SC; Scale bar: 20 µm in A and C; 5 µm in B and D.

Fine structure (TEM)

Longitudinal (Fig. 3 A) and serial (Fig. 3 B-D; Fig. 4 A) cross sections through the sensory peg of Sc were used to describe the fine structural characters. Cross sections in the middle of the peg revealed that it is double-walled (Fig 3 C). Both walls have no pores and appear to share the same cuticular structure in terms of cuticle electron density and thickness (about 50 nm). The two cuticular walls define two distinct cavities, an innermost and an outermost cavity. The innermost cavity (diameter about 200 nm) is filled by the densely packed outer dendritic segments of the sensory neurons and by the sensillum lymph (Fig. 3 B-D). Cross sections close to the tip revealed the stellate characteristic of the sensory peg (Fig. 3 B). The inner and outer wall merge towards the tip, thereby forming 8 – 12 subconical (in cross section) cuticular fingers which are separated by spoke canals. The thickness of the cuticular wall of the cuticular fingers differs, being thinner on the side facing the peg lumen (about 75 nm) and thicker towards the outside (about 150 nm).

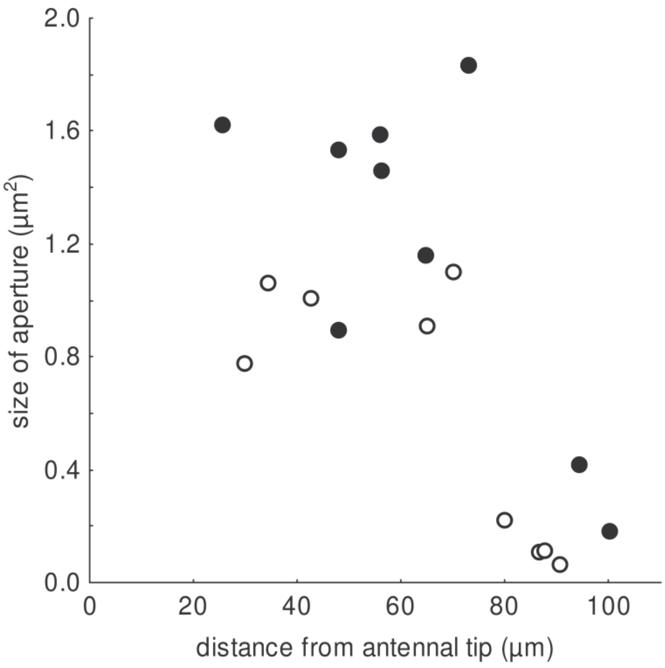

Figure 2: Location and aperture size of peg-in-pit sensilla: Size of the aperture is related to the location of the peg-in-pit sensilla: large apertures (> 0.5 µm^2) of the sensilla coeloconica located distal compared to the small apertures (< 0.5 µm^2) of the sensilla ampullacea located more proximal. Two apical antennomeres of different workers, indicated with black and open circles, were examined.

The outermost cavity in the middle of the peg and within each cuticular finger is filled with electron dense tubular-like material (Fig. 3 B, C). In contrast to the cuticular fingers, at the base of the peg electron dense tubular-like material is almost completely absent, while clusters of electron lucid vesicles (diameter ranging from 80 to 400 nm) are found (Fig. 3A inset; Fig. 3D). These vesicles surround the inner cuticular wall deep into the sensillar sinus, where they are also found within the cytoplasm of the trichogen cell (Fig. 4A, B). The inner cuticular wall originates from the dendritic sheath which is formed by the thecogen cell (Fig. 4 B).

Since the inner wall is preserved even after KOH treatment, it was also observed in the SEM study (Fig. 1 D). The Sc is innervated by three sensory neurons without variation in the number across investigated specimens (n = 13; several sensilla per specimen). It is noteworthy that one dendrite is always remarkably larger (diameter 1 µm) than the other two (diameter 0.5 µm) which is the case at both the outer and the inner dendritic segment level (Fig. 4 A-D). The three outer dendritic segments enter the cuticular peg and extend unbranched up to the tip of the peg. The outer as well as the inner dendritic segment is surrounded by the thecogen cell which forms the inner sensillum lymph space (Fig. 4 B-D). The thecogen cell has numerous microvilli and is filled with a large number of elongated mitochondria (Fig. 4 B). No tubular bodies were found in the sensory neurons associated with the Sc. The fine structural characters of the Sc are summarized in the schematic drawing (Fig. 5).

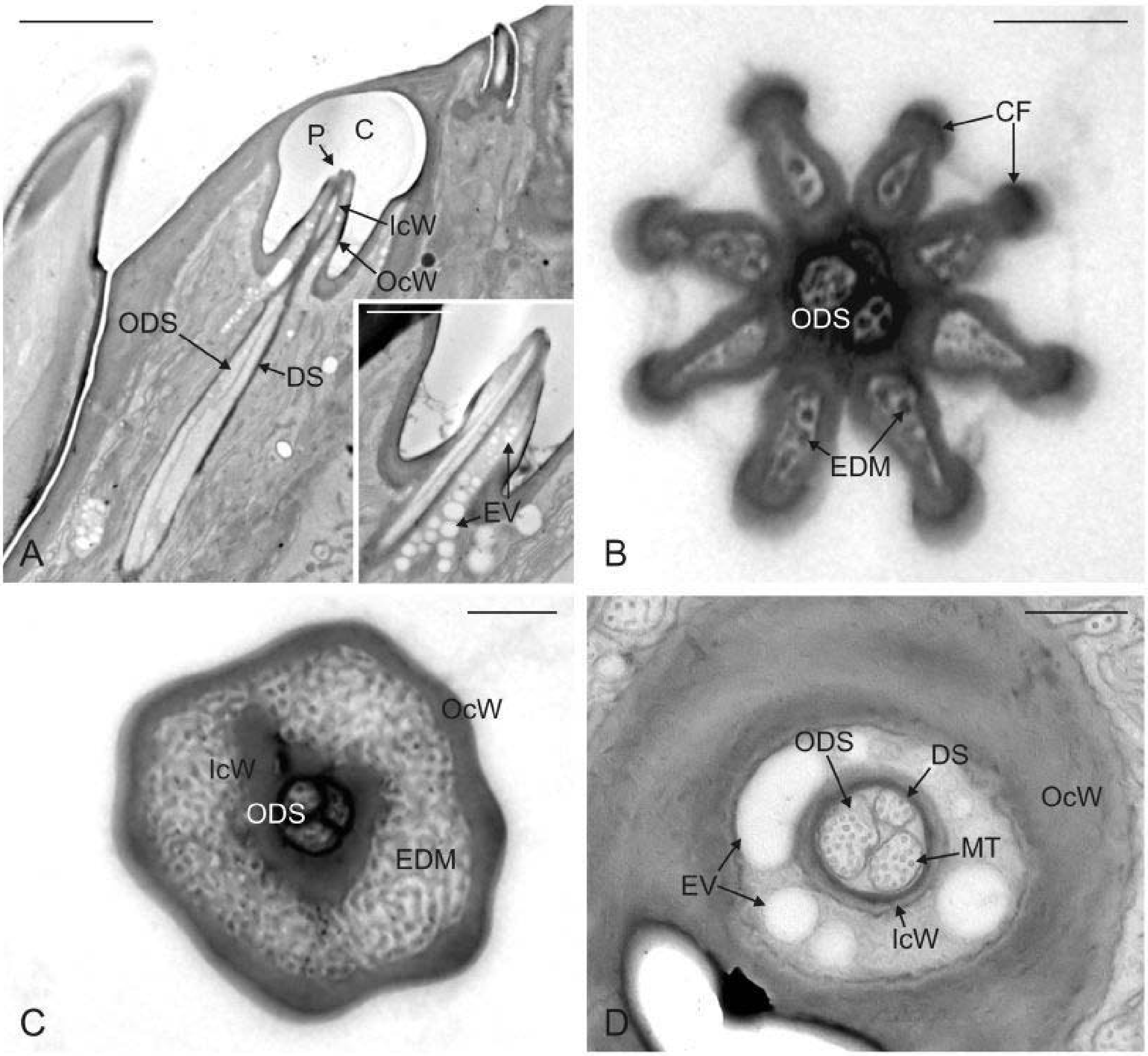

Fig. 3: TEM micrographs of sensilla coeloconica in *Atta vollenweideri:*

A: Longitudinal section of a sensillum coeloconicum showing the cuticular chamber with the peg and the outer dendritic segments enveloped by the dendritic sheath. At the peg level the inner and the outer cuticular wall are visible. **B-D:** Cross sections of a sensillum coeloconicum close to the tip (**B**) and to the base (**D**). In **B** the stellate arrangement of the cuticular fingers and in **C** and **D** the double-walled structure of the sensillum are visible. The internal lumen of the peg is filled with electron-dense material in the distal half (**B** and **C**), while electron lucid vesicles are found in the proximal half (**D**, inset in **A**). Scale bar: A: 5 µm; inset: 2 µm; B-C: 250 nm; D: 500 nm. C, chamber of a sensillum coeloconicum; CF, cuticular fingers; DS, dendritic sheath; EDM, electron-dense material; EV, electron lucid vesicles; IcW, inner cuticular wall; IDS, inner dendritic segment; MT, microtubules; OcW, outer cuticular wall; ODS, outer dendritic segment; P, peg

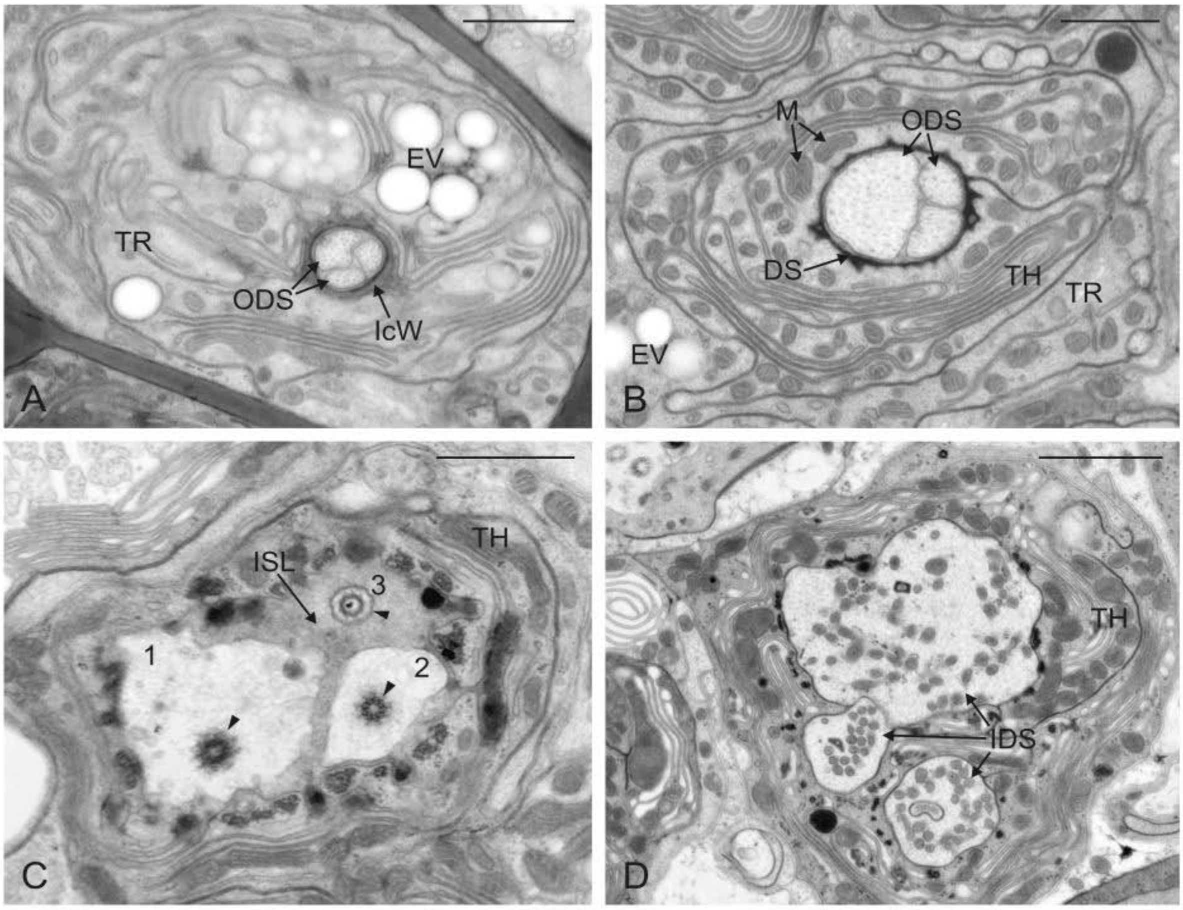

Figure 4: TEM micrographs of the cellular components of the sensilla coeloconica:

A: Cross section through the proximal end of the inner cuticular wall. At this level, three outer dendritic segments are clearly visible, as well as cluster of electron lucid vesicles produced by the trichogen cell. **B**: Cross section of the three outer dendritic segments encircled by the dendritic sheath. The bundle of outer dendritic segments is surrounded by the thecogen cell. The trichogen cell is also visible, as well as a small cluster of electron lucid vesicles. **C**: Cross section of the three dendrites at the level of the ciliary segments. Neuron 1 and 2 are sectioned at the level of the basal bodies (arrowheads), while neuron 3 is sectioned more distally at the ciliary collar level (arrowhead). The dendrites are surrounded by an inner sensillum lymph space. **D**: Inner dendritic segments of the three sensory neurons surrounded by the thecogen cell. One of the dendrites is remarkably larger than the others. Scale bar: A-C: 1 µm; D: 2 µm.

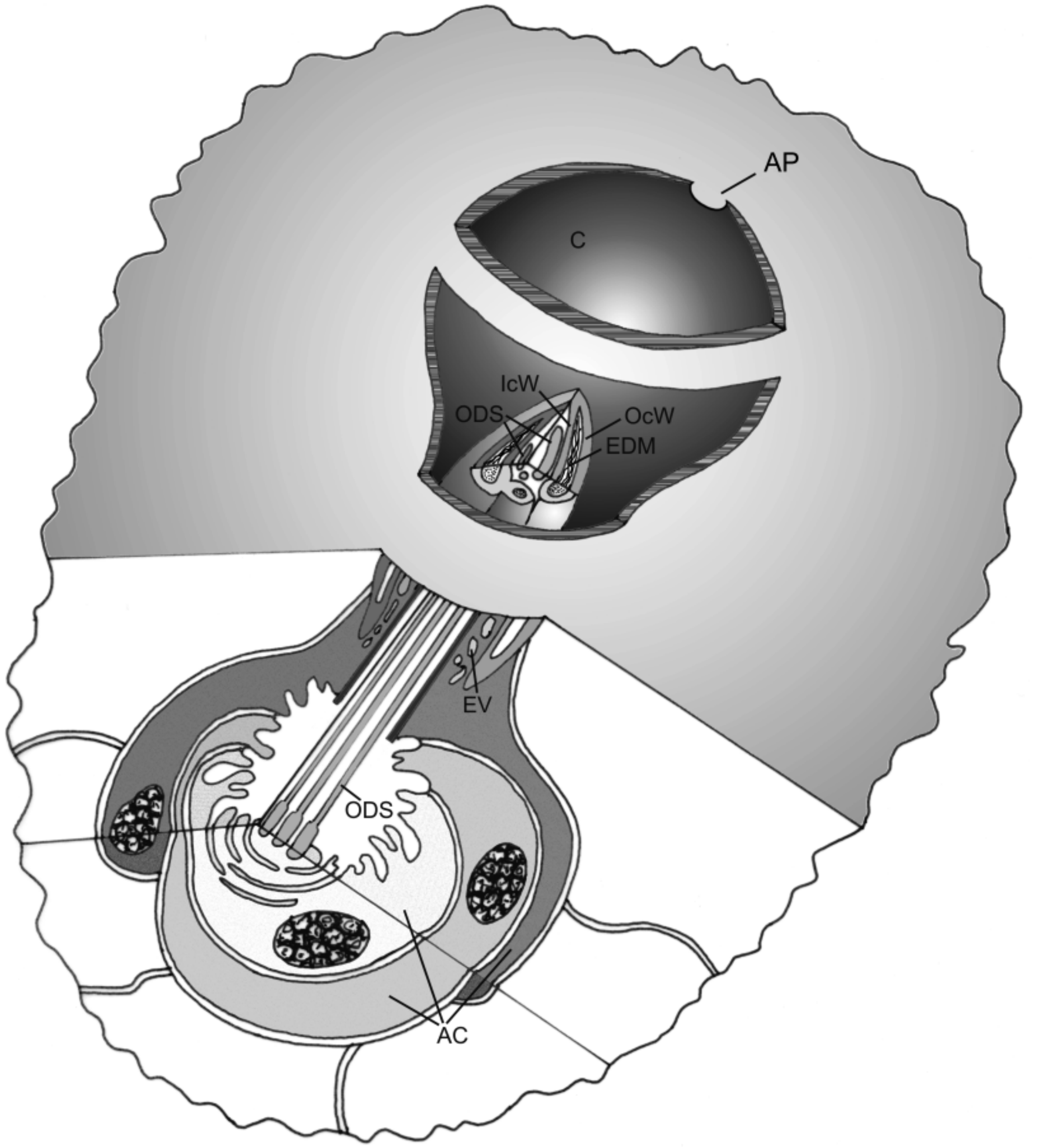

Figure 5: Schematic 3-dimensional reconstruction (not in scale) of a sensillum coeloconicum: AC, auxiliary cells; AP, aperture; C, chamber; DS, dendritic sheath; EDM, electron-dense material; IcW, inner cuticular wall; OcW, outer cuticular wall; ODS, outer dendritic segment; EV, electron lucid vesicles.

Electrophysiology

The neuronal activity of up to three sensory neurons was recorded when the electrode was placed close to the aperture of a Sc. The sensory neurons can easily be distinguished by the different amplitudes of the extracellular measured action potentials (spikes). In most cases, the neuron with the largest spike amplitude responds to an injection of a cold air stimulus into the continuous flow with a phasic-tonic response (Fig. 6 A, B).

Based on approximately 100 recordings of thermosensitive neurons, the neuronal activity ranges between 8 Imp/s to 140 Imp/s (peak frequency) at room temperature (RT = 24°C). Using a stimulus air flow (convective heat) at 11.2 °C below the temperature of the continuous air flow, the peak frequency ranges from 177 Imp/s to 690 Imp/s (n = 16) within 200 ms after stimulus onset. In response to an increase in air temperature (Δ T= 6°C) after stimulation (continuous air flow), the thermosensitive neurons cease the generation of action potentials for sometimes longer than 10 seconds.

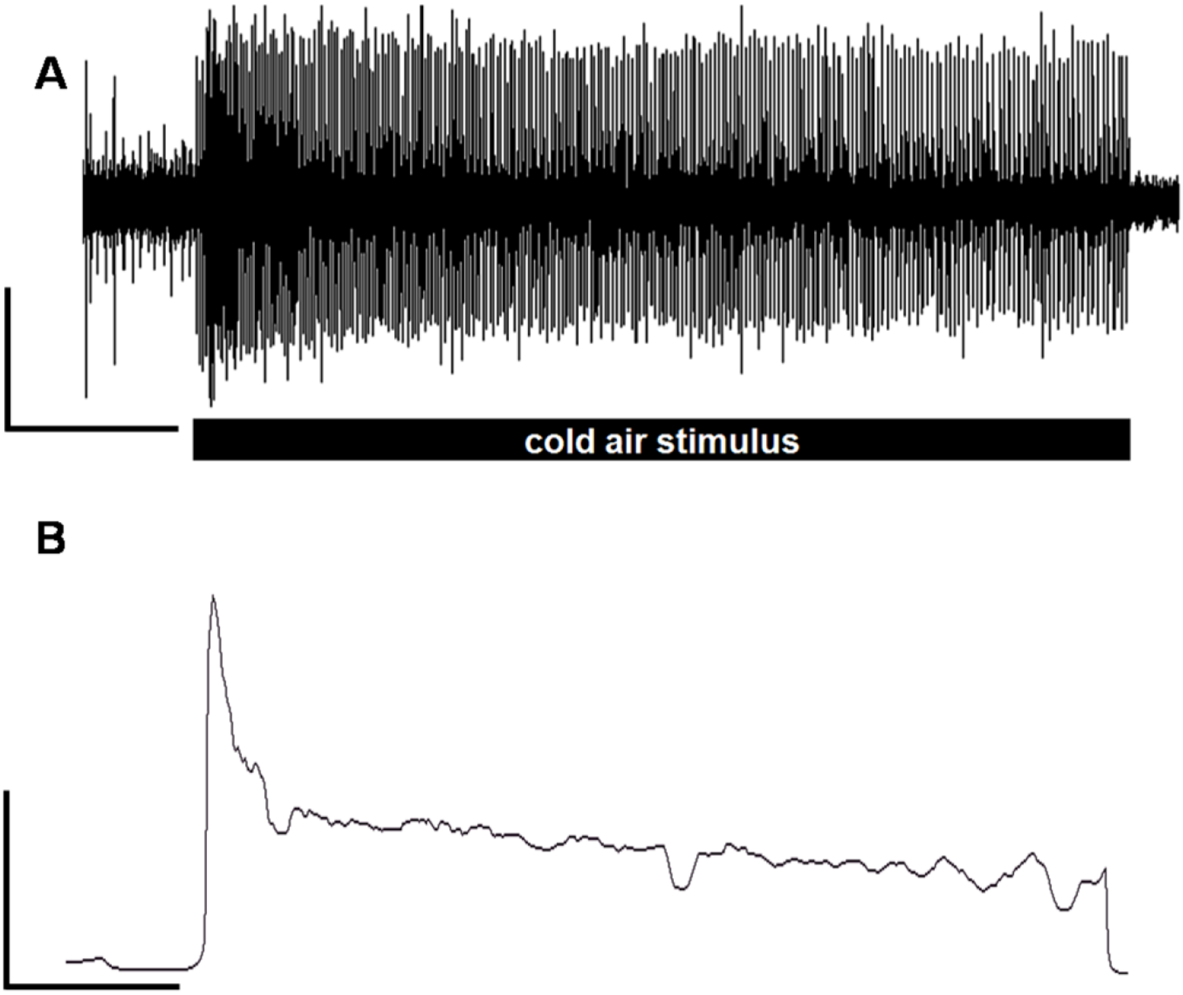

Figure 6: Response of a thermosensitive neuron to a drop in air temperature of 11.2°C:
A: After stimulus onset, the neuron responded with a phasic-tonic increase in activity, thus qualifying as a cold-sensitive neuron. Scale bar: horizontal: 2 s; vertical: 2 mV. **B:** Instantaneous frequency (reciprocal of the time between two spikes) shows two distinct phases of the response. An initial phasic response is followed by a tonic but adapting response. Scale bar: horizontal: 2 s; vertical: 100 Imp/s.

Using an IR-Emitter pulsed at 0.25 Hz, the thermosensitive neuron shows a reduced activity during a radiant heat stimulus of 133 mW/cm^2 compared to the neuronal activity when the IR-emitter was turned-off (Fig. 7). Thus, radiant heat is an effective stimulus although the sensory peg is sheltered in an almost enclosed cavity. Interestingly, the response characteristic differs in comparison to the described phasic-tonic response to convective stimuli. The phasic part of the off-response to IR stimulation is reduced (Fig. 7).

In addition to warm and cold air applied with the stimulus air flow, and to radiant heat applied with the IR-emitter, an ordinary red laser pointer (<1 mW) and even a human finger in close vicinity to the antenna sufficed as thermal stimulus to diminish the neuronal activity of the thermosensitive neuron. Based on these experiments, the investigated neuron of the Sc is considered as a cold-sensitive neuron. In response to prolonged stimulation (10 min) with either 30°C or 18°C, the cold-sensitive neuron shows complete physiological adaptation, i.e. there is no difference in neuronal activity before and after 10 min of stimulation (Fig. 8 A-B) (n = 10). Although neuronal activity reaches 80 Imp/s (mean instant frequency, bin 1s) after onset of the cold stimulus air flow (18°C), an activity comparable to pre-stimulation condition (23 Imp/s) is reached within one minute (Fig 8 A). Similar recovery of the neuronal activity is observed after onset of the warm stimulus air flow (30°C) with an initial reduced activity (0.7 Imp/s) and an activity comparable to pre-stimulation condition within one minute of stimulation (Fig. 8 B).

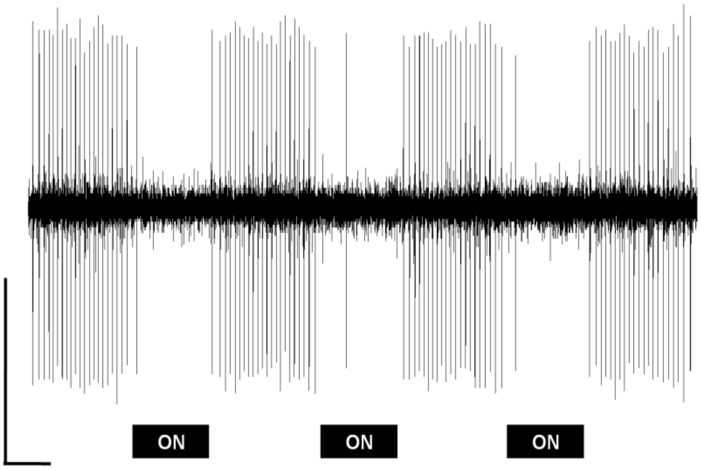

Figure 7: Response to IR stimulation:
Immediately after stimulus onset (IR-emitter, 133 mW/cm^2) the neuronal activity ceased. The mean instant frequency dropped from 11 Imp/s to almost zero during IR stimulation. Compared to stimulation with a convective stimulus (Fig. 6), the neuron showed a different response characteristic, lacking a phasic response after the cessation of IR-stimulation. Scale bar: horizontal: 2 s; vertical: 4 mV.

Discussion

Morphology (SEM)

Sensilla coeloconica (Sc) are peg-in-pit sensilla with a sensory peg located in a chamber connected to the environment by an aperture (Altner and Loftus 1985). In several ant species, small apertures with similar diameters have already been described (Hashimoto 1990; Jaisson 1970; Prellinger 1940). Based on the size of the aperture of different peg-in-pit sensilla, Sc and Sa can be discriminated in some ant species, e.g. *Solenopsis invicta* (Renthal et al. 2003) and *Atta vollenweideri* (this study), but are indistinguishable in other species, e.g. *Atta sexdens* (Kleineidam et al. 2000; Renthal et al. 2003). Compared to the abundance of hair sensilla, Sc have been found in low numbers on the antenna of ants and other insects as well (Dumpert 1972; Hashimoto 1990; Itoh et al. 1984; Iwasaki et al. 1995; Nishikawa et al. 1985; Renthal et al. 2003; Tominaga and Yokohari 1982).

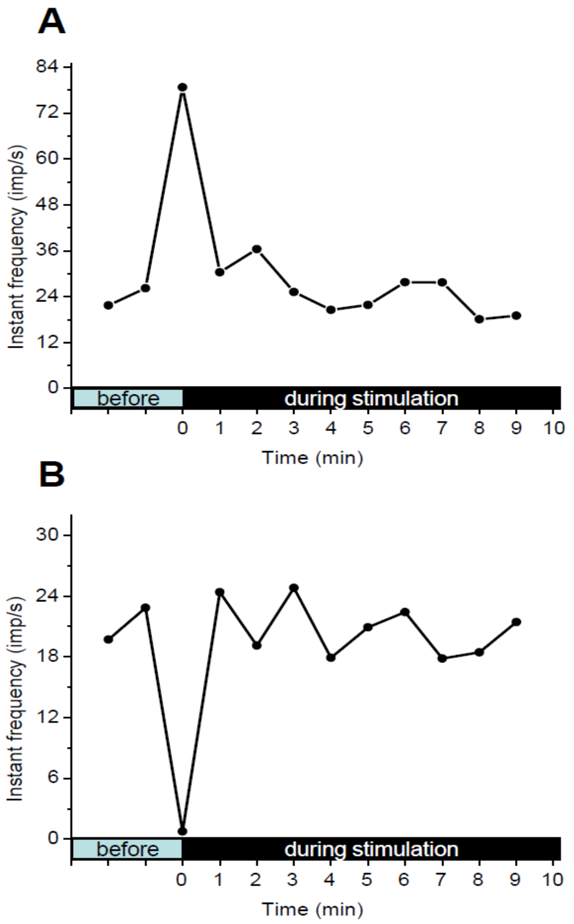

Figure 8: Physiological adaptation of a cold-sensitive neuron to prolonged stimulation with a convective stimulus. **A:** During stimulation with cold air (18°C) the cold-sensitive neuron resumed to pre-stimulus activity within the first minute of stimulation. **B:** During stimulation with warm air (30°C), a similar result was found. This rapid adaptation was observed for all the cold-sensitive neurons investigated (n= 10).

The peg-in-pit morphology protects the sensory peg against harsh mechanical contact, for instance during antennal grooming, and probably avoids evaporative water loss and evaporative cooling (Altner et al. 1977; Shanbhag et al. 1995). Mechanical protection might be important in particular for thermosensitive neurons whose mechanical sensitivity has previously been described (Gödde and Haug 1990).

Fine structure (TEM)

Altner et al (1977) introduced a classification of sensilla, in order to predict the function of a sensillum based on its fine structure characters, rather than on gross morphology alone. The wall structures of the sensory pegs (single-walled or double-walled), and the types of exclusive pore structures are the major traits for classification. The combination of sensory neurons for different modalities e.g. in thermosensitive sensilla challenge the significance of this classification with respect to a structure-function analysis.

Across insect orders, thermosensitive neurons were found in sensilla with different fine structural traits, e.g. single- or double-walled, and porous or non-porous pegs. Most often, thermosensitive neurons have been found in association with two hygro-sensitive neurons forming a sensory triad that consists of a moist-sensitive neuron, a dry-sensitive neuron and a cold-sensitive neuron (MDC-triad) (Altner and Loftus 1985; Altner and Prillinger 1980). MDC-triads have been described mostly for single-walled peg-in-pit sensilla with a non-porous peg on an inflexible socket (Altner and Prillinger 1980; Altner et al. 1981; Altner et al. 1977; Altner et al. 1983; Ameismeier and Loftus 1988; Gödde and Haug 1990; Haug 1986; Nishikawa et al. 1985; Tichy 1979; Tominaga and Yokohari 1982; Waldow 1970; Yokohari 1978; 1981; Zimmermann 1992). For the honeybee, the MDC-triad has been described for sensilla coelocapitula with a putative single-walled structure (Yokohari 1983; Yokohari et al. 1982). However, the MDC-triad has also been found in sensilla with a double-walled peg, e.g. in the cockroach *Periplaneta americana* (Altner et al. 1977). For the honeybee, a thermosensitive neuron has been found in the Sc, which is also probably a double-walled sensillum, but unfortunately information on detailed fine structure and the attribution of physiological data to this sensillum are inconclusive (Lacher 1964; Slifer and Sekhon 1961).

As shown in this study, the Sc of *Atta vollenweideri* are double-walled sensilla housing a cold-sensitive neuron. Double-walled sensilla with spoke channels commonly house chemosensitive neurons (Steinbrecht 1997) and in case of the locust (*Locusta migratoria*), a thermo-chemosensitive combination has been described (Altner et al. 1981). The two neurons beside the cold-sensitive neuron in the Sc of *Atta vollenweideri* did not respond to any of the thermal stimuli tested, and whether these are chemosensitive remains unresolved.

Lamellation of the outer dendritic segment is a trait that has been described for some cold-sensitive neurons, e.g. in the MDC-triad of the stick insect (*Carausius morosus*) (Altner et al. 1978). Lamellated outer dendritic segments also have been described for the 'black hair sensillum' of the cave beetle (*Speophyes lucidulus*) which carries two antagonistic thermosensitive neurons (cold- and warm-sensitive neurons) (Loftus and Corbière-Tichané 1981). In contrast, exclusively chemosensitive sensilla with lamellated outer dendritic segments of sensory neurons have been described e.g. on the palps of *Pieris rapae* (Lee et al. 1985) Thus, lamellation of the outer dendritic segment of a sensory neuron does not necessarily predict its function as cold-sensitive neuron.

In the Sc of *Atta vollenweideri*, electron lucid vesicles were found at the base of the sensory peg, close to the outermost cavity. Vesicles with similar electron density have been reported in the sensillum lymph of double-walled sensilla of several insect species (Altner et al. 1977; Ameismeier 1985; Cribb and Jones 1995). For the chemosensitive Sc described in *Bombyx mori*, the content of these vesicles has been discussed as precursor of the electron dense filaments in the lumen of the cuticular fingers. However, their possible function for sensory transduction remained elusive (Hunger and Steinbrecht 1998).

Electrophysiology

A cold-sensitive neuron was identified in the Sc by extracellular recordings of neuronal activity. Following a drop in temperature, the cold-sensitive neurons respond with increased neuronal activity (phasic-tonic response), irrespective whether cold air or radiant heat (off-response) were used as stimuli. Cold-sensitive neurons with a phasic-tonic response characteristic are widespread in insects (Ameismeier 1985; Lacher 1964; Loftus 1968; Merivee et al. 2003; Must et al. 2006a; Must et al. 2006b; Nishikawa et al. 1992; Tichy 1979). Due to the chemo-physical properties of air, any change in air temperature goes along with a change in relative humidity. The use of a heat-exchanger implies that besides heat also the relative humidity of the air is manipulated. It is argued that the measured responses of the cold-sensitive neurons were specific to temperature, and not associated to the differences in relative humidity of the air used for stimulation.

Two lines of indirect evidence support this view. First, radiant heat is unlikely to noteworthy influence the humidity of the continuous air blown over the preparation, and was shown to be effective as thermal stimulus. Second, a very basic experiment using human breath emphasized the specificity of the response. The neurons always responded with increased neuronal activity to cold air (and increased relative humidity) but with a decreased neuronal activity to human breath (warm air and increased relative humidity; data not shown). This result also suggests that humidity has little influence on the response of the investigated cold-sensitive neurons, an interaction that has been shown for the thermosensitive neurons of the stick insect *Carausius morosus* and a cave beetle (Loftus and Corbière-Tichané 1981; Tichy 2007; 1978).

A remarkable result of this study is the sensitivity of the cold-sensitive neurons to radiation emitted e.g. from an IR-emitter, a laser pointer, or fingertips. It has been shown by several authors that the sensitivity of cold-sensitive neurons to radiant stimuli is low or negligible (Davis and Sokolove 1975; Gingl and Tichy 2001), and the peg-in-pit morphology has been discussed as a feature that leads to a reduced IR-sensitivity (Gingl et al. 2005).

The phasic part of the response is reduced or even absent in response to stimuli generated with the IR-Emitter compared to the response to convective stimuli. This conspicuous difference in response characteristics is probably due to the slow passive cooling of the IR-emitter filament after switch-off. The neuronal response to rapid loss in radiant heat, e.g. during antennal scanning movements in front of a warm object might be more pronounced than that measured in the present experiments.

Several studies on pyrophilous beetles and flat bugs of the genus *Aradus* showed that morphologically specialized sensilla house warm-sensitive neurons with a phasic response characteristic upon IR stimulation (Müller et al. 2008; Schmitz and Bleckmann 1997; 1998; Schmitz et al. 1997; Schmitz et al. 2000a; Schmitz et al. 2000b; Schmitz et al. 2002; Schmitz and Trenner 2003). Besides these highly adapted sensilla, warm-sensitive neurons have been rarely described in other insect species. Such neurons have only been identified in larvae of cave beetles and in blood sucking arthropods, and in most cases they are associated with a cold-sensitive neuron in the same sensillum (Davis and Sokolove 1975; Gingl et al. 2005; Hess and Loftus 1985; 1984; Loftus and Corbière-Tichané 1981).

The cold-sensitive neuron of the Sc in *Atta vollenweideri* shows rapid adaptation to prolonged stimulation. Unfortunately, cold-sensitive neurons in the honeybee were not tested under prolonged stimulation (Lacher 1964), so that comparative data for other Hymenoptera are lacking. A comparable response characteristic as found in *Atta vollenweideri* was recently described for cold-sensitive neurons in a cave beetle (Must et al. 2006a). Adaptation to continuous stimulation is a common property in sensory systems and has been well described for olfactory neurons (Kaissling et al. 1987). Sensory adaptation increases the working range in which changes in intensities can be measured but reduces the ability to measure absolute intensities. Thus, the cold-sensitive neurons of *Atta vollenweideri* are tuned to track rapid changes in convective and radiant heat at different ambient temperatures.

Sensory transduction

The sensory transduction mechanism of thermal stimuli in the investigated Sc is still unknown. However, two different transduction mechanisms have been discussed for various other thermosensitive neurons. First, mechanosensitive transduction has been described for highly specialized thermosensitive sensilla in buprestid beetles and in a flat bug (Müller et al. 2008; Schmitz et al. 2007; Schmitz and Bleckmann 1997; Vondran et al. 1995).

Second, absorption of thermal energy at the membrane of the sensory neuron has been discussed to directly lead to an opening of ion channels (Gödde and Haug 1990). Across animal clades, the occurrence of thermosensitive ion channels of the **T**ransient **R**eceptor **P**otential family (TRP- family) is highly conserved (McKemy 2007). These channels either respond to heat and capsaicin (McKemy et al. 2002) or cold and menthol (Rosenzweig et al. 2005). *Drosophila* larvae lacking a specific TRP channel showed an impairment of the thermo-tactic behavior implying a specific role of TRP channels for the thermal sense of insects. It remains to be shown whether ion channels of the TRP-family are involved in the sensory transduction of thermal stimuli in the investigated cold-sensitive neurons.

Functional morphology of sensilla coeloconica for thermoreception

Based on the ground bauplan of insect sensilla, the most basic organization is found in mechanosensitive sensilla (Keil 1997). Although the origin of the Sc described in the present study is unknown, morphological characters indicate derived traits not found in mechanosensitive sensilla. All traits found in a given sensillum might be constrained by its evolutionary origin, and additionally shaped by evolution to meet the function of any of the associated sensory neurons. The Sc of *Atta vollenweideri* appear to be a good example illustrating the interdependency of origin, adaptation and multi-function. Based on the abundance of thermosensitive neurons found in peg-in-pit sensilla, the general morphology of the Sc in *Atta vollenweideri* is probably an adaptation for thermoreception. In contrast, a double-walled sensory peg is a rare trait described for thermosensitive sensilla and suggests that the two physiologically undescribed sensory neurons of the sensillum are chemosensitive.

The functional significance of the pit suggest itself a protection against mechanical stress, and the shield with the small aperture might reduce evaporation from the peg. The low impact of changing humidity on the response of the cold-sensitive neuron is likely brought about by the almost closed chamber within the antennal cuticle. In addition, rapid changes of air temperature (convective stimuli) might be attenuated due to the enclosure of the sensory peg, thereby acting as a low-pass filter. However, this idea remains to be investigated.

Radiant heat proved to be an effective stimulus for the cold-sensitive neuron, and it is argued that the shield has probably little impact on IR-reception. For infrared radiation of 3 µm wavelength, Schmitz et.al (2007) estimated a penetration depth of approximately 6 µm into cuticle. Thermal energy is absorbed at the sensillum and the electron dense filaments of the sensory peg might improve the absorption. If this is the case, the outermost cavity of the sensory peg is important for thermal stimulus transduction and the wall structure (double-walled) can be considered as adapted for thermoreception. It has been shown in behavioral experiments that leaf-cutting ants are able to use radiant heat for orientation, an ability that requires the spatial detection of thermal objects (Kleineidam et al. 2007). Antennal scanning can be observed during this orientation task, and the Sc are good candidate sensilla to receive spatial information about the thermal environment.

Acknowledgements

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VI. Detection of minute temperature transients by thermosensitive neurons in ants

Abstract

The antennae of leaf-cutting ants are equipped with sensilla coeloconica that house three receptor neurons, of which one is thermosensitive. Using convective heat (air at different temperatures), we investigated the physiological characteristics of the thermosensitive neuron associated with the sensilla coeloconica in the leaf-cutting ant *Atta vollenweideri*. The thermosensitive neuron very quickly responds to a drop in temperature with a brief phasic increase (50ms) in spike rate and hence classifies as cold receptor (ambient temperature = 24°C). The short latency and the brief phasic response enable the thermosensitive neuron to follow temperature transients up to an estimated frequency of around 5 Hz. Although the neuron responds as a cold receptor, it is extremely sensitive to warm stimuli. Already a temperature increase of only 0.005°C leads to a pronounced decrease in the resting activity of the thermosensitive neuron (ambient temperature = 24°C). Through sensory adaptation, the sensitivity to temperature transients is maintained over a wide range of ambient temperatures. We conclude that the thermosensitive neuron of the sensilla coeloconica is adapted to detect minute fluctuations in temperature, providing the ants with thermal information of their microenvironment, which they may use for orientation.

Introduction

The assessment of environmental temperature is crucial for small, ectotherm animals like insects, because they have to respond immediately to unfavorable thermal conditions. Due to their low mass, insects heat up or cool down quickly. For instance, a 10mg fly exposed to direct sunlight heats up 10°C in ten seconds (Heinrich 1993). Most insects exhibit a pronounced thermal preference that allows them to avoid hyper- or hypothermia. In particular, social insects show an amazingly precise thermal preference e.g. during brood care. Already an offset of only 0.2°C from optimal brood temperature provokes the relocation of brood in ant colonies (Roces and Núñez 1989; 1995; Weidenmüller et al. 2009). Other insects, like blood-sucking bugs are also highly sensitive to thermal stimuli, which they use to find their hosts (Wigglesworth and Gillet 1934); and even species without obvious specialization to host detection show strict thermal preferences, e.g. cockroaches (Murphy and Heath 1983) or flies (Sayeed and Benzer 1996; Yamamoto 1979). For most temperature-guided behaviors of different insect species that have been studied so far, it is unclear how the temperature stimulus is transferred from the thermal source to the insects' receptors. Insects may use radiant heat (thermal radiation) as cue for orientation, or the changing air temperature around their body (convective heat) may trigger the behavioral response.

Several physiological studies have shown that thermosensitive neurons of antennal sensilla in insects respond to both, convective heat and thermal radiation (Gingl and Tichy 2001; Gingl et al. 2005; Kreiss et al. 2007; Ruchty et al. 2009). However, some insect species are known to rely mainly or even exclusively on thermal radiation as orientation cue. In these cases, specific properties of the thermosensitive sensilla are expected to serve as adaptation to the detection of thermal radiation. One well-investigated example is the pit organ of Australian fire beetles that carries up to 80 thermosensitive sensilla. Accessory structures of these sensilla change upon absorption of thermal radiation emitted at wavelengths of 3-5 µm from forest fires resulting in a photo-mechanical stimulus transduction (Müller et al. 2008; Schmitz et al. 2007; Schmitz and Bleckmann 1997; Vondran et al. 1995).

In the physiological temperature range of 30-35°C (corresponding to longer wavelengths of around 10 µm), however, thermal radiation has much less power as indicated by the Stefan–Boltzmann law. Therefore, the detection of differences in thermal radiation in that particular temperature range poses high demands on a sensory system.

The blood-sucking bugs *Triatoma infestans* and *Rhodnius prolixus* are guided solely by the thermal radiation emitted from their endothermic hosts (Lazzari and Núñez 1989; Schmitz et al. 2000).These bugs seem to measure the distance to their host based on temperature gradients along their antennae (Lazzari 2009) and probably evaluate the size of the object by saccadic antennal movements (Flores and Lazzari 1996).

Like the triatomine bugs, leaf-cutting ants can use thermal radiation as cue for orientation. Similar to the detection system in the bugs, ants scan their environment by fast antennal movements to detect heated objects and use them as learned landmarks (Kleineidam et al. 2007). During antennal scanning, the antennae of leaf-cutting ants can reach angular velocities of up to 3.6°ms^{-1} (Ehmer and Gronenberg 1997) that may generate rapid, minor temperature transients at thermosensitive sensilla.

As the sensilla coeloconica (Sc) of leaf-cutting ants house a thermosensitive neuron, they are good candidates for providing the ants with the underlying thermal information used for orientation (Ruchty et al. 2009). Irrespective of whether thermal radiation and/or convective heat act on the antennal sensilla, the resulting temperature transients are expected to be minute and brief. In order to detect such stimuli, thermosensitive neurons with high temporal resolution and outstanding sensitivity are required. In this study, we investigate in detail the physiology of the thermosensitive neuron of Sc in the leaf-cutting ant *Atta vollenweideri* and ask whether the neuron is adapted to detect rapid and minute temperature transients. To this aim, we stimulated the neuron with brief air pulses of different temperatures and investigated whether different ambient temperatures influence its response characteristics. Based on these measurements, we were able to evaluate whether the neurons are suited to detect temperature transient, which, e.g., occur during the thermal landmark orientation.

Material and Methods

Animals

Workers of *Atta vollenweideri* were obtained from a laboratory colony collected in 2002 at the Reserva Ecológica El Bagual, Formosa, Argentina. The colony was reared at the Biozentrum , University of Würzburg at 25°C and 50% relative humidity in a 12h/12h photoperiod, and fed mainly with leaves of privet (*Ligustrum vulgaris*) and dog rose (*Rosa canina*). As for the experiments only medium sized workers from the feeding site were collected, it is assumed that only foragers were investigated.

Physiology of the thermosensitive neuron

Preparation and recording

The physiology of the thermosensitive neuron associated with a sensillum coeloconicum (Sc) was investigated by extracellular recording of its neuronal activity. To generate independent measurements, only one sensillum per specimen was investigated. Workers were mounted on a plastic holder with adhesive tape and the scapus was glued onto the holder with white-out correction fluid (Tipp-Ex, Bic, France). Under visual control and at a magnification of 390x (Leitz microscope equipped with NPL-Fluotar L25/0.35, Leitz, Germany), an electrolytically sharpened tungsten electrode was superficially inserted into the cuticle next to a single Sc using a manual micromanipulator (HS-6, Märzhäuser, Germany). In order to achieve a good signal to noise ratio during such an extracellular recording, the reference electrode was inserted deep into the flagellum in close vicinity of the recording electrode. Recordings were band-pass filtered (60 Hz-3 kHz) and amplified 1000x (Neuroprobe Amplifier 100, A-M-Systems, USA and VBF8, Kemo, Great Britain). The electrical noise was significantly reduced using an additional digital filter (Humbug, Quest Scientific, Canada). Data were digitized at a sampling rate of 10 kHz and recorded on a PC using custom-made software (LabView, National Instruments Corporation, Austin, Texas, USA).

To describe the physiology of the thermosensitive neurons in terms of general response properties, response dynamics, sensitivity and adaptation to ambient temperature, we used convective heat as stimulus.

Step changes in air temperature were used to assess the threshold sensitivity to temperature transients and based on the responses to step changes of different amplitude (ΔT), dose response curves were measured. During all measurements, a continuous airflow (flow rate 1.5 l/min) was blown via a glass tube (2 cm inner diameter at a distance of 1 cm from the antenna) over the preparation. The stimulus airflow (5mm inner diameter, at a distance of 1 cm from the antenna) was positioned directly opposite to the tube providing the continuous airflow (Fig. 1A). This arrangement allowed the injection of the stimulus airflow into the laminar continuous flow resulting in a rapid change in temperature at the investigated sensillum (Kleineidam et al. 2000). Air temperature in both airflows were controlled by heat exchangers and measured with thermocouples (NiCr-Ni, Type K, Conrad Electronics, Germany).The continuous airflow was always kept at a temperature of 24°C, except during the adaptation experiments. A thermocouple was placed on the plexiglass holder close to the investigated antenna to estimate the actual stimulus temperature.

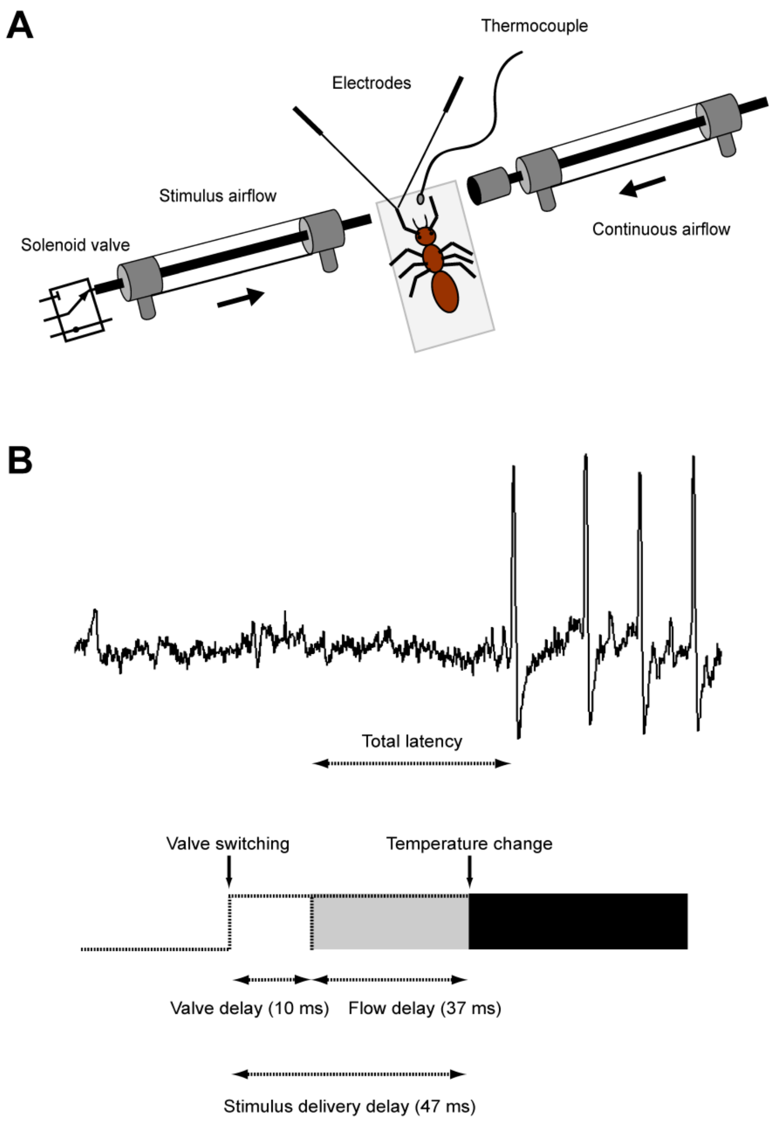

Figure 1: Stimulation setup and response latency calculation
A: The thermosensitive neuron was stimulated with rapid changes in temperature by switching the stimulus airflow into a laminar continuous airflow. The resulting change in temperature was measured with a thermocouple. **B:** Based on the position of the solenoid valve and its switching delay of 10 ms, a total stimulus delay of 47 ms occurred until the warm or cold air puff reached the preparation.

The heat exchangers could be heated or cooled via two silicon tubes connected to two independent water baths (DC1, Haake, Germany). During the experiments the stimulus airflow was switched on for 5 s using a solenoid valve (Lee, USA, valve delay 10 ms) controlled by the data capture software (LabView, National Instruments Corporation, Austin, Texas, USA).
We analyzed the response of the thermosensitive neurons to temperature stimuli in the range of ΔT= +8°C to ΔT= -14°C around an ambient temperature of 24°C. In the range close to ambient temperature we used 0.1°C steps to describe the neurons sensitivity in greater detail. To investigate the neurons' adaptation characteristics and the effect of changes in ambient temperature on the physiology of the thermosensitive neurons, we described dose-response curves at ambient temperatures of 18°C, 24°C and 30°C, respectively.

Data analysis

During extracellular recordings from single Sc the recording electrode picked up voltage signals from one to three receptor neurons simultaneously. The thermosensitive neuron could be easily distinguished from the other neurons by the higher amplitude of its recorded potentials. This fact facilitated the analysis of the response characteristics of that particular neuron. We used a voltage spike detection based on threshold, programmed in LabView, to selectively detect action potentials generated by the thermosensitive neuron. From these data, we calculated the instantaneous frequency (reciprocal of the time elapsed between two consecutive action potentials). The maximum response of a neuron is described by the peak-frequency that is the highest instantaneous frequency during stimulation.
We used a very conservative measure to calculate how fast the neurons respond to a step change in temperature (total latency). We calculated the flow velocity at the end of the stimulation tube being 27.37 cm/s (8 bar pressure, diameter: 5 mm, length: 40 cm), which results in a delay of 37 ms caused by the stimulus airflow (flow delay). Together with the valve delay of 10 ms, the calculated stimulus delivery delay is 47 ms (Fig. 1B). The flow delay highly depends on the distance between the outlet of the tube and the antenna, and at our preparation this distance could not be assessed with high accuracy. Therefore, we subtracted only the valve delay and not the flow delay to calculate the total latency of the response. Thus, the neuronal latency that is the time between stimulus onset and peak frequency is expected to be considerably smaller as indicated by our measure of the total latency.

In order to assess the highest sensitivity of the thermosensitive neurons, we choose the stimulus delivery with the fastest temperature steps we could generate. However, such temperature steps cannot be monitored by a thermocouple system, because of the mass of the thermocouple that is larger than the tip of an antenna, and because the voltage signal has to be low-pass filtered to obtain an accurate measure.

We described the duration of the phasic response by subsequently measuring the time until the instantaneous frequency decreased to 66% of the peak-frequency. The response characteristics of sensory neurons can be well described by a power function (Drew and Abbott 2006). We used a model according to Stevens' Power law (Stevens 1957) to iteratively calculate the dose-response relationship of the thermosensitive neurons. The function used allows us to compare the sensitivity of the thermosensitive neurons with other sensory systems, across modalities. For statistical analysis and to plot data, we used the software STATISTICA 8 (Statsoft, Tulsa, USA) and ORIGIN 8 (OriginLab, USA).

Results

The thermosensitive neurons of sensilla coeloconica (Sc) in *Atta vollenweideri* have a resting activity (RA) of around 10 imps/s (mean=9.30, s.d. =4.05, n=34, at 24°C; independent measurements). As an example of one neuron, a minute temperature increase (ΔT= 0.1°C; Fig. 2A) results in a phasic-tonic drop in activity and vice versa with a phasic-tonic increase in neuronal activity to a temperature drop (ΔT=-5°C; Fig. 2B). A change of ΔT= -5°C from ambient temperature (24°C) results in a peak-frequency of 105 imps/s and a maximum response of around 290 imps/s at ΔT=-14°C (Fig. 2B). Thus, these neurons classify as being cold-sensitive (Ruchty et al. 2009). The neurons' responses to temperature transients seem to be immediate, since the peak frequency is reached already at a time when the thermocouple first detects a change in temperature (Fig. 2C). The almost instantaneous temperature transient we applied with our stimulus delivery system to measure the neurons' response onset is much faster than any technical device. Such temperature steps cannot be monitored with conventional thermocouples at a temporal resolution necessary to measure the actual temperature at the receptive site, as it is illustrated with the temperature measurement shown in Fig. 2C.

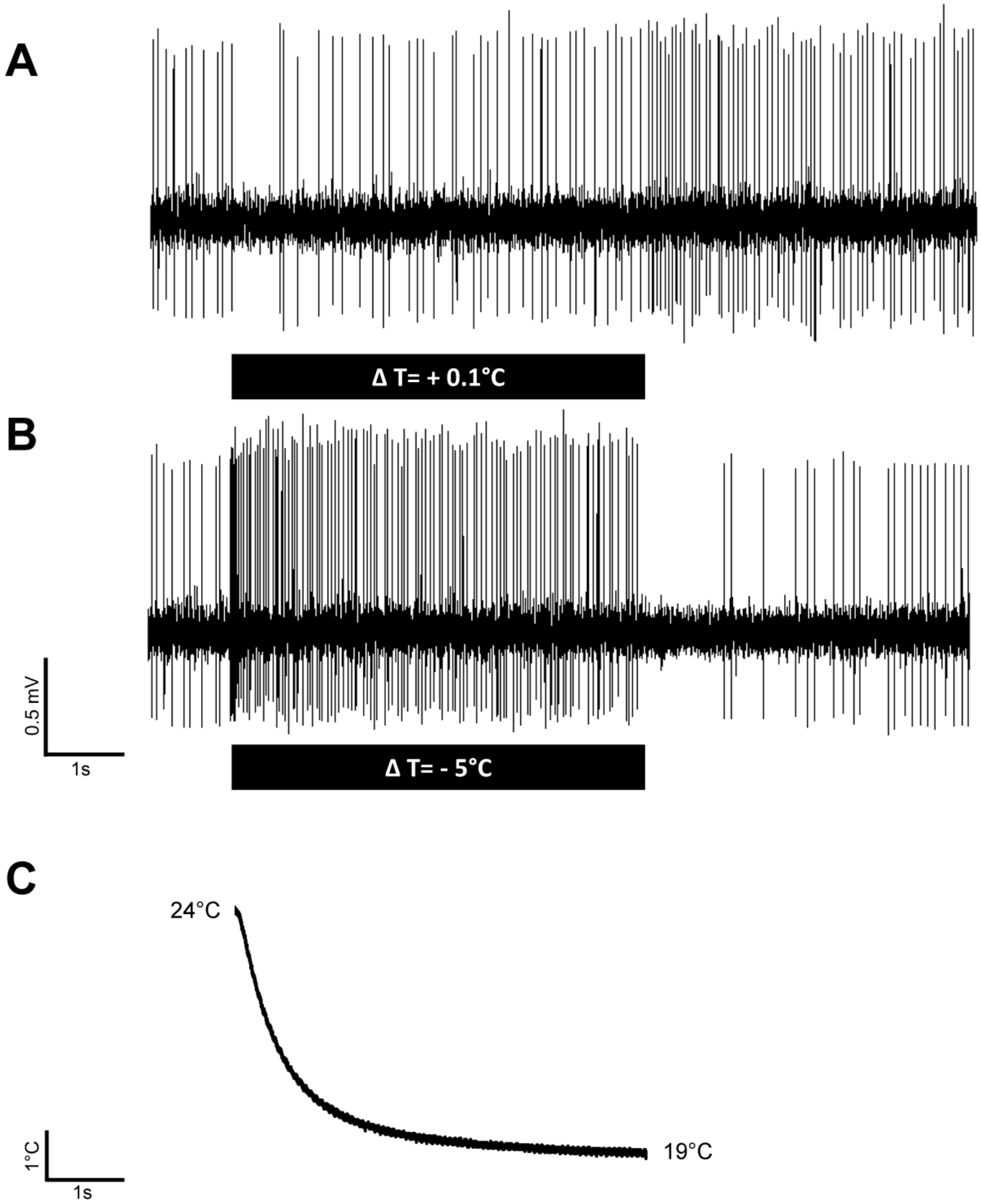

Figure 2: General response characteristic

A: Example of an extracellular recording of a thermosensitive neuron associated with a sensillum coeloconicum. The thermosensitive neuron has a resting activity of about 10 imps/s and responds to a temperature increase of ΔT=0.1°C with an initial cessation of neuronal activity. **B:** A drop in temperature of ΔT= -5°C (ambient temperature in both examples 24°C) results with a phasic-tonic increase in neuronal activity. The highest instantaneous frequency (peak frequency) during the phasic part of the response was 105 imps/s. Subsequently to stimulation (5s), the neuron ceases firing and recovers to resting activity approximately 3 s later. **C:** The voltage signal of the thermocouple indicates how rapid the temperature stimulus was applied however it allows no conclusion about the time course of the actual temperature at the receptive site.

Sensitivity

The thermosensitive neurons are extremely sensitive to temperature transients. An example of a dose-response curve is given in Fig. 3. The slope of the dose-response curve is low for large ΔT, and thus the neuron's ability to discriminate between two large but different cooling temperature steps is low. Sensitivity for small temperature steps and is much higher around ambient temperature (24°C; marked with an arrow in Fig. 3) as shown by the steep slope of the dose-response curve.

In response to warming temperature steps of more than 1°C above ambient temperature, this neuron does not generate any action potentials during the time window analyzed (2 s) and even steps as little as 0.1°C result in cessation of neuronal activity for about 600 ms (Figs. 2A, 3). The dose-response curve is well described by the power function: $y=a*x^k$ with k=0.52 (R^2=0.996). Based on the model used, the steepest slope of the dose-response curve is at stimulus intensities slightly higher than ambient temperature (<0.01°C; calculated for all cooling and the smallest warming temperature steps used in the experiments). In the ΔT>0 range neuronal activity drops almost to zero. In the neuron shown in Fig. 3, a calculated temperature step of only ΔT=+0.005°C leads already to a decrease of 1 imp/s (10%) in neuronal activity (200 imp/s per 1°C).

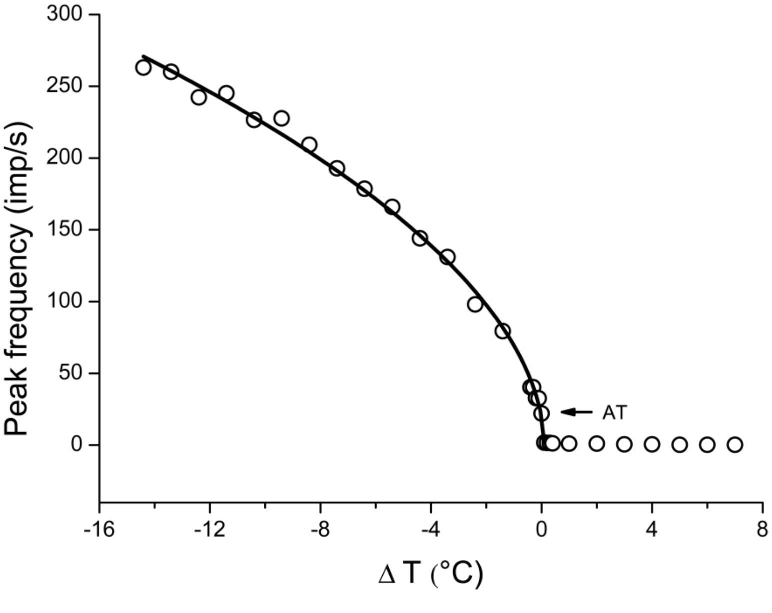

Figure 3: Sensitivity of the thermosensitive neuron
Example of a dose-response curve recorded at 24°C (= 0°C at abscissa) ambient temperature (AT, indicated by an arrow). The peak frequency is shown as a function of step changes from ambient temperature. The sensitivity of the neuron, indicated by the slope of the curve, is highest around ambient temperature. Using an iterative fitting function (Stevens' Power function: $y=a*x^K$; k=0.52; R^2= 0.996), the sensitivity of the neuron was quantified.

We calculated the power function for 24 neurons in order to quantify and compare the sensitivity of several neurons. At an ambient temperature of 24°C we found *k*-values as low as 0.26, and up to 0.71 (mean *k*=0.52; s.d. =0.28; n=24).

The approach using a power function to fit the dose-response curves allows the use of the exponent *k*, i.e. the slope of the curve, as a measure of sensitivity that can be used to compare the sensitivity of the thermosensitive neuron of Sc with receptor neurons for other sensory modalities across systems.

Response latency and duration

The response of the neurons to temperature stimulation is remarkably fast. Using our conservative measure for a number of neurons, we obtained about 46 ms total latency for temperature steps of ΔT=-14°C (Fig. 4; mean=46.02 ms; s.d. =5.30; n=7). By subtracting the flow delay (37 ms) estimated from the flow velocity of the stimulus air flow (see Fig 1B), this would result in a neuronal response latency of only 9 ms. The response latency depends on the strength of stimulation and increases with decreasing temperature steps. About 61 ms pass until the peak frequency is reached at a temperature step of ΔT = -9°C (Fig. 4; mean = 60.63 ms; s.d. = 7.95; n = 7) and it takes even longer at ΔT = -4°C (mean = 72.25 ms; s.d. = 11.68; n = 7).

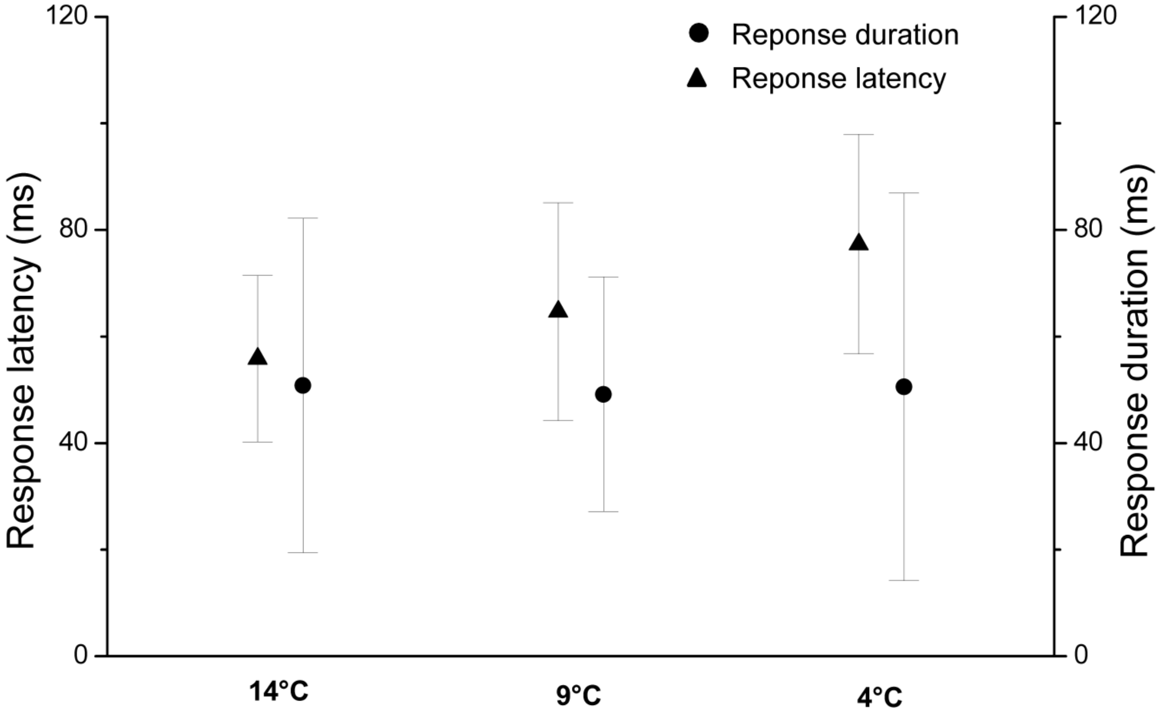

Figure 4: Response dynamics
The phasic part of the response starts with a short latency and is brief. This indicates a high temporal resolution of the thermosensitive neuron to detect thermal fluctuations around ambient temperature. Response latency depends on stimulus intensity (Black diamonds, n=7, mean with s.d. as whisker), whereas the duration of the phasic response seems to be independent of stimulus intensity (Black squares, n=11, mean with s.d. as whisker).

The neurons' phasic response upon temperature stimulation is brief. About 51 ms after the peak frequency is reached, the instant frequency already decreases to 66% of the peak frequency (ΔT = -14°C: mean = 50.81 ms; s.d. = 20.94; n = 12). The length of the phasic response seems to be independent of the strength of stimulation, since both smaller steps in temperature elicit a phasic response of a similar duration (Fig. 4; ΔT = -9°C: mean = 53.06ms; s.d. = 18.99; n = 12; ΔT = -4°C: mean = 50.55 ms; s.d. = 24.23; n = 11). The short latency until the peak frequency is reached and in particular the short phasic part of response enables the thermosensitive neuron to follow fluctuations in temperature up to an estimated frequency of around 5 Hz.

Adaptation

In order to investigate how ambient temperature affects the physiology of the thermosensitive neurons, we used three ambient temperature levels (18°C, 24°C and 30°C), and determined dose-response curves. The thermosensitive neurons (n=3) adapt to the ambient temperatures in the range tested, and maintain a high sensitivity to minute temperature transients (Fig. 5). Following a change in ambient temperature, the neurons always adapt to the same RA as before (RA_{before} and RA_{after}: 9-13 imps/s, n=3).

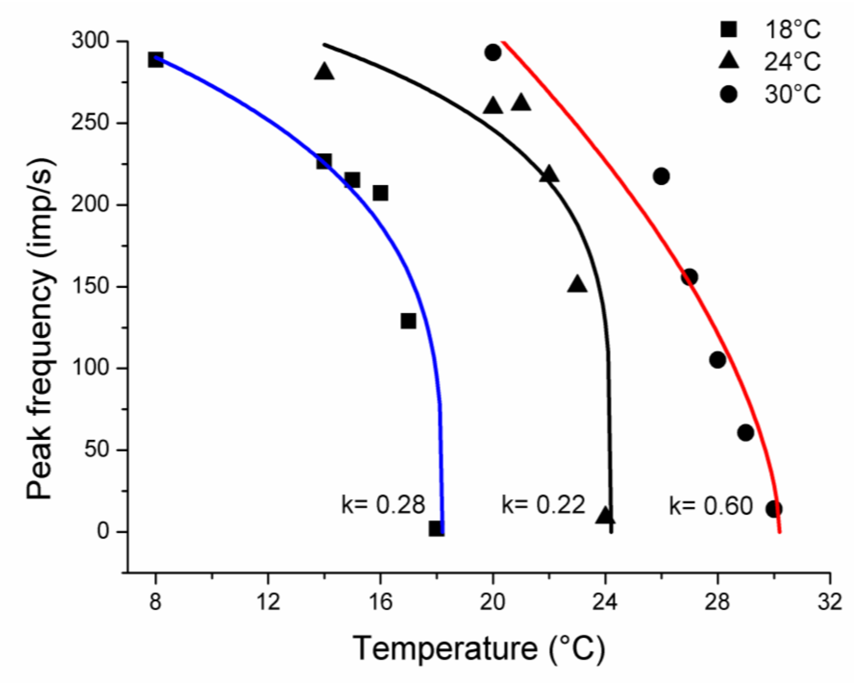

Figure 5: Adaptation

Example of dose-response curves of one thermosensitive neuron recorded at different ambient temperatures (18°C, 24°C and 30°C). The thermosensitive neuron is able to detect rapid temperature transients at different ambient temperatures; however the three calculated dose-response curves differ in their sensitivity (*k*-value). The resting activity as well as the range of neuronal activity remains unaffected. The x-axis shows the stimulus temperature applied to the preparation. Prior to stimulation the neuron was adapted to either 18°C (squares), 24°C (diamonds), or 30°C (circles) ambient temperature.

In addition, the range of neuronal activity (maximum of 280 to 290 imp/s) seems not to be affected by the ambient temperature. However, the slope of the dose-response curves differs across the three different ambient temperatures tested. We found the highest sensitivity at an ambient temperature of 24°C (*k* at 24°C: 0.22-0.24; n=3) followed by a slightly higher *k*-value at 18°C (*k* at 18°C: 0.28-0.31; n=3). At an ambient temperature of 30°C, we measured notably higher *k*-values (lowest sensitivity; *k* at 30°C: 0.57-0.63; n = 3).

Discussion

The thermosensitive neuron of the sensilla coeloconica (Sc) in *Atta vollenweideri* ants responds to a drop in temperature with a brief phasic-tonic increase in neuronal activity. The sensitivity of the neuron to temperature transients around various ambient temperatures is remarkably high. Therefore, this thermosensitive system probably allows the ants to acquire information about their thermal environment by antennal scanning during thermal orientation.

Sensitivity

The course of the dose-response curves shows that the thermosensitive neuron of a Sc is extremely sensitive to temperature transients. Across the different neurons investigated, their sensitivity differs to some extend (different *k* values), and this might lead to range fractioning of the thermosensitive sensilla. Compared to thermosensitive neurons of other insects, the sensitivity is higher by at least one order of magnitude (discrimination ability in the cave beetle and the stick insect: 0.7°;(Loftus and Corbière-Tichané 1981; Tichy and Loftus 1987)).

We used a power function to describe the dose-response curve that allows us to quantify the neuron’s sensitivity. The exponent of the power function (*k*) is a measure of sensitivity and enables comparisons across thermosensitive neurons as well as across modalities and species. In comparison to the key senses in humans, the thermosensitive neuron of a Sc is as sensitive as photosensitive neurons for brightness (*k*=0.33), and even more sensitive than odor-sensitive neurons (*k*=0.6). Human’ perception of temperature and thermal radiation is, with a *k*-value of around 0.7 considerably less sensitive than the ants’ sensory neurons (Stevens 1970).

As can be derived from the good fit of the power function to our measurements, the sensitivity of the thermosensitive neuron is highest for small steps of increasing temperature (0.005°C result in 1imp/s change; warm-sensitivity). However, the rate of action potential decreases in this case and thus the thermosensitive neuron has to be classified as cold receptor. At first glance, cold- and warm-sensitivity seem to be inconsistent, but in fact they have to be considered as adaptation to detect minute fluctuations in temperature. The response property described for the thermosensitive neurons is comparable to that of the photosensitive neurons of the vertebrate visual system. Photosensitive neurons of the vertebrate retina are depolarized in the resting state that causes a 'dark-current', and hyperpolarize when stimulated by light. This physiological property, together with the signal amplification through a second messenger cascade, results in a highly sensitive and low noise sensory system that is able to detect low stimulus intensities (Lagnado and Baylor 1992). The range in which the thermosensitive neurons can code for warming temperature steps is very limited. In response to a temperature transient of only +0.1°C the neuron does not generate any action potentials for sometimes several hundred milliseconds (600 ms in Fig. 2B).

Response latency and duration

The response of the thermosensitive neuron to temperature transients occurs very fast. The response latency (total latency) is only about half the latency measured e.g. for the thermosensitive neurons of the "little ash beetle" *Acanthocnemus nigricans* (Kreiss et al. 2007). However, the beetles' thermoreceptors are very different from other insect thermoreceptors. They are adapted to receive infrared radiation with a spectral sensitivity around 2.8-3.5 µm (Hammer et al. 2001).The fire beetle pit organs with the thermosensitive sensilla are located at the thorax of the animals and house warm-sensitive neurons (Evans 1964; Schmitz and Bleckmann 1997; Vondran et al. 1995). Each thermosensitive sensillum contains a specialized structure (cuticular sphere), which absorbs heat or thermal radiation at a particular wavelength and acts as an outer pressure vessel. Inside the sphere, a microfluidic component transduces the internal pressure onto the dendritic tip of a mechanoreceptor, and therefore this mechanism is termed photo-mechanic transduction (Müller et al. 2008; Schmitz et al. 2007; Schmitz and Bleckmann 1997; Vondran et al. 1995). In contrast, the Sc in leaf-cutting ants are antennal sensilla, which house a cold-sensitive neuron (Ruchty et al. 2009).

In a previous study of the cellular and cuticular morphology of the Sc in leaf-cutting ants, we found neither a cuticular sphere in analogy to the sensilla of the pit organ in fire beetles, nor did we discover any other accessory structures that indicate a photo-mechanic transduction mechanism (Ruchty et al. 2009). Whether the Sc of leaf-cutting ants are also adapted to detect infrared radiation, as it has been discussed previously, remains to be shown (Ruchty et al. 2009).

The discrepancy in response latency between the thermosensitive neurons in fire beetles and leaf-cutting ants is probably due to differences in the transduction mechanisms. The much faster response to temperature transients indicates a more direct stimulus transduction at the thermosensitive neurons associated with Sc in leaf-cutting ants. In the malaria mosquito *Anopheles gambiae*, it has recently been shown that a thermosensitive receptor channel of the TRP family (TRPA1) is expressed in Sc neurons (Wang et al. 2009). We speculate that a transmembrane ion channel probably mediates the stimulus transduction at the thermosensitive neurons of the Sc in leaf-cutting ants as well. It has to be shown in comparative studies on the expression of TRP ion channels whether the molecular entities of thermoreception are conserved across insect species as suggested by McKemy 2007.

The phasic response of the investigated thermosensitive neurons to temperature transients is very brief and both, the short response latency and the short response duration result in an estimated temporal resolution of 5 Hz. This response property aids to detect rapid fluctuations in temperature e.g. generated by the antennal movements shown by the ants during the performance of the thermal orientation behavior (Kleineidam et al. 2007).

Adaptation

In a recent study, we could show that the thermosensitive Sc neuron rapidly adapts to long lasting changes in ambient temperature (Ruchty et al. 2009). In the present study we show that the thermosensitive neurons work as receptors for temperature transients within range of at least 12°C around an ambient temperature of 24°C, and that neither their resting activity nor their activity range is influenced substantially. After adaptation to 18°C, the sensitivity of the thermosensitive neurons is still comparable to the sensitivity that we found at an ambient temperature of 24°C. The investigated subtropical leaf-cutting ant species encounters the ambient temperature range of 18-24°C in various different situations like foraging, nest digging, as well as fungus- and brood-care (Bollazzi et al. 2008; Bollazzi and Roces 2002).

During summer, when daytime temperatures are high, the colonies avoid hot and dry conditions by shifting their foraging activity from daytime to exclusive nighttime foraging. Thus, temperatures above 30°C are encountered less frequently and sensory adaptation of the here described thermosensitive neurons to high temperature is probably less important. In contrast to leaf-cutting ants, other thermosensitive systems of insects that are tuned to sense temperature are less independent of ambient temperature.

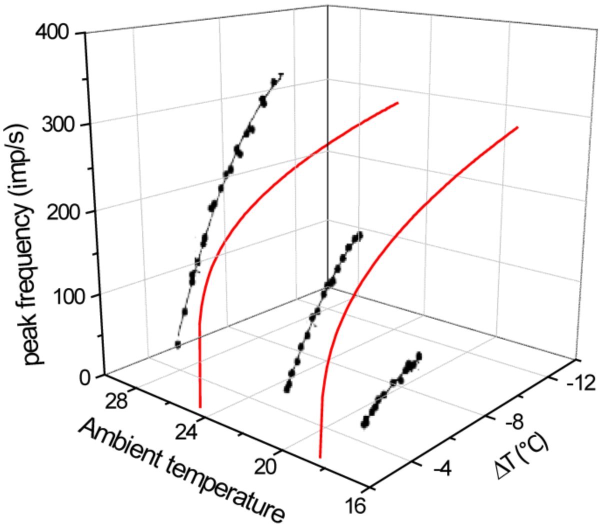

Figure 6: Comparison of thermosensitive neurons in leaf-cutting ants and locusts.
Data of the present account (*Atta vollenweideri*; red curves) are compared to data from a study on the locust *Locusta migratoria*(black curves; (Ameismeier and Loftus 1988). The ants' thermosensitive neurons are more sensitive to temperature transients (ΔT) than the comparable thermosensitive neurons in the locust. While the ants' thermosensitive neurons compensate well for different ambient temperatures, in *Locusta migratoria* both, the sensitivity as well as the range of neuronal activity, depend on ambient temperature.

For example thermosensitive neurons in locusts show increasing sensitivity with increasing temperature (Ameismeier and Loftus 1988)(Fig. 6), and in cockroaches high sensitivity is restricted to a narrow range of ambient temperatures (Loftus 1968).

The adaptation properties of the thermosensitive neurons described in the present study do not provide the insects with a measure of average or steady ambient temperature. However, this important information is provided by other thermosensitive neurons, which show neuronal activity that is closely related to ambient temperature levels. Such neurons have been found in various types of sensilla, and have been described for example in the migratory locust *Locusta migratoria* (Ameismeier and Loftus 1988; Waldow 1970), the cockroach *Periplaneta americana* (Nishikawa et al. 1992;Waldow 1970) and the honeybee *Apis mellifera* (Lacher 1964; Nishikawa et al. 1992). Ants also have a very accurate measure of average ambient temperature as shown by their precise brood translocation to places with favorable thermal conditions (Roces and Núñez 1989; 1995; Weidenmüller et al. 2009).

In the case of leaf-cutting ants, the symbiotic fungus is also translocated in response to unfavorable temperature conditions (Bollazzi and Roces 2002). The corresponding receptor neurons that are required for such thermal behavior of ants are not yet described, nor are their sensilla identified unambiguously. Likely candidate sensilla are peg in pit sensilla of the type sensilla ampullacea (Kleineidam and Tautz 1996; Kleineidam et al. 2000) or sensilla coelocapitula (Yokohari 1983).

Functional adaptation for orientation behavior

When ants move through their environment, they constantly face temperature transients either self-generated by their antennal movements, or resulting from thermal discontinuities of the surroundings. The thermosensitive neurons described in this study are extremely sensitive and even minute transients can be detected. Their fast and brief response, together with antennal scanning allows the ants to assess different temperatures within their microenvironment. Due to solar radiation, the microenvironment of ants is thermally structured. Unfortunately, no data are available describing the thermal microenvironment of leaf-cutting ants with high spatial resolution.

The sensitivity of the ants' thermosensitive neurons is comparable to the most sensitive animal system for detection of temperature transients hitherto described: the pit organ of snakes. It has been calculated that temperature differences as minute as 0.003°C can be detected by boids and pit vipers (Bullock and Diecke 1956). Thus, these neurons are only marginally more sensitive than the thermosensitive neurons we described in the present study (detection of 0.005°C). The snakes use their pairs of pit organs (Newman and Hartline 1982) to detect the thermal radiation emitted by their endothermic prey (Buning et al. 1981a; b) and for thermoregulatory purposes (Krochmal and Bakken 2003). Leaf-cutting ants as well use thermal radiation for orientation (Kleineidam et al. 2007) and thermal radiation has been shown to be an effective stimulus for the thermosensitive neuron associated with the Sc (Ruchty et al. 2009).

In both, the pit organ of snakes and the Sc of insects, the receptor neurons respond to local temperature transients irrespective of the physical property of the stimulus (convective or radiant heat). The morphology of the pit organ of snakes serves as adaptation to the detection of thermal radiation: the pit shields the thermosensitive neurons from scattered radiation.

We speculate that the tiny pit of the Sc in ants serves a similar function and together with the remarkable physiological characteristics discovered in the present study provides the ants with spatial information about their thermal microenvironment. Therefore, we suggest that the Sc may play a prominent role in the detection of spatially distributed warm objects during foraging (Kleineidam et al. 2007).

Acknowledgements

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VII. Representation of thermal information in the antennal lobe of leaf-cutting ants

Abstract

Insects are equipped with various types of antennal sensilla, which house thermosensitive neurons adapted to receive different parameters of the thermal environment for a variety of temperature-guided behaviors. In the leaf-cutting ant *Atta vollenweideri*, the physiology and the morphology of the thermosensitive sensillum coeloconicum has been thoroughly investigated; however, the central projections of its receptor neurons are not known. Here we selectively stained the three neurons found in single sensilla coeloconica and tracked their axons into the brain of *Atta vollenweideri* workers. Each of the three axons terminated in a single glomerulus of the antennal lobe. Two of the innervated glomeruli were adjacent to each other (“Gemini glomeruli”) and are located lateral, while the third one was clearly separated and located medial in the antennal lobe. Using two-photon Ca^{2+} imaging of antennal lobe projection neurons, we studied, where in the antennal lobe thermal information is represented. In the 11 investigated antennal lobes, we found up to 6 different glomeruli in a single specimen responding to temperature stimulation. Both, warm- and cold-sensitive glomeruli could be identified. All thermo-sensitive glomeruli were located in the medial half of the antennal lobe. Based on the general representation of temperature stimuli and functional data of the “Gemini” glomeruli, we conclude that thermal information received by sensilla coeloconica is processed in the medial of the three target glomeruli. Our study links a specific thermosensitive neuron to its central target (a single glomerulus) and evidences an important role of the antennal lobe in temperature processing in insects.

Introduction

Insects possess a highly developed thermal sense to assess various parameters of their thermal environment. Apart from temperature avoidance or preference, required for successful thermoregulation of the own body temperature, some insects utilize their thermal sense for amazingly sophisticated temperature guided behaviors. Blood-sucking bugs, for instance, detect thermal radiation emitted by their endothermic prey to receive a blood meal (Lazzari, 2009; Lazzari and Núñez, 1989; Schmitz et al., 2000), Australian fire beetles locate forest fires to encounter mates and to reproduce (Evans, 1964; Schmitz et al., 1997; Vondran et al., 1995), and leaf-cutting ants can use thermal radiation as an orientation cue (Kleineidam et al., 2007). Most of the thermo-sensitive neurons that receive thermal information about the environment are located on the antennae within cuticular structures termed sensilla.

In the leaf-cutting ant *Atta vollenweideri* one antennal thermosensitive sensillum has been studied in great detail. This sensillum coeloconicum (Sc) has a remarkable morphology with a sensory peg deeply sunken in a cuticular pit, which is separated from the environment by a tiny aperture (Ruchty et al., 2009). The sensillum houses three receptor neurons of which one is thermosensitive (cold-sensitive neuron) while the others are putatively chemosensitive (Ruchty et al., 2009). Recent electrophysiological investigations revealed an extreme sensitivity and high temporal resolution of the thermosensitive neuron (Ruchty M., in revision). Based on the response characteristic to thermal stimuli and due to the extraordinary morphology of the sensillum, it has been suggested as a candidate for detecting spatially distributed heated objects during thermal landmark orientation (Kleineidam et al., 2007; Ruchty et al., 2009, Ruchty M., in revision). In order to understand how insects orient in their thermal environment, it is necessary to know where and how thermal information is processed in the central nervous system. Until now, the central projections of thermosensitive neurons have been investigated only in few studies and all of them revealed the antennal lobe as the first integration centre (Nakanishi et al., 2010; Nishikawa et al., 1995; Nishino et al., 2009). The tracing study of Nishikawa et al. (1995) nicely shows where thermal information of specific thermosensitive neurons is further processed. The other two studies show, in which part of the antennal lobe thermal information might be represented, however based on the tracing technique used it cannot be excluded that other neuropils are innervated as well.

In the present study we investigate the representation of thermal information in the antennal lobe of the leaf-cutting ant *Atta vollenweideri*. Using two-photon-laser-scanning microscopy, we measure the spatiotemporal neuronal activity upon temperature stimulation throughout the whole antennal lobe. In particular, we focus on the thermal information received by the thermosensitive neuron from the Sc. By selectively staining single sensilla (Sc), we investigate the central projections of the receptor neuron axons into the antennal lobes of the ants. Finally, we correlate the pattern of neuronal activity obtained during the Ca^{2+} imaging experiments with the target regions of the Sc receptor neurons to determine which of the Sc receptor neurons display thermosensitivity.

Material and Methods

Animals

Workers of *Atta vollenweideri* were obtained from a laboratory colony collected in 2002 at the Reserva Ecológica El Bagual, Formosa, Argentina. The colony was reared at the Biozentrum of the University of Würzburg at 25°C and 50% relative humidity in a 12h/12h photoperiod, and fed mainly with leaves of privet (*Ligustrum vulgaris*) and dog rose (*Rosa canina*). The performed experiments comply with the current laws of the Federal Republic of Germany.

Central projections from sensilla coeloconica

We selectively stained single sensilla coeloconica (Sc) and investigated the anterograde projection of the receptor neurons into the ants' brain. Ants were mounted onto plexiglass holders with dental wax (surgident periphery wax, Heraeus Kulzer, Germany) and a strap of adhesive tape. Following the immobilization procedure, the scapus of each antenna was glued onto the holder by using white-out correction fluid (Tipp-Ex, Bic, France). Under visual control and at a magnification of 390x (Leitz microscope equipped with NPL-Fluotar L25/0.35, Leitz, Germany), an electrolytically sharpened tungsten electrode was inserted into a single Sc. The sensory peg was deliberately injured by carefully moving the electrode into the sensillum, which caused the permeation of receptor lymph into the chamber as observed under the microscope.

The injured dendrites were then stained via a droplet of dextran-biotin (D-7135, Molecular Probes) dissolved in distilled water and applied onto the antennal tip for 10 minutes using a Hamilton syringe (single-staining).

To investigate whether the target region in the ant's brain was exclusively innervated by receptor neurons of the Sc we, subsequently to the single sensillum staining, extensively cut antennal hair sensilla with a razorblade and stained the neurons with tetra-methyl-rhodamine-dextran + biotin (3,000 MW, lysine-fixable; Microruby, D 7162, Molecular Probes) dissolved in distilled water (double-staining). All individuals from the single- and double-staining experiments were then kept separately in Petri dishes in order to avoid social grooming that could have decreased the staining success. After 24 hours, the brains of the ants were dissected under ant saline solution, transferred into ice-cold fixative (4% formaldehyde in phosphate buffered saline (PBS, PH 7.2) and stored over night in the fridge. Brains were then rinsed in PBS (3 times à 10 minutes) and subsequently incubated in Alexa 488 conjugated streptavidin (S-11226, Molecular Probes, Eugene, OR) for 48 hours. After being rinsed again in PBS (3 times à 10 minutes), the brains were dehydrated in an ascending ethanol series (50, 70, 90, 95 and 3 times 100%, each 10 minutes), transferred into methyl-salicylate (M-2047, Sigma-Aldrich, Steinheim, Germany) and finally investigated at a confocal-laser-scanning microscope (Leica TSC SP 2, Leica Microsystems, Wetzlar, Germany). The software AMIRA 3.1 (Mercury Computer Systems, Berlin, Germany) was used to analyze the confocal image stacks and to reconstruct the innervated neuropils as well as the axons of the labeled receptor neurons.

Two-photon Ca^{2+} imaging

Animal preparation

In order to study where in the antennal lobe thermal information is represented, we retrogradely labeled projection neurons that connect the antennal lobe via two antenno-protocerebral tracts (APTs) to the mushroom bodies and the lateral horn (Galizia and Rössler, 2010; Kirschner et al., 2006; Mobbs, 1982). Single ants were mounted in plexiglass holders using dental wax (surgident periphery wax, Heraeus Kulzer, Germany).

Subsequently, a small window was cut into the head capsule and tracheae, glands and muscles were gently moved aside in order to access the dye injection site in the lateral protocerebrum, close to the vertical lobe (alpha-lobe) of the right mushroom body. Prior to the dye injection, a sharp glass electrode was used to perforate this region and to injure the projection neurons of the APTs.

Afterwards a second glass electrode coated with some crystals of Fura-2 dextran (potassium salt, 10000 MW, F3029, Molecular Probes) dissolved in aqua dest. was inserted in the perforated region and remained there for approximately ten seconds. The brain was then covered by two-component glue (KWIK-SIL, World Precision Instruments, Berlin, Germany) to avoid desiccation, and the animals were kept in darkness for a staining period of around 3-4h. Prior to the functional imaging experiments, we first detached the KWIK-SIL from the brain, and then glands, tracheae and muscles covering the antennal lobe ipsilateral to the injection site were removed. Subsequently, we excised the esophagus at the mouthparts, pulled it for a few millimeters out of the head capsule and fixed it with some dental wax aside the head capsule. This procedure was done to reduce brain movement during data acquisition. In order to protect the preparation and the brain, we again covered it with KWIK-SIL before mounting the plexiglass holder with the ant at the imaging setup.

Imaging setup

In vivo Ca^{2+} imaging was performed using a custom-built two-photon-laser-scanning microscope equipped with a Ti:sapphire laser system (MaiTai HP, Spectra-Physics, Newport, Santa Clara, USA) as light source (about 100 fs output). To excite the Fura-2 dye we used a wavelength of 810 nm. The laser intensity was adjusted with a Pockel's cell (Conoptics, Danburry, USA). The microscope was equipped with two galvanometric scan mirrors (Model 6210; Cambridge Technologies, Lexington, USA) for scanning the specimen in the *x*-*y* axis, and a piezoelectric focusing element (P-725.4CD PIFOC, Physik Instrumente, Karlsruhe, Germany) for *z*-scanning along the optical axis. A water immersion objective (25X; XPlan MP, 1.05 NA; Olympus) was mounted onto the piezo element for both, focusing the laser beam onto the antennal lobe and collecting the emitted fluorescence after dye excitation. To control the microscope and for data acquisition, custom-written software was used (LabView, National Instruments Corporation, Austin, Texas, USA).

Odor and temperature stimuli

As a pre-test to check for vital preparation and successful staining procedure, we induced neuronal activity by stimulating the right antenna with two air puffs containing an odor mixture of citral and octanol (Aldrich Chemical Company, Inc.). The odors were loaded on a stripe of filter paper (10 µl of each odor at a concentration of 10^{-2}, with hexane as solvent; Aldrich Chemical Company, Inc.).

After evaporation of the solvent for 4 min, the filter paper was placed into a syringe that was connected to a silicon tube used for stimulus application. The odor stimuli were applied manually 5 and 15 seconds after start of the recording.

Only those animals in which odor-elicited neuronal activity could be measured were subsequently used in the experiments designed to investigate the representation of thermal information within the antennal lobe. To generate warm (30°C) or cold (15°C) air stimuli, the airflow was channeled through a custom-made heat exchanger connected to a water bath via two silicon tubes (DC1, Haake, Germany). The outlet of the air tube was positioned close to the ant's right antenna (2 cm distance) and air temperature was controlled by the water bath and measured by a thermocouple (ALMEMO, Ahlborn Messtechnik, Holzkirchen, Germany). During both, odor and temperature stimulation a constant airflow (ca. 1.5 l/min) was blown over the preparation.

Optical recording of temperature evoked activity

Functional data were collected by acquiring images with 128x128 pixel resolution and at a frame rate of 4 Hz. To screen the entire antennal lobe with all glomeruli for responses to temperature changes, we conducted 10-15 consecutive framescans (trials) in 10 µm steps along the *z*-axis. In order to prevent neuronal adaptation in the thermosensitive neurons, we spaced the trials with a time interval of two minutes. During each trial the antenna was stimulated four times, every 20 frames (5s) for a period of 2s, by switching the stimulus airflow onto the antenna using a solenoid valve (Lee, USA) that was controlled by the data capture software. Having acquired the functional data, we recorded image stacks of the whole antennal lobe volume with a higher optical resolution (256x256, 2µm step size).

Data processing and analysis

For data processing, the raw fluorescence images (frame scans) were read into ImageJ. To calculate the relative change in fluorescence *(ΔF/F)* we first subtracted the background fluorescence. The mean of the first 10 images was used as reference (F_0) that was subtracted from each image in the sequence in order to obtain *ΔF*. The result (*ΔF*) was then divided by F_0, which results in an image showing the relative fluorescence change *(ΔF/F)*. An increase in cytosolic Ca^{2+}, and thereby an increase in neuronal activity, causes a reduction in *ΔF/F* of the Fura-2 dye at an excitation wavelength of 810 nm (Grynkiewicz et al., 1985).

An average image of all frames of each trial (100 frames) was calculated and used to select regions of interest (ROI) that corresponded to single glomeruli. Each and every recognizable glomerulus at each scan-depth was labeled with an individual ROI, and the relative fluorescence change (*ΔF/F*) over time (response kinetic) was analyzed. Ca^{2+} transients of all responding and some non-responding glomeruli were then illustrated using ORIGIN 8.1 (OriginLab, USA) and Adobe Illustrator (Adobe, San Jose, USA). The 3D-reconstruction of glomeruli was accomplished by using the software AMIRA 5 (Mercury Computer Systems, Berlin, Germany).

The spatial representation of temperature stimuli in the antennal lobe was analyzed by measuring the *x-y-z* coordinates of every thermosensitive glomerulus in each of the investigated antennal lobes. The 3D positions of the thermosensitive glomeruli were mapped into a mean antennal lobe which was calculated based on the mean volume of all investigated antennal lobes (n= 11). For the visualization of these data we used ORIGIN 8.1 (OriginLab, USA).

Results

Central projections from sensilla coeloconica

As result of numerous attempts, we selectively stained the receptor neurons of single sensilla coeloconica (Sc) in 19 independent preparations (antennae). In all of these cases, we found three sensory neurons, each innervating a single glomerulus in the ipsilateral antennal lobe (Fig. 1A, B; n = 19). All three innervated glomeruli are located in the dorsal part of the antennal lobe, at a distance of 80-110 µm (depth) from its anterior surface and close to the dorsal lobe.

Two of the innervated glomeruli are located at a lateral position, whereas the third glomerulus is located medially and is larger than the adjacent glomeruli in this region (Fig. 1B). Because of their side-by-side position we termed the two lateral glomeruli “Gemini glomeruli”. All three Sc-glomeruli are innervated by a single axon with a large diameter (mean = 1.12 µm, s.d. = 0.089; n = 6, measured at the unbranched axon) that branches within the glomerulus and densely innervates the whole glomerular lumen (Fig. 1B, D). The diameter of these receptor neuron axons is significantly larger in comparison to the axons of olfactory hair sensilla (mean = 0.62 µm, s.d. = 0.049, n = 8, p< 0.01; Kelber C., unpublished data; Fig. 1C). However, the shape and the size of the three glomeruli is similar to adjacent, putatively olfactory glomeruli (Fig. 1C). Double labeling of receptor neuron axons from Sc (green) and from different types of hair sensilla (magenta) revealed that their glomerular innervation pattern are not overlapping within the three Sc-glomeruli (double-staining, n = 3). Thus, we conclude that the receptor neurons associated with Sc terminate in glomeruli that are exclusively innervated by these neurons (Fig. 1C).

Two-photon Ca^{2+} imaging

In 257 out of 810 investigated animals, projection neurons from the two APTs could be stained successfully. Two-photon image stacks through the antennal lobes (ipsilateral to the injection site) revealed that in the successful attempts the overall majority of glomeruli is stained with calcium sensitive dye and thus amenable for functional imaging (Fig. 2A). The spatial resolution of the obtained image stacks is exceptional in all three spatial dimensions and enables to reconstruct glomeruli and the whole antennal lobe in 3D, on the basis of *in vivo* morphological data (Fig. 2A, B). Landmarks like the macroglomerulus (Kleineidam et al. 2005) or the antennal nerve and its tracts within the antennal lobes can be used to locate the area where the receptor neurons of the Sc terminate. Based on their position within the antennal lobe and their characteristic shape and arrangement, the two lateral, “Gemini glomeruli” can be identified and serve as landmark glomeruli to localize the medial glomerulus. Fig. 2B depicts the positions of the three glomeruli that are putatively innervated by receptor neurons from the Sc (solid spheres).

In a total of 20 specimens, we recorded odor evoked neuronal activity, as indicated by a sudden drop in fluorescence (*ΔF/F*), which could be assigned to individual glomeruli of the antennal lobe. As an example, in Fig. 2C an average image (from 100 frames) of an optical section through the antennal lobe is shown (depth = 45 µm). Upon stimulation with the odor mixture (10^{-2} citral and 10^{-2} octanol), a single glomerulus responds with an increase in neuronal activity. The resulting increase of cytosolic Ca^{2+} concentration in the projection neurons leads to a rapid (within one frame) drop in fluorescence (*ΔF/F* ≈ 11%; purple ROI and purple trace; Fig. 2D). The other glomeruli in the same optical section do not respond to this odor mixture and thus leave their sensory modality unspecified.

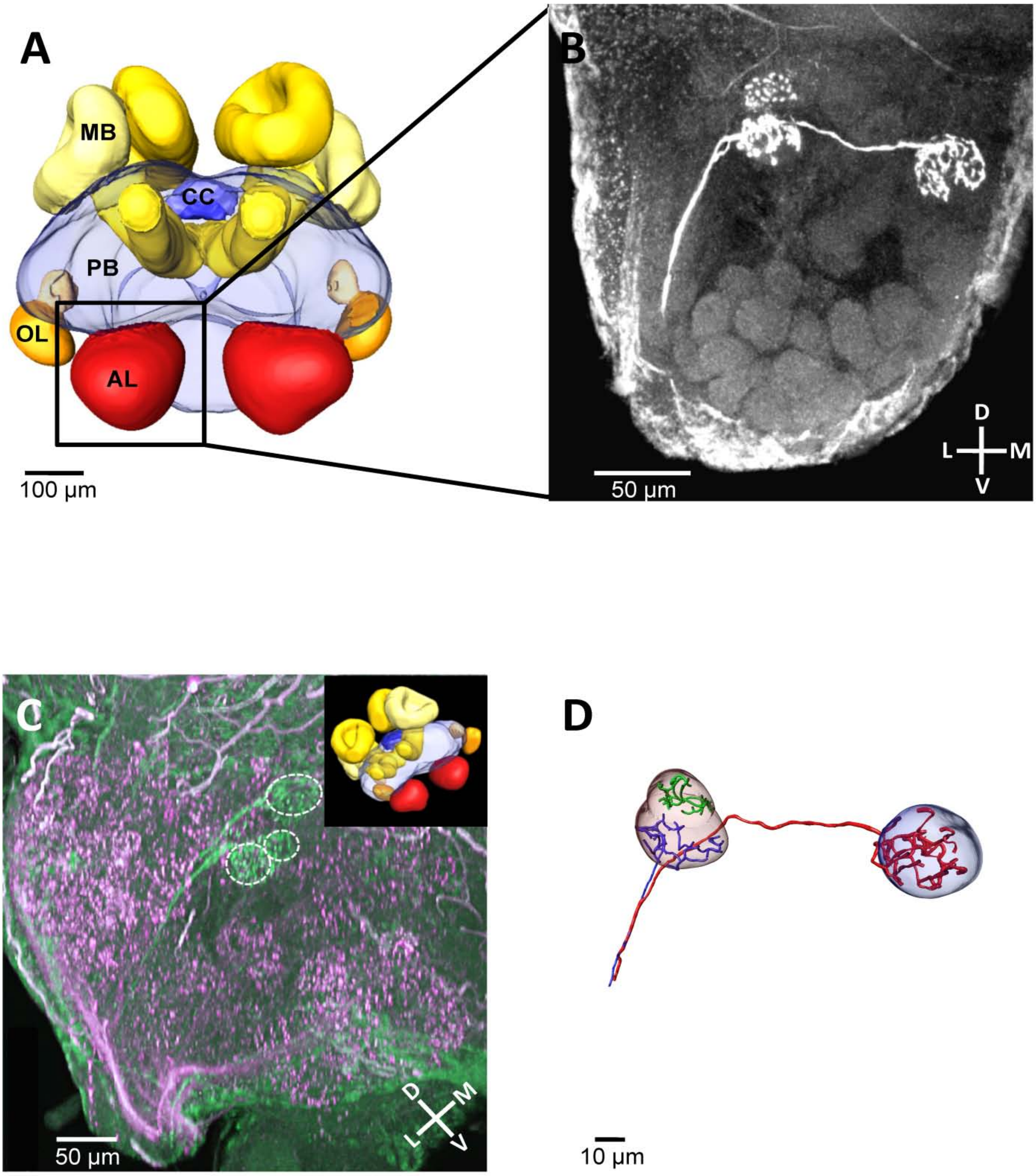

Figure 1: Central projections from sensilla coeloconica
A: 3D reconstruction of the major neuropils of a brain of *Atta vollenweideri* (courtesy of C. Kelber). AL: antennal lobe, CC: central complex, MB: mushroom bodies, OL: optic lobes, PB: protocerebrum. **B:** Projection view of a confocal image stack. The three receptor neurons of a single sensillum coeloconicum terminate each in a single glomerulus, and all three glomeruli are located at the dorsal part of the antennal lobe (two lateral glomeruli (“Gemini”) and a medial one). **C:** Double staining of a single sensillum coeloconicum (green) and various hair sensilla (magenta) show no overlap in the three target glomeruli of sensilla coeloconica (n = 3; inset picture illustrates the orientation of the investigated antennal lobe by using the exemplary brain shown in A). **D:** 3D Reconstruction of the receptor neurons and the glomeruli innervated by the sensilla coeloconica of the specimen shown in Fig. 1B revealed that each neuron innervates a single glomerulus. All three neurons (axon diameter 1-1.4 µm, n = 6) establish extensive arborizations in their glomeruli. D: dorsal, L: lateral, M: medial, V: ventral.

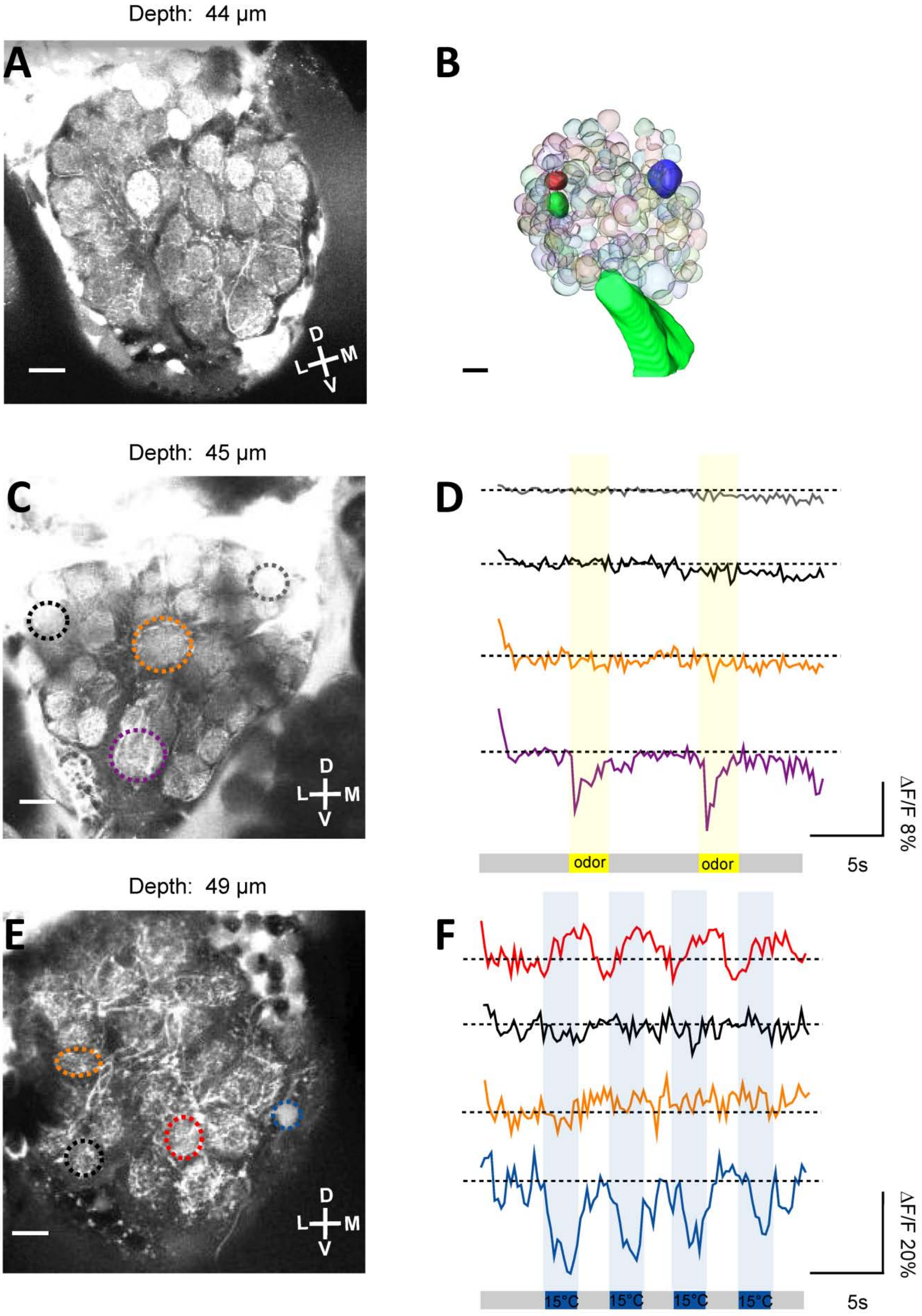
Depth: 44 µm
A
D
L
M
V
B
Depth: 45 µm
C
D
L
M
V
D
ΔF/F 8%
5s
odor
odor
Depth: 49 µm
E
D
L
M
V
F
ΔF/F 20%
5s
15°C
15°C
15°C
15°C

Figure 2: Two-photon imaging of antennal lobe projection neurons
A: Based on the good optical resolution of the images obtained with the two-photon microscope, single glomeruli could be discriminated, some landmark glomeruli could be identified and neuronal activity could be measured selectively. **B:** 3D reconstruction of single glomeruli in the antennal lobe based on *in vivo* morphological data. Individual glomeruli, in this case the three glomeruli that are putatively innervated by the receptor neurons of the sensilla coeloconica, could be identified (solid glomeruli). **C:** The average image (100 frames) of a Ca^{2+}imaging trial was used to select regions of interest (ROI, encircled by dotted lines). The relative fluorescence change (*ΔF/F*) in single glomeruli upon stimulation. **D:** In this optical section (depth = 45 µm), only a single glomerulus (purple ROI) responded to odor stimulation (two air puffs of a mixture of 10^{-2} citral and 10^{-2} octanol at second 5 and 15 of the recording) with a drop in *ΔF/F* of about 11%. Other glomeruli, e.g. the orange ROI did not respond to the odor stimulation. **E-F:** Upon stimulation with 15°C cold air in another specimen two glomeruli respond in this optical section. Based on their response properties, these glomeruli classify as warm- and cold-sensitive, respectively (red and blue ROI). The repeated stimulation (n = 4) induced changes in *ΔF/F* of up to 20% in the cold-sensitive glomerulus and about 10% in the warm-sensitive glomerulus. D: dorsal, L: lateral, M: medial, V: ventral. Scale: 10 µm

Temperature evoked responses in projection neurons

Using the two-photon microscope, we screened the whole antennal lobe and investigated where thermal information is represented. In 11 out of 31 specimens we successfully recorded changes in neuronal activity within antennal lobe glomeruli in response to temperature stimuli. By using repeated stimulation with temperature steps, both, warm- and cold-sensitive glomeruli could be identified. An example of two thermosensitive glomeruli in an optical section through the antennal lobe (depth = 49 µm) is shown in Figs. 2E, F. Both glomeruli respond to the four repeats of cold stimulation (step change to 15°C; ambient temperature = 24°C) with different response properties. A medial glomerulus (blue ROI and blue trace in Fig. 2E, F) responds with a drop in fluorescence upon stimulation (cold-sensitive glomerulus). In contrast, a central glomerulus (red ROI and red trace in Fig. E, F) seemed to be inhibited during the cold-stimuli and responded with increased activity after termination of the cold-stimuli (warm-sensitive glomerulus). There is a stronger Ca^{2+} signal in response to the temperature change in the cold-sensitive glomerulus (*ΔF/F*≈ 20%) than in the warm-sensitive glomerulus (*ΔF/F*≈ 10%).

Spatial distribution of temperature evoked glomerular activity

Across all 11 investigated specimens we detected one to six thermosensitive glomeruli per antennal lobe. Among the 38 thermosensitive glomeruli we investigated in total, the majority (n = 36) respond with an increase in neuronal activity to cold stimuli (15°C) whereas only two glomeruli respond with an increase in neuronal activity to warm stimuli (30°C). Without any exception, all thermosensitive glomeruli are located in the medially oriented half of the antennal lobe. Only seven of the medial thermosensitive glomeruli are located at the upper 50 µm from the anterior surface of the antennal lobe whereas most of them are located at distances between 70 and 120 µm (n = 29), (ventral view, Fig. 3A). Among the 38 thermosensitive glomeruli six glomeruli are clustered medially (100-120 µm from the lateral margin, ventral view, Fig. 3A) at a distance of 80-110 µm along the anterior/posterior axis. This position corresponds to where we also found one of the three innervated glomeruli in our receptor neuron staining from sensilla coeloconica (ventral view, Fig. 3A, black spheres). Most often (23 times) we detected thermosensitive glomeruli in the dorsal part of the antennal lobe at a distance of approximately 20 to 80 µm from the dorsal margin (anterior view, Fig. 3A).

Functional imaging of the Sc target glomeruli

In a number of specimens, we could identify the “Gemini glomeruli” by focusing through the lower (posterior) part of the antennal lobe. We specifically investigated their potential temperature sensitivity in two examples. Our investigations revealed that the “Gemini glomeruli” are insensitive to temperature stimuli irrespective of whether warm (30°C) or cold (15°C) temperature stimuli are presented (Fig. 3B-E). In Figs. 3B, C, the two lateral “Gemini glomeruli” can clearly be distinguished, and they do not respond to stimulation with cold stimuli (15°C, green and gray ROIs, Fig. 3B, C, depth = 95 µm). In the medial part where the third target glomerulus of the Sc is located, we identified two glomeruli as candidates based on their relative position to the “Gemini glomeruli” (black and blue ROIs, Fig. 3B-E). The dorsal glomerulus (black ROI) is stained only weakly and does not exhibit any substantial activity changes upon temperature stimulation (15°C). However, the second candidate glomerulus (30µm ventrally, blue ROI) responds with stimulus-correlated oscillations in fluorescence (cold-sensitive glomerulus; blue ROI, *ΔF/F*≈ 10%).

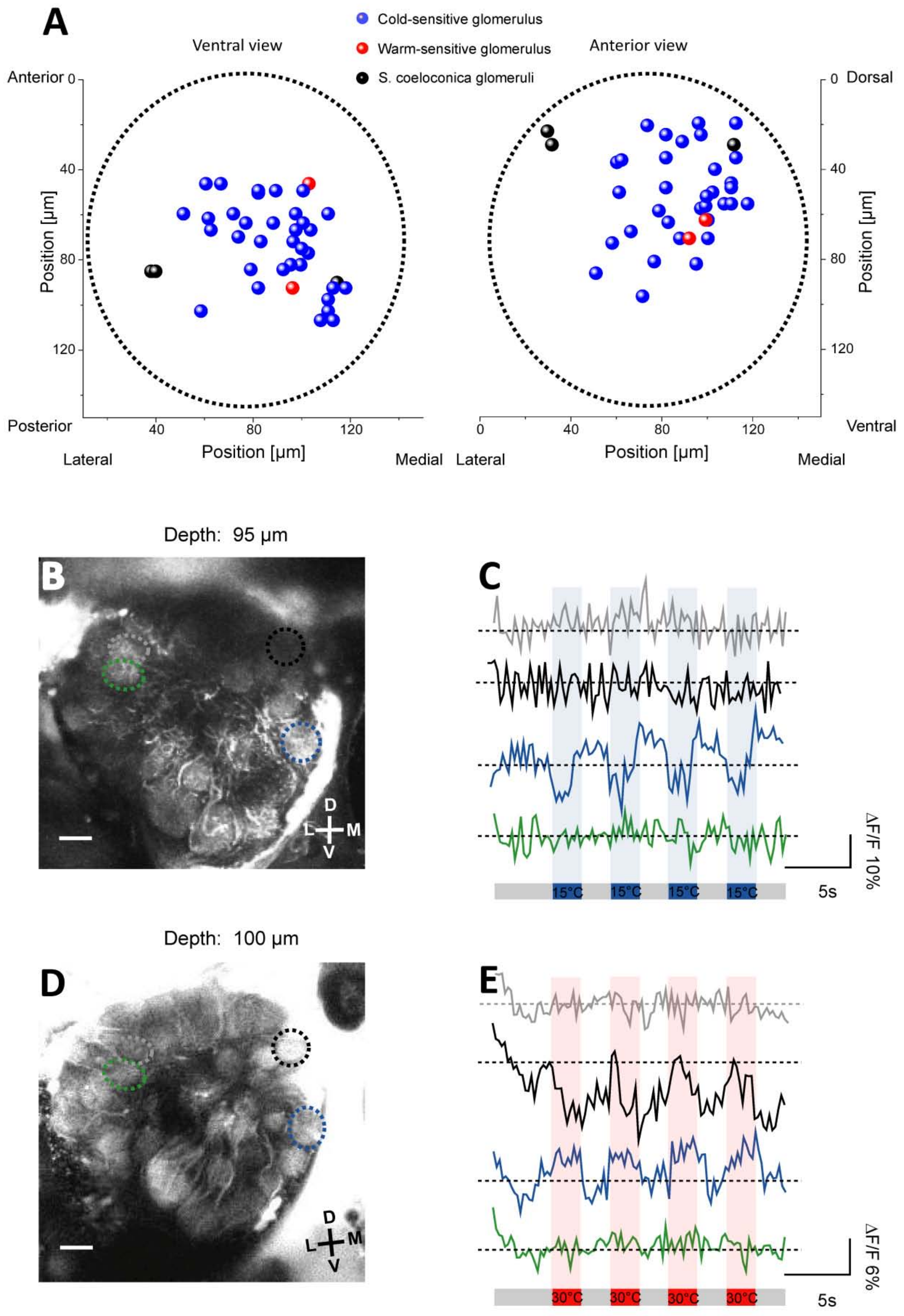
A
Cold-sensitive glomerulus
Warm-sensitive glomerulus
S. coeloconica glomeruli
Ventral view
Anterior view
Anterior
Posterior
Dorsal
Ventral
Position [µm]
Lateral
Medial
0
40
80
120
Depth: 95 µm
B
D
L
M
V
C
ΔF/F 10%
5s
15°C
Depth: 100 µm
D
E
ΔF/F 6%
30°C

Figure 3: Representation of thermal information in the antennal lobe
A: Localization of 36 cold-sensitive (blue) and 2 warm-sensitive glomeruli (red) recorded in 11 different antennal lobes. The three target glomeruli of the sensilla coeloconica (black spheres; see also Fig. 1B) are included in this mean antennal lobe that is based on the mean volume of all investigated antennal lobes (dotted lines). The two lateral “Gemini glomeruli” are located in a region where no thermosensitive glomeruli could be determined. **B-E:** The identified “Gemini glomeruli” are insensitive to temperature stimulation (gray and green ROIs), irrespective of whether cold- (**C**) or warm stimuli were applied (**E**). In the medial portion of this optical section we found between one and two thermosensitive glomeruli in both examples (in **B and C,** blue ROI: *ΔF/F*≈ 10%**;** in **D** and **E**, blue ROI: *ΔF/F*≈ 6% and black ROI, *ΔF/F*≈ 12%). D: dorsal, L: lateral, M: medial, V: ventral.

The second example also shows that the “Gemini glomeruli” are not responding to the temperature stimuli (Fig. 3D, E, 30°C, gray and green ROIs). The medial glomeruli, of which one supposedly is the third target glomerulus of the Sc receptor neurons, both respond to the warm stimuli in this example (blue ROI, *ΔF/F*≈ 6%, black ROI, *ΔF/F*≈ 12%). Based on its response characteristics, the ventral glomerulus (blue ROI) classifies as cold-sensitive glomerulus. The dorsal candidate glomerulus (black ROI, Fig. 3E) responds to the temperature stimuli as well but the phase relation of the temperature stimuli and the changes in neuronal activity did not allow a final conclusion about whether it classifies as warm- or cold-sensitive. Throughout all recordings, we never found glomeruli, which we respond to temperature stimuli, located in the lateral half of the antennal lobe where the two “Gemini glomeruli” are located (Fig. 3A, black spheres). The functional imaging data and the innervation pattern of the Sc provides correlative evidence that the temperature information received by these particular sensilla is sent to a medial glomerulus in the antennal lobe.

Discussion

In the present study we show that the thermosensitive sensilla coeloconica (Sc) project to the first olfactory neuropil, the antennal lobe. Within the antennal lobe, each of the three receptor neurons innervates a single glomerulus. Two of these three glomeruli are located side-by-side at a lateral position in the antennal lobe, while the third one is located medially. Using Ca^{2+} imaging of antennal lobe projection neurons, we identified up to six thermosensitive glomeruli within a single antennal lobe. We found both warm and cold-sensitive glomeruli, which all are located in the medial half of the antennal lobe. The two lateral “Gemini glomeruli” targeted by receptor neurons of the Sc are insensitive to temperature stimuli.

Based on the restriction of the thermosensitivity to the medial part of the antennal lobe and our Ca^{2+} imaging data of the ”Gemini glomeruli”, we thus conclude that temperature information received by the thermosensitive neuron of the Sc is represented in the medial target glomerulus.

Central projections from sensilla coeloconica

The selective staining of single Sc resulted in three labeled neurons, which all projected to the antennal lobe. This number corresponds to the number of receptor neurons identified in a previous study on the fine-structure of the sensillum (Ruchty et al., 2009). All three labeled neurons densely innervated a single glomerulus each. The three target glomeruli were located in the dorsal part of the ipsilateral antennal lobe at a distance between 80-110 µm from the anterior surface of the antennal lobe. The entire antennal lobe spans approximately 150 µm from anterior to posterior. In the cockroach, two types of thermosensitive sensilla send their axons to a single glomerulus at a similar (dorsal/posterior) region of the antennal lobe. Thus, the thermosensitive neurons (one per sensillum) of both sensilla converge in a single glomerulus (Nishikawa et al., 1995). Electrophysiological recordings in the same species revealed thermosensitive projection neurons and interneurons in the medial/posterior part of the antennal lobe (Fischer and Tichy, 2002; Nishikawa et al., 1995; Nishino et al., 2003; Waldow, 1975; Zeiner and Tichy, 2000). Studies on the central projections from Sc on the antennae of honeybees (Nishino et al., 2009) and carpenter ants (Nakanishi et al., 2010) suggest similar target regions, although due to the experimental approach used in these two studies, other target region e.g. the dorsal lobe cannot be excluded.

Based on the similar position of the target glomeruli of thermosensitive neurons in the leaf-cutting ant and the cockroach, as well as the putative region in honeybees and carpenter ants, we suggest that in insects in general antennal thermosensitive neurons terminate in specific glomeruli located in similar regions within the antennal lobe. These findings suggest a conserved representation of thermosensitive glomeruli in the antennal lobe across insect species.

Compared to olfactory sensilla, thermosensitive sensilla occur in low numbers on the antenna of insects (Dumpert, 1972; Hashimoto, 1990; Itoh et al., 1984; Renthal et al., 2003; Tominaga and Yokohari, 1982), and in the leaf-cutting ant *Atta vollenweideri* only around 12 Sc have been discovered on each antenna (Ruchty et al., 2009).

It has repeatedly been shown for olfactory receptor neurons that the size of a glomerulus is related to the number of receptor neurons terminating in it (Berg et al., 1998; Hansson et al., 1995; Kelber et al., 2010; Kleineidam et al., 2005). The present study shows that the three Sc-glomeruli have a similar volume compared to the adjacent glomeruli, which are targeted by receptor neurons from olfactory sensilla (Kelber et al., 2010). Based on the relation between glomerular volume and number of innervating olfactory receptor neurons, we expected a considerably smaller volume of the Sc-glomeruli. To explain our contrasting finding, we hypothesize that the Sc-neurons occupy a larger volume of their glomeruli because of the larger axon diameters we found, compared to olfactory receptor neurons. This seems to be a common property of receptor neurons associated with Sc in Hymenoptera, since similar results have also been shown recently for honeybees and carpenter ants (Nakanishi et al., 2010; Nishino et al., 2009). Large axon diameters result in fast information propagation and thus may facilitate rapid behavioral responses to e.g. temperature changes.

Functional analysis of temperature-sensitivity in the antennal lobe

Two-photon microscopy was applied in insects for physiological studies of the olfactory system in the fruit fly (Jayaraman and Laurent, 2007; Root et al., 2007; Sachse et al., 2007; Suh et al., 2004; Wang et al., 2003) and the migratory locust (Moreaux and Laurent, 2007) and of the visual system in the blowfly (Haag et al., 2004; Kalb et al., 2004; Kurtz et al., 2006; Spalthoff et al., 2010). In the present study, we use the two-photon microscope for the first time to acquire functional and morphological data of the antennal lobe in a social insect. The technique proved to be suited to study the spatiotemporal activity of projection neurons innervating single glomeruli and to reconstruct the glomeruli directly after the functional data have been acquired. The major advantages of this imaging technique, compared to conventional imaging, is the reduced dye bleaching and tissue damage and the enhanced penetration depth of the laser (Denk and Svoboda, 1997; Helmchen and Denk, 2005) which allow physiological access to the whole antennal lobe in this ant species. Due to the high *z*-resolution, the 3D reconstruction of single glomeruli and the whole neuropil based on *in vivo* data is possible in parallel to the physiological investigation. This is a major advantage compared to conventional methods like confocal laser scanning microscopy used to obtain morphological data of insect brains or brain compartments. Before data acquisition, the brains have to be dissected and treated with dehydrating chemicals and clearing solutions. Especially the dehydration of neuronal tissue leads to shrinking artifacts, which bias volume based data acquisition (Bucher et al., 2000).

Spatial representation of thermal information in the antennal lobe

We screened the whole antennal lobe volume in search for thermosensitive glomeruli by performing 10-15 consecutive framescans each shifted by 10 µm in *z*-axis. Although we did not quantify the number of glomeruli that were accessible, we are confident that we investigated the great majority of all glomeruli. However, because of the step size chosen (10 µm), it cannot be excluded that we may have missed to record from a small percentage of thermosensitive glomeruli during data acquisition. The mean diameter of glomeruli in the antennal lobe of *Atta vollenweideri* is about 6.30 µm (Kelber et al., 2010), and an adequate step size to securely capture all glomeruli would have resulted in an experimental protocol that exceeds the life time of the preparation. In addition to this experimental limitation, the variances in dye loading of projection neurons possibly resulted in an oversight of thermosensitive glomeruli. Our dye application resulted in some projection neurons loaded heavily with calcium sensitive dye and others which were stained only weakly. Exceedingly high dye concentrations in projection neurons lead to considerable buffering of Ca^{2+}, and weak staining may result in only marginal fluorescence changes. In both cases the applied temperature stimulus may well have elicited neuronal activity without causing a measurable change in fluorescence (example Fig. 3B, black ROI).

Number of thermosensitive glomeruli

The number of thermosensitive glomeruli in the antennal lobe of the leaf-cutting ant possibly reflects the number of different types of thermosensitive receptor neurons in different sensilla on the antenna. Thermosensitive neurons, which differ in their physiological response characteristics are necessary to assess different parameters of the thermal environment which is prerequisite for a variety of temperature-guided behaviors. It has recently been shown that the thermosensitive neuron housed in a Sc is physiologically adapted to detect rapid and minute temperature changes (0.005°C) (Ruchty et al., 2009; Ruchty et al., in revision).

Through sensory adaptation the sensitivity of the neuron is maintained over a wide range of ambient temperatures and a role of this sensillum during the thermal landmark learning in leaf-cutting ants has been suggested (Kleineidam et al. 2007; Ruchty et al., 2009; Ruchty M, in revision). Based on the stimulation protocol used in the present study we were not able to investigate the physiological characteristics of the projection neurons and to compare them with the receptor neuron physiology. It has to be shown whether the receptor neuron physiology predicts the projection neuron physiology on which they synapse.

Neurons that do not adapt as strong as the thermosensitive neurons of Sc, thereby providing the animals with information about the actual ambient temperature in their direct surrounding, have been described in antennal sensilla of the migratory locust *Locusta migratoria* (Ameismeier and Loftus, 1988; Waldow, 1970)*,* the cockroach *Periplaneta americana* (Nishikawa et al., 1992) and the honeybee *Apis mellifera* (Lacher, 1964). In ants, neurons with such non-adapting physiological characteristics are expected as well, since ants can asses and respond to steady temperatures with very high accuracy, e.g. during brood care (Roces and Núñez, 1989, 1995; Weidenmüller et al., 2009). Possible candidate sensilla to house thermosensitive neurons with that particular physiological characteristics are e.g. the sensilla coelocapitula which have been recently identified on the antenna of different ant species including leaf-cutting ants (Kleineidam CJ., unpublished data) and which house a thermosensitive neuron in honeybees (Yokohari, 1983). It is assumed that these putative antennal thermosensitive sensilla send their axons to the antennal lobe as well and thereby adding to the number of up to six glomeruli discovered per antennal lobe.

In 11 different individuals we found a total of 38 thermosensitive glomeruli with a maximum of six in a single antennal lobe. Most of the discovered glomeruli were cold-sensitive glomeruli, which respond with increasing neuronal activity upon negative temperature steps. In general, every neuron is, at least to some extend sensitive to temperature stimuli and increases its neuronal activity with increasing temperature. Based on this relation we conclude that the cold-sensitive glomeruli responded specifically to the temperature change, however unspecific temperature responses cannot be excluded for the two warm-sensitive glomeruli discovered in the present study. If the responses were indeed unspecific we would expected a considerably higher number of warm-sensitive glomeruli.

Apart from an unspecific thermosensitivity, there are several other possibilities which potentially result in warm-sensitive projection neurons. The most obvious explanation is that warm-sensitive receptor neurons occur in addition to the already discovered cold-sensitive ones on the antennae of the investigated species.

Until now, there is no data, which evidences warm-sensitive neurons on the antennae of the leaf-cutting ant (*Atta vollenweideri);* however, in a closely related species warm-sensitive neurons were discovered in an antennal sensillum (Kleineidam and Tautz, 1996). Future experiments have to show whether this is also the case in *Atta vollenweideri*.

Furthermore, the presence of warm-sensitive projection neurons as discovered in the present study not necessarily requires warm-sensitive receptor neurons in the periphery. In the cockroach, warm-sensitive projection neurons and interneurons were found although no antennal warm-sensitive neurons have been discovered so far (Fischer and Tichy, 2002; Nishikawa et al., 1995; Zeiner and Tichy, 2000). Fischer and Tichy 2002 speculated that the response of warm-sensitive projection neurons is the result of the antennal lobe network where the input from cold-sensitive receptor neurons to their target glomeruli may lead to a disinhibition of other glomeruli (Fischer and Tichy, 2002). Another possible explanation could be that the respective projection neurons are not at all driven by antennal neurons but rather excited via central thermosensitive neurons comparable to the warm-sensitive anterior cell neurons involved in thermoregulation in *Drosophila* (Hamada et al., 2008). Whether the recorded increase of neuronal activity in projection neurons to warm stimuli is the result of neuronal activity in a peripheral or a central thermosensitive neuron or whether the neuronal network within the antennal lobe triggers the response has to be shown in future experiments.

Processing of thermal information provided by sensilla coeloconica

The comparison of receptor neuron staining and Ca^{2+} imaging experiments let us to conclude that the thermosensitive neuron of the Sc innervates the medial of the three Sc-glomeruli. Two lines of evidence allow this conclusion. The screening of the antennal lobe for thermosensitive glomeruli clearly showed that this modality is processed within the medial part of the neuropil. The lateral part, in which two of the Sc-glomeruli are located, was insensitive to temperature stimulation in all specimens investigated (Fig. 3A).

Among the 11 investigated specimens we found six times cold-sensitive glomeruli in a medial position, in a region where also the medial Sc-glomerulus is positioned. Our conclusion is further supported by our functional imaging data of the two "Gemini glomeruli", which did not exhibit any sensitivity to thermal stimuli (Fig. 3B-E). Unfortunately, we could not identify the medial Sc-glomerulus unambiguously during the two-photon microscopy. Using the "Gemini glomeruli" as landmarks, we assume that one of the two thermosensitive glomeruli we identified in the medial part is the target glomerulus of the thermosensitive neurons from the Sc (Fig. 3B-E). Future experiments have to reveal which of the two medial candidates is indeed targeted by the thermosensitive neuron of the Sc.

The combination of the selective staining and the state of the art imaging technique enabled us to investigate the processing of temperature information within the antennal lobe and link the obtained results to a specific thermosensitive neuron which has been suggested to play a key role during thermal orientation in the leaf-cutting ant. It is now possible to investigate the underlying neuronal mechanisms of specific temperature guided behaviors as well as to study the role of the first olfactory neuropil in temperature processing in insects in general.

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VIII. Direction sensitivity of sensilla coeloconica to thermal radiation

Abstract

It has recently been shown that leaf-cutting ants are able to use thermal radiation as orientation cue outside their nest in the context of foraging. The antennae of *Atta vollenweideri* are equipped with thermosensitive sensilla of which the sensilla coeloconica were suggested as possible candidate to detect the underlying stimuli during that particular thermal orientation behavior. The sensilla are clustered at the antennal tip and have an extraordinary morphology with a sensory peg sunken in a pit, separated from the environment by a tiny aperture. In the present study we investigate whether the morphology of the sensilla serves as adaptation to detect thermal radiation (infrared radiation).

The sensory pegs of the sensilla coeloconica are oriented with similar angles within the cuticular pit, irrespective of the position of the sensilla in the apical cluster. Based on the equally oriented sensory pegs, we suggest that all sensilla coeloconica together function as one single unit. The eye-catching peg-in-pit morphology of the sensilla may separate the peg from side radiation which could result in a direction sensitivity of a single sensillum. This hypothesis was tested by stimulating the sensillum with thermal radiation from different angles. Single sensilla coeloconica are indeed direction sensitive and thus morphologically adapted to infrared detection. We found the highest sensitivity to thermal radiation emitted at an angle 60° towards the antennal tip. We suggest that both the equal peg orientation and the peg-in-pit morphology are functional adaptations to detect spatially distributed heated objects during thermal landmark orientation in *Atta vollenweideri* ants.

Introduction

Central place foragers such as social Hymenoptera possess well developed orientation abilities to successfully find their way to a food source and back to their nesting site. For orientation, these insects use a multitude of cues that can basically be divided into egocentric and geocentric cues. Egocentric cues play a major role during e.g. the assessment of the distance traveled by the insects. For instance, walking Hymenoptera like desert ants count their strides using proprioceptive information of mechanosensitive sensilla (Collett and Collett 2000; Wittlinger et al. 2006; 2007). Flying hymenoptera like honeybees, however, use visual odometry, driven by the optic flow experienced during flight to travel between food source and nest hive (Srinivasan et al. 2000). Apart from these egocentric cues, geocentric cues like landmarks, the earth's magnetic field or the sun's polarization pattern on the sky provide social insects with information about their current position relative to the nest (Banks and Srygley 2003; Collett et al. 1998; Collett et al. 1999; Collett 1996; Labhart 1986; Labhart and Meyer 2002; Rossel and Wehner 1986; Wehner et al. 1996).

Most ants, including the leaf-cutting ants use trail pheromones to travel between food sources and their nest hive (Hölldobler and Wilson 1990; Weber 1972). Additional geocentric visual cues provide the ants with directional information on the otherwise directionless trails (Vilela et al. 1987). Although olfaction is the dominant sense during orientation of ants, a recent study has shown that thermal radiation (infrared radiation) emitted by warm objects close to the foraging trails can serve as orientation cue in the leaf-cutting ant *Atta vollenweideri* (Kleineidam et al. 2007; Chapter IV). It is assumed that during this behavioral task, the ants assess the intensity of thermal radiation successively when scanning with the antenna (klinotaxis) or by comparing the intensity between their two antennae to orient towards the heated object (tropotaxis). In insects, tropotactic orientation based on thermal radiation is rarely described and has only been shown in Australian fire beetles and blood-sucking bugs. Fire beetles detect thermal radiation emitted from forest fires which they approach to encounter mates for reproduction (Evans 1964; Schmitz et al. 1997; Vondran et al. 1995). In blood-sucking bugs thermal radiation emitted from endothermic prey is both necessary and sufficient for host detection (Lazzari 2009; Lazzari and Núñez 1989; Schmitz et al. 2000).

To assess the position of the radiant source (forest fire, host or thermal landmark) specific adaptations of the thermosensory system in these insects are expected. Fire beetles of the genus *Melanophila* possess two abdominal pit organs, each containing up to 80 thermosensitive sensilla that are morphologically adapted to detect thermal radiation. Accessory structures of the sensilla change upon absorption of thermal radiation resulting in a photomechanic transduction (Müller et al. 2008; Schmitz et al. 2007; Schmitz and Bleckmann 1997; Vondran et al. 1995). *Triatomine* bugs and leaf-cutting ants utilize their antenna to locate the position of the radiant source (Flores and Lazzari 1996; Kleineidam et al. 2007; Lazzari 2009). The bugs probably measure a thermal gradient along their antenna to determine the distance to their host (Lazzari 2009) whereas saccadic antennal movements are probably used to assess the size of the radiant object (Flores and Lazzari 1996). In contrast, ants establish non-saccadic antennal movements when scanning their surrounding for radiant objects during orientation (Kleineidam et al. 2007; Ruchty et al. 2009; Chapter IV).

In both cases, antennal thermosensitive sensilla are expected to receive the underlying thermal stimuli. The thermosensitive sensillum coeloconicum (Sc) was identified on the antenna of *Atta vollenweideri* and extensively studied (Ruchty et al. 2009). Sc are peg-in-pit sensilla which house three receptor neurons of which one is thermosensitive (cold-sensitive) (Ruchty et al. 2009). The thermosensitive neuron is extremely sensitive to temperature transients and can resolve temperature stimuli at a high temporal resolution, a precondition to perform the klinotactic orientation behavior (Ruchty et al., in revision; Chapter VI). The Sc are clustered at the antennal tip and the sensory peg is deeply sunken in a cuticular chamber connected to the environment via a tiny aperture (Ruchty et al. 2009). The aperture may shield the sensory peg from side radiation resulting in a direction sensitivity of a Sc. This idea is supported by the clustered arrangement and the eye-catching morphology of the sensilla and a functional significance during the detection of thermal radiation has been suggested (Ruchty et al. 2009).

Here we investigate whether the Sc may function as one single unit by measuring the orientation of the sensory peg with respect to the flagellar axis of the antenna. In order to test whether the peg-in-pit morphology of the Sc results in direction sensitivity of a single sensillum, we selectively stimulated the thermosensitive neuron with thermal radiation from various angles around the sensillum and analyzed its response to the respective stimulation angle.

Materials and Methods

Animals

All experiments described in the present study were accomplished with media workers of the leaf-cutting ant *Atta vollenweideri* obtained from a laboratory colony. The colony was reared at the Biozentrum, University of Würzburg at 25°C and 50% relative humidity in a 12h/12h photoperiod, and fed mainly with leaves of privet (*Ligustrum vulgaris*) and dog rose (*Rosa canina*).

Orientation of the sensory peg

To investigate how the sensory pegs of the sensilla coeloconica (Sc) are oriented with respect to the flagellar axis of the antenna, we used scanning electron microscopy (SEM). Prior to the morphological measurements, the apical antennomere was sectioned obliquely with a razor blade followed by tissue removal with warm 3M KOH-solution and 10 min cleansing in an ultrasonic bath. The specimens were then dehydrated in a graded ethanol series (50%, 70%, 80%, 90% 95% and 100%; 10 min each) and aceton (100% for 10 min), and critical point-dried (CPD030, Balzers Union, Liechtenstein). Subsequently, the antennomeres were glued onto SEM-supports, gold-palladium-coated (MED010, Balzers Union, Liechtenstein) and examined with a SEM (DSM962, Zeiss, Germany).

In order to describe the orientation of the sensory peg of the Sc within the antenna, the inclination angle and the rotation angle of the cup-shaped base of 5 to 7 Sc in 7 specimens were measured (Fig. 1C/D). For this purpose, the cup-shaped base of the sensillum was positioned such that the view was perpendicular to its surface. Since the peg stands upright in the base of the sensillum, this position describes in which direction the peg is oriented. By tilting the specimen to a perpendicular view of the flagellar axis, the inclination angle was measured. The rotation of the flagellar axis with respect to the body could not be assessed, therefore only a relative measure of the rotation angle was measured. This was done by measuring how much a given sensillum has to be rotated around the flagellar axis to meet the orientation of one reference sensillum. The variances of inclination and rotation angles within each specimen were assessed by calculating the standard deviation (SD) of both measures. The SD values of inclination- and rotation angles were compared across 7 specimens using the t-test.

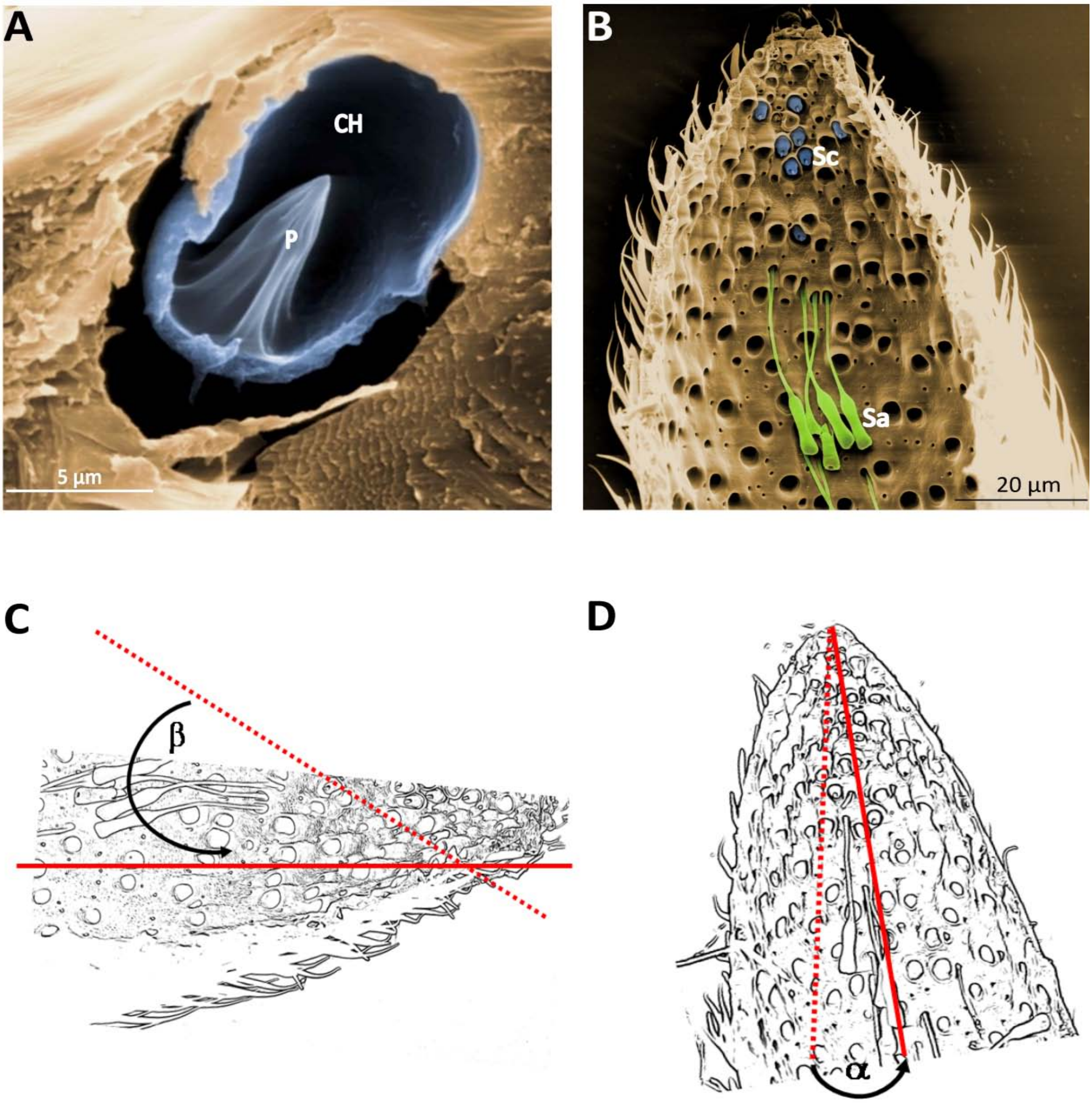

Figure 1: Morphology of sensilla coeloconica and quantification of their peg orientation
A: SEM image of a longitudinally sectioned Sc. The sensory peg (P) is deeply embedded in the cuticular chamber (CH) (modified from Ruchty et al. 2009). The aperture is not visible in the present example. **B:** Internal view of the last antennomere with the clustered sensilla coeloconica (Sc, blue) and sensilla ampullacea (Sa, yellow). **C:** The inclination angle of the sensory peg was measured by tilting the specimen from the perpendicular view at a cup-shaped base of a sensillum to a perpendicular view of the flagellar axis. **D:** To investigate the peg orientation in terms of rotation angle we measured how much a given sensillum has to be rotated around the flagellar axis to meet the orientation of one reference sensillum.

Direction sensitivity

In order to investigate whether the peg-in-pit morphology of the Sc results in direction sensitivity of the thermosensitive neuron, we accomplished extracellular recordings of single Sc. The recording technique is similar to the one introduced in the chapters V and VI. Workers were mounted on a plastic holder with adhesive tape and the scapus and the flagellum of each antenna were glued onto the holder with white-out correction fluid (Tipp-Ex, Bic, France). Under visual control and at a magnification of 390x (Leitz microscope equipped with NPL-Fluotar L25/0.35, Leitz, Germany), an electrolytically sharpened tungsten electrode was superficially inserted into the cuticle next to a single Sc using a manual micromanipulator (HS-6, Märzhäuser, Germany). In order to achieve a good signal to noise ratio, the reference electrode was inserted deep into the flagellum in close vicinity of the recording electrode. Recordings were band-pass filtered (60 Hz-3 kHz) and amplified 1000x (Neuroprobe Amplifier 100, A-M-Systems, USA and VBF8, Kemo, Great Britain). The electrical noise was significantly reduced using an additional digital filter (Humbug, Quest Scientific, Canada). Data were digitized at a sampling rate of 10 kHz and recorded on a PC using custom-made software (LabView, National Instruments Corporation, Austin, Texas, USA).

For stimulation of the thermosensitive neuron we used a miniature Peltier element (surface 3 mm^2) mounted on a modified micromanipulator which allowed us to position the Peltier from 0° to 180° longitudinally to the antenna (longitudinal angle) and from 0° to 360° around the preparation (rotation angle) at a distance of 3 cm. The Peltier was driven with oscillating voltage (+- 1.5V) at a frequency of 0.2 Hz thereby generating oscillating surface temperature with an amplitude of 4°C (+-2°C around ambient temperature). At each stimulation angle we stimulated with 10 temperature oscillations (each oscillation with 2.5 s warm and cold phase, respectively; 50 s total recording time).

At the beginning of the recording sessions we positioned the Peltier in a rectangular position above the preparation (90° longitudinal angle) at 30° rotation angle. From this angle as starting point we conducted recordings from proximal (180°) to distal (0°) along the flagellar axis (step size of 30°). Subsequently, the Peltier was moved 30° further in rotation angle and again data was obtained along the longitudinal axis.

To exclude convective heat as possible stimulus during the experiments, a continuous airflow (flow rate 1.5 l/min) was blown via a glass tube (2 cm in diameter, at a distance of 1 cm from the antenna) over the preparation. The continuous airflow was channeled through a custom made heat exchanger (see chapters V, VI or VII) to keep the ambient temperature at a value of 24°C.

Data analysis

The recording electrode picked up neuronal activity (spikes) of up to three neurons simultaneously. The thermosensitive neuron could be easily distinguished by its larger amplitude and we used spike detection software based on voltage threshold written in LabView (National Instruments Corporation, Austin, Texas, USA) to analyze the neuronal activity of the thermosensitive neuron. To calculate the sensitivity of the thermosensitive neuron at various stimulation angles, we separated the recorded potentials in two classes: 1) spikes during warm-stimulation ($\Delta T= +2°C$) and 2) spikes during cold-stimulation ($\Delta T= -2°C$). We calculated the mean activity during these two phases for the 10 repeated stimulation cycles (temperature oscillations). The ratio between the mean activity during cold and warm phase was used as a measure for sensitivity of the thermosensitive neuron to that particular stimulation angle. We calculated the sensitivity for each stimulus cycle and plotted the results as the mean of 10 repeated measurements for each angle along the respective longitudinal axis. In each specimen, the axis containing the highest sensitivity was analyzed statistically using an ANOVA (LSD post-hoc test, $p < 0.05$). We subsequently pooled the sensitivity values of these longitudinal axes across all investigated specimens ($n= 13$) and analyzed the results with an ANOVA (LSD post-hoc test, $p < 0.01$). To visualize the sensitivity subject to the stimulation angle, we normalized the calculated values and plotted color and length coded vectors using the 3D rendering freeware POVRAY. Based on the known location of the Sc on the antennae of the ants (lateral/ventral; chapter V) we estimated where on the antenna the vectors are supposedly located in a freely moving animal.

Results

Peg orientation

The sensilla coeloconica (Sc) are clustered at the tip of the apical antennomere (Fig. 1B; see also chapter V). The sensory peg of Sc is completely embedded in a cuticular chamber (Fig. 1A) and only a tiny aperture connects it to the environment. The peg (its tip) is oriented directly towards the aperture with the same orientation angles of all Sc pegs of the apical cluster. The mean inclination angle of 5 to 9 Sc within each cluster was 41° and has a low standard deviation which was used to determine the variation in this measure. The mean standard deviation was 3.4° (n = 7) indicating that all Sc of the apical cluster are orientated with similar inclination angles. For the rotation angle, only a relative measure could be assessed because the rotation angle between flagellum and the ant body could not be measured. The mean standard deviation of rotation angles was 7.9° and significantly larger than that of the inclination angles (t-test, n = 7, $p < 0.05$; Fig. 2). Thus, all Sc of the apical antennomere are oriented with a similar inclination angle (41°) frontally, along the flagellar axis and with a larger variance to the side (lateral or latero-ventral).

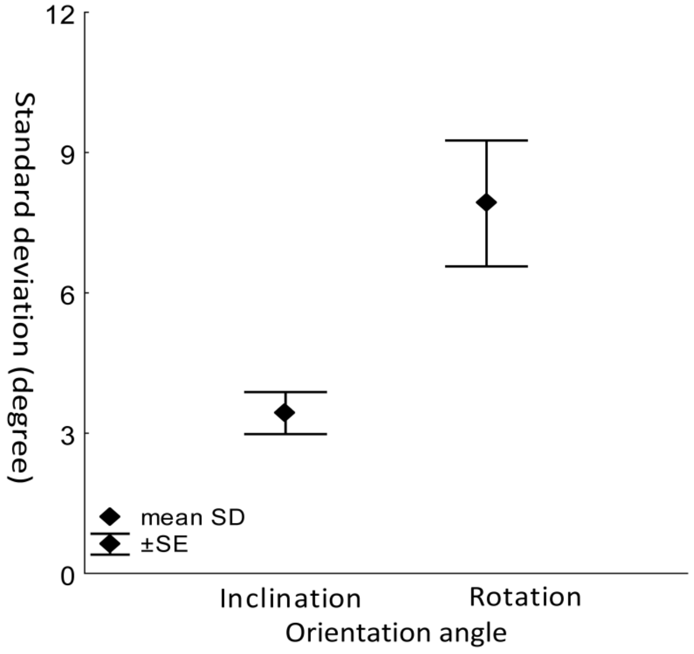

Figure 2: Peg orientation
Mean variance (standard deviation, SD) of inclination- and rotation angle measured for 5 to 9 sensilla coeloconica in 7 different apical antennomeres. The variance of the inclination angle (which is 41° to the flagellar axis) is significantly lower than the variance of the rotation angle (t-test, n = 7, $p < 0.05$). Whiskers indicate the standard deviation of the mean standard deviation.

Direction sensitivity

The thermosensitive neurons of the Sc are extremely sensitive to thermal radiation. The neurons' activity precisely follows the temperature oscillation of the Peltier element and a stimulus amplitude of only 4°C results in a change of neuronal activity of up to 260% (Fig. 3A). The extent to which the thermosensitive neuron changes its neuronal activity depends on the stimulation angle. This results in a direction sensitivity of a single sensillum. The highest sensitivity values are found at angles towards the antennal tip. In the example of Fig. 3B each vector visualizes the stimulation angle, and the color and length of the vectors code for the neurons' sensitivity (with red as the highest sensitivity). In half of the investigated specimens (n = 6), the highest sensitivity was found along the flagellar axis (Fig. 3C). In the other specimens the highest sensitivity was found along a longitudinal axis shifted between -30° and +30° from the flagellar axis. In the example of Fig. 3C, the thermosensitive neuron is most sensitive when stimulated with a longitudinal angle of 30° from distal, with a significantly higher sensitivity compared to the other angles investigated (ANOVA, LSD post-hoc test, $p < 0.05$, Fig. 3D). To investigate whether the different Sc establish the highest sensitivity towards stimuli from similar longitudinal angles we pooled the data of the longitudinal axes containing the angle of highest sensitivity of all 13 individuals analyzed. We found a significantly higher sensitivity towards the distal tip of the antenna at an inclination angle of 60° (ANOVA, LSD post-hoc, $p < 0.01$, Fig. 4).

Discussion

The peg-in-pit morphology and the similar peg orientation and the highest sensitivity of the thermosensitive neuron to a certain angle, that the sensilla coeloconica (Sc) are direction sensitive to thermal radiation and functionally one unit. We thus conclude that the Sc are radiant heat receptors which are morphologically adapted to detect spatially distributed radiant objects. We propose that the Sc play a prominent role during the thermal orientation behavior of *Atta vollenweideri.*

Peg orientation

The Sc are clustered on the apical antennal segment and it was suggested that the total of around 12 sensilla function as one unit (Ruchty et al. 2009). The similar peg orientation of the Sc we describe in the present study supports this hypothesis. Although the sensilla are located at a region where the antennal tip is curved, the inclination angle of the measured sensilla has a variance of less than 4°. This indicates that the orientation of sensory pegs does not follow the curved antennal surface and that the chamber is embedded more or less oblique into the antennal cuticle, depending on the distance to the antennal tip. Further evidence for our functional-unit-hypothesis was recently found when studying the central projections of the three receptor neurons of the Sc. Independent of the position of the sensillum in the apical cluster, the thermosensitive neurons converge in a single glomerulus (Ruchty et al., in revision; Chapter VII). Such convergent stimulus detection is expected to result in an improved signal to noise ratio and enables the detection of minute stimuli in a noisy environment (Anton and Tichy 1994).

Functional morphology

The present study revealed that single Sc are direction sensitive to thermal radiation. The thermosensitive neuron of Sc is most sensitive to stimulation from distal angles. Based on the peg orientation, which was at a mean inclination angle of 41°, we expected the highest sensitivity of the thermosensitive neuron to thermal radiation from a similar angle. However, our direction sensitivity experiments revealed the highest sensitivity of the thermosensitive neurons at a longitudinal angle of 60° (pooled data, Fig. 4, $n = 13$). A possible explanation for this discrepancy between morphology and physiology could be that the cuticle does not shield the peg from scattered radiation as completely as hypothesized in the introduction of the present chapter. For thermal radiation at a wavelength of 3 µm, a penetration depth of approximately 6 µm into the cuticle of fire beetles was estimated (Schmitz et al. 2007). It is assumed that the cuticle of ants has similar properties and thus the thin cuticle covering the chamber of the Sc would only marginally absorb the applied radiation.

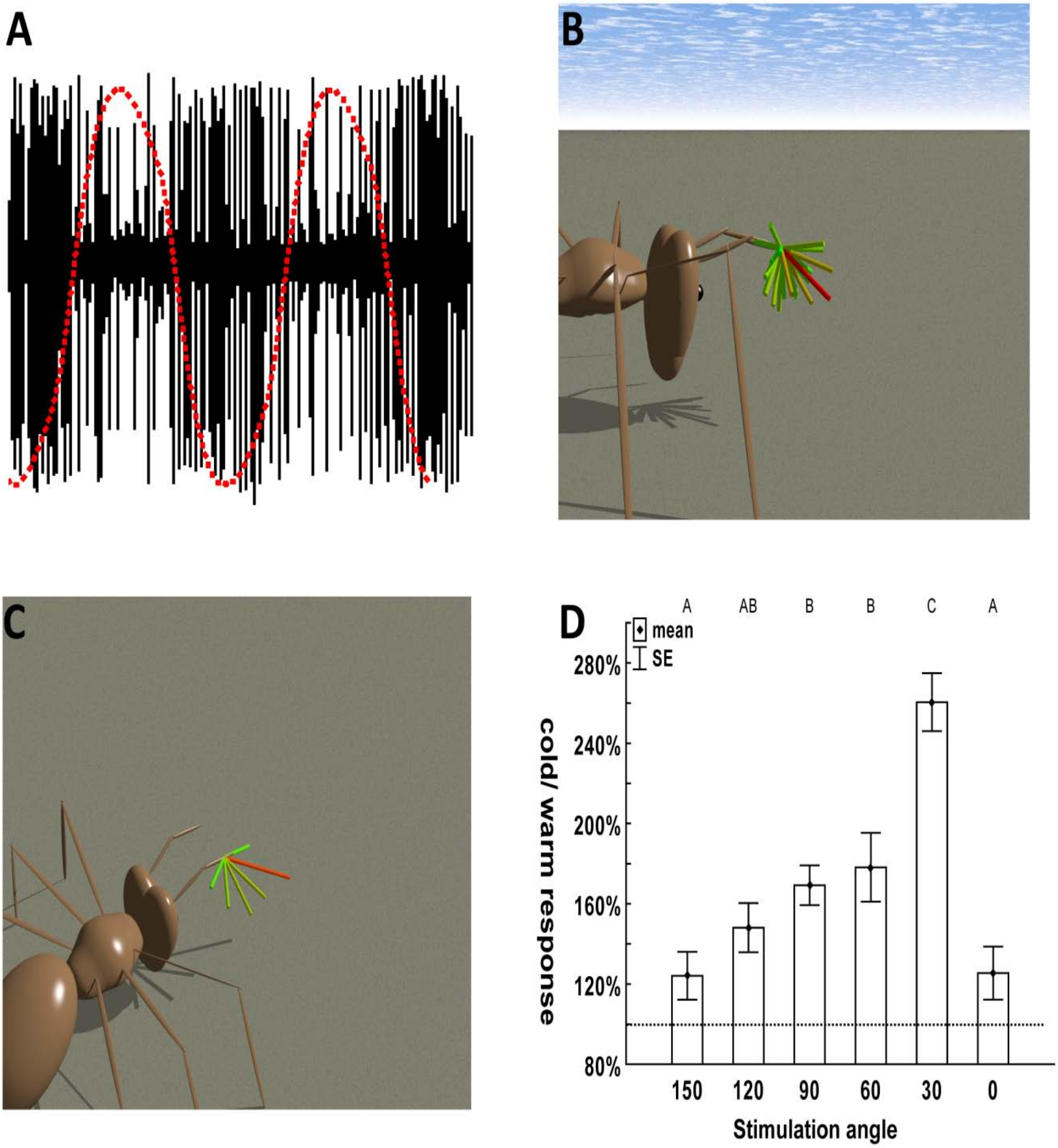

Figure 3: Direction sensitivity of single sensilla coeloconica
A: In order to investigate the direction sensitivity of the sensilla coeloconica, the neuron was stimulated with thermal radiation emitted by a Peltier element positioned at various angles around the sensillum. The thermosensitive neurons followed the applied temperature oscillations (0.2 Hz) and the amplitude (4°C) resulted in precise changes of neuronal activity. **B:** Color and length of the vectors indicate the sensitivity of the thermosensitive neuron at the particular angle, with red as the highest sensitivity. **C:** The resulting vectors are shown for the flagellar axis where the highest sensitivity was measured. **D:** Repeated stimulation revealed a statistically significant difference between the stimulation angles, with the highest sensitivity 30° towards the antennal tip. (ANOVA, LSD post-hoc test, p=0.05, single sensillum).

Based on the orientation angle and the putative permeability of the antennal cuticle, the largest surface of the sensory peg should be irradiated by thermal radiation emitted at an angle between 60° and 90° along the longitudinal axis. This expectation correlates with our physiological data where the highest sensitivity of the thermosensitive neurons was found at 60° followed by 90° (right above the preparation). As mentioned in the result section, the highest sensitivity was most often measured along the flagellar axis (n = 6), however some variance in terms of the rotation angle of the longitudinal axes occurred. These differences could partly be the result of the higher variance in rotation angle of the sensory pegs (S.D.: 7.9°). The results on the rotation angles in addition depend on how accurately the flagellum is positioned and turned during the preparation of the specimen. Unfortunately, it was impossible to position the flagellum exactly parallel to the plexiglass holder. Already minor variations during the fixation of the antenna could have had a considerable affect on the obtained results of the direction sensitivity experiments. To counteract this variance only the axis containing the highest sensitivity was chosen for data analysis.

The morphology of the Sc resembles the pit organs of snakes "en miniature"

Probably the best known example for a functional morphology similar to the Sc is known in snakes. Boids and pit vipers are equipped with pairs of pit organs (Newman and Hartline 1982), which the snakes mainly use to receive thermal radiation emitted by endothermic prey during foraging (Buning et al. 1981a; b) but also for thermoregulatory purposes (Krochmal and Bakken 2003). The pit organs are equipped with thermosensitive neurons (Bullock and Diecke 1956) with a similar extreme sensitivity as the neurons located in the Sc of leaf-cutting ants (Ruchty et al., in revision; chapter VI). The receptor neurons located in both the pit organ and the Sc are primarily temperature receptors that respond to a local temperature change in the pit membrane and the peg surface, respectively. Accessory structures of both the pit organ and a single Sc tune the thermosensitive neurons to the detection of thermal radiation. In the sensilla, the sensory peg is sunken in a pit that is separated from the environment by a tiny aperture (Ruchty et al. 2009). The cuticle in which the peg is embedded partially shields the peg from scattered thermal radiation which results in direction sensitivity of each sensillum (this study).

The pit organs in snakes have a larger aperture and the thermal environment is imaged topographically onto the pit membrane and each thermosensitive neuron is sensitive to a temperature stimulus from a certain direction (Bakken and Krochmal 2007). In leaf-cutting ants each Sc is direction sensitive and the associated thermosensitive neuron responds dependent on the stimulus angle. All Sc detect thermal radiation from the same direction, thus no topographical detection is expected based on the arrangement of sensilla to each other.

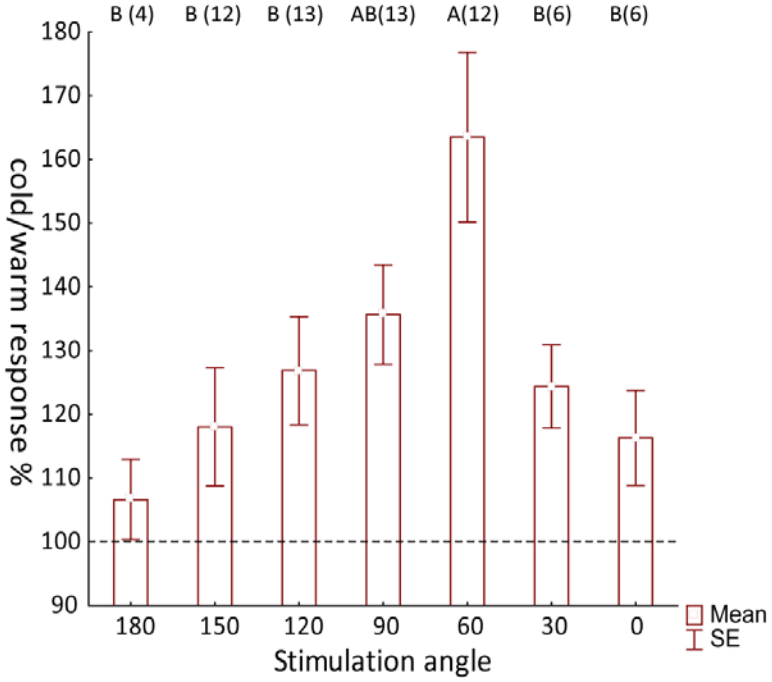

Figure 4: Pooled sensitivity
Pooled data of all thermosensitive neurons (number of neurons shown in brackets). The investigated neurons are most sensitive to stimulation at an angle of 60° towards the antennal tip. (ANOVA, LSD post-hoc test, p=0.01).

The common morphology of both, sensillum and pit organ enables the animals to assess the position of a radiant source and use the gained information to e.g. orient in their thermal environment. During the thermal orientation behavior of individual ants, we observed rapid antennal movements and it was suggested that the movements result in rapid temperature transients at the thermosensitive neurons. In chapter VI it was shown that the thermosensitive neuron associated with the Sc can cope with these rapid temperature transients. Based on the physiological data and the functional morphology of the Sc, we conclude that the Sc play a key role in detecting spatially distributed objects during the thermal orientation of leaf-cutting ants (Kleineidam et al. 2007).

Although being equipped with direction sensitive Sc and thermosensitive neurons which fulfill the physiological requirements, it is still puzzling how the ants actually determine the position of radiant objects during thermal orientation. As the ants move their antenna they have to integrate thermal information received by the Sc with the actual position of their antennae to accurately localize a radiant object.

To date it is unknown how leaf-cutting ants integrate proprioceptive information with thermal information and at what level of the central nervous system the processing of these two sensory modalities takes place. Mechanosensitive sensilla at the joint between head and scapus of the antenna serve as proprioceptive organ to assess the position of the antennae and its movements (Ehmer and Gronenberg 1997). It has been shown that the neurons associated with these sensilla exclusively project to the dorsal lobe in the ant brain. However, the thermosensitive neurons of Sc innervate a single glomerulus in the dorsal part of the first olfactory neuropil, the antennal lobe. It is thus assumed that thermal and proprioceptive information is processed in higher brain centers. Candidate neuropils are, for instance, the mushroom bodies and the lateral protocerebrum where the thermal information is transmitted via antennal lobe projection neuron tracts (Nishino et al. 2003; Zeiner and Tichy 2000; chapter VII).

Thermosensitive peg-in-pit sensilla like the Sc of *Atta vollenweideri* are widespread in insect species (Altner and Loftus 1985; Tichy 1979; Waldow 1970; Yokohari 1983) although only few have the same eye-catching morphology with a deeply sunken peg and a tiny aperture separating the peg from the environment. Sc with a similar morphology are known in honeybees, desert ants and carpenter ants (Ruchty M., unpublished data). The Sc in these insects have a similar morphology compared to the Sc in the leaf-cutting ants. It is thus assumed that the sensilla could be functionally adapted to the detection of thermal radiation as well and serve a similar purpose during thermal orientation. Until now it is not known whether the detection of spatially distributed radiant objects is a general ability of social insects. First evidence for this hypothesis is provided by classical conditioning experiments, which show the ability of honeybees to learn the association between thermal radiation and a food reward in the context of foraging (Hammer et al. 2009; Heran 1952; Menzel et al. 2001). The present study discovered a novel sensory system in leaf-cutting ants which the animals can use for orientation. Future investigations have to show whether Sc are morphological infrared detectors across Hymenoptera or even across insects in general.

IX. Outlook

The present thesis provides an in-depth analysis of the sensory basis of the thermal orientation behavior in the leaf-cutting ant *Atta vollenweideri*. In chapter IV it was shown that leaf-cutting ants are able to learn the association between a food reward and a heated object, which can be used as thermal landmark for orientation. Although the ants established the thermal orientation behavior in the context of foraging, the actual role of the thermal landmark learning is still unclear. Leaf-cutting ants are central place foragers and use an elaborated trail-pheromone system to travel between food sources and their nest hive (Hölldobler and Wilson 1990; Weber 1972). The ability to detect thermal radiation as orientation cue in the same context might serve as e.g. backup system in situations when the trail-pheromone is absent. To determine their position relative to their nest the ants may then use discontinuities in their thermal environment as geocentric landmarks.

The morphological and the physiological investigations presented in the chapters V and VI revealed that, in addition to the investigated thermosensitive neuron (cold-sensitive neuron), two other receptor neurons with unknown sensory modality are associated with the sensilla coeloconica. The fine-structure of the sensory peg of the sensilla indicated a chemosensitivity of those particular neurons (chapter V). In the migratory locust (*Locusta migratoria*) it has been shown that apart from a thermosensitive neuron (Ameismeier and Loftus 1988) the same sensillum houses two chemosensitive neurons which respond to plant odors (green leaf volatiles), and thus a role of the sensillum coeloconicum in the context of foraging was suggested (Boeckh 1967; Kafka 1970).

Future electrophysiological investigations have to show whether the associated neurons of the sensilla coeloconica in the leaf-cutting ant respond to plant odors as well. If this is the case, the sensilla coeloconica would be adapted to receive foraging specific stimuli (leaf volatiles) in the context of the thermal environment. This could serve as adaptation to e.g. locate sun exposed leafs or trees which have been shown to be highly palatable for the ants and which are preferred by the workers during foraging (Folgarait et al. 1996).

Based on the extraordinary morphology of the sensilla coeloconica and the physiological characteristics of the associated thermosensitive neuron described in chapters V, VI and VIII, a main conclusion of this thesis is that the sensilla coeloconica are adapted to the detection of spatially distributed radiant objects.

The morphology of this sensillum type is very similar in other Hymenopteran species like honeybees. Based on the conserved property of the sensilla coeloconica across social insects, one can speculate that the sensilla serve a similar purpose during thermal orientation in these species as well. However, to unambiguously link the morphology and the physiology to the thermal orientation behavior, further experiments have to evidence whether the sensilla are necessary and sufficient for a behavioral response. A possible approach to address this question is to block or knock-out the function of this sensillum type specifically by e.g. covering the sensilla with radiation absorbing material. The selective blocking of the sensilla is expected to impair the orientation ability when the ants are confronted with radiant objects in the test situation after the classical conditioning.

The extreme sensitivity and the extraordinary response dynamics discovered in the thermosensitive neuron associated with the sensilla coeloconica indicate a fast stimulus transduction. In comparison to fire beetles, the thermosensitive neurons in leaf-cutting ants are almost twice as fast (Kreiss et al. 2007) (see chapter VI for details). Over the last couple of years it has been repeatedly shown that temperature stimuli are transduced via ion channels of the transient receptor potential (TRP) family, which are embedded in the terminals of the afferent fibers of mammals or insects. These type of receptors are highly conserved across animal species (McKemy 2007). In general, 3 TRP families are of particular interest as thermo-receptors: The vanilloid TRP channels (Caterina et al. 1997; Jordt and Julius 2002; Smith et al. 2002), the melastatin TRP channels (McKemy et al. 2002; Peier et al. 2002) and the ankyrin TRP channels. In *Drosophila,* behavioral and genetic data demonstrate that a TRP channel, orthologous to the ankyrin 1 TRP channel (TRPA1) in mammals, plays a major role during thermosensation and nociception (Al-Anzi et al. 2006; Tracey et al. 2003). Just recently, the same channel (TRPA1) was verified in the sensilla coeloconica of the mosquito *Anopheles gambiae* (Wang et al. 2009). As the molecular entities of thermoreception are highly conserved across species, it is suggested that a TRP channel is expressed in the sensilla coeloconica of leaf-cutting ants as well. A helpful molecular tool to analyze whether this assumption is the case could be the *in situ* hybridization technique that, for example, was used by Wang et al. 2009. With this technique it is possible to discover all neurons in the antennae that express TRP channels and thus serve a potential thermosensitive function.

Chapter VII provides first details on the representation and processing of thermal information in the central nervous system of leaf-cutting ants. Based on the results obtained in the present thesis, it is now possible to link the receptor physiology of a specific thermosensitive neuron to its central processing within a single glomerulus in the antennal lobe. An open question is, whether the physiological characteristics of the projection neurons reflect the characteristics of the receptor neurons that synapse on them. It is of particular interest whether the number of thermosensitive glomeruli predicts the number of thermosensitive neuron types in the periphery to obtain a comprehensive knowledge about where temperature is received and later processed in the central nervous system.

A surprising result of chapter VII is that temperature information is processed along the olfactory pathway, form antennal receptors via the antennal lobe towards the mushroom bodies. Several studies in vertebrates (Mamasuew et al. 2008; Schmid et al. 2010) and invertebrates (Fischer and Tichy 2002; Hamada et al. 2008; Nishikawa et al. 1995; Waldow 1975; Zeiner and Tichy 2000) revealed similar results in the first olfactory neuropil. In cockroaches it has been shown that the temperature information is relayed to the mushroom bodies (Nishino et al. 2003; Zeiner and Tichy 2000). These findings indicate a putative co-processing of olfactory stimuli with thermal information. An interesting question to address is whether the perception of odors is influenced by e.g. the ambient temperature in the leaf-cutting ant, as it has recently been shown in *C. elegans* (Adachi et al. 2008). A possible experimental approach is, to analyze the behavioral response of the ants to odor stimuli at different ambient temperatures and the comparison of the neuronal activity in antennal lobe projection neurons upon odor stimulation at varying ambient temperatures.

With the integrative approach of the present thesis, using different techniques from electron microscopy via electrophysiology to a state of the art imaging technique, one can now better understand how temperature as sensory modality is received and processed on various levels from behavior to the central nervous system. This thesis will lead to a better understanding of the thermal sense and its biological significance in social Hymenoptera.

X. Literature

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XII. Scientific Publications

Lopez-Legentil S., **Ruchty M**., Domenech A. and Turon X. (2005):

Life cycles and growth rates of two morphotypes of *Cystodytes* (Ascidiacea) in the western Mediterranean.
Marine Ecology-Progress Series **296**, 219-228

Kleineidam C. J., **Ruchty M**., Casero-Montes Z. and Roces F. (2007):

Thermal radiation as a learned orientation cue in leaf-cutting ants (*Atta vollenweideri*).
Journal of Insect Physiology **53**, 478-87

Ruchty M., Romani R., Kübler L. S., Ruschioni S., Roces F., Isidoro N. and Kleineidam C. J. (2009):

The thermo-sensitive sensilla coeloconica of leaf-cutting ants (*Atta vollenweideri*).
Arthropod Structure Development **38**, 195-205

Ruchty M., Roces F. and Kleineidam C. J.:

Detection of minute temperature transients by thermo-sensitive neurons in ants
Journal of Neurophysiology (in review)

Ruchty M., Helmchen F., Wehner R. and Kleineidam C. J.:

Representation of thermal information in the antennal lobe of ants
Frontiers in Behavioral Neuroscience (in review)

Kleineidam C. J., Roces F. and **Ruchty M.:**

Infrared sensing in ants
(in preparation)

XIII. Conference contributions

Ruchty M., Romani R., Roces F. and Kleineidam C. J. (2007):
“Structure and function of a cold receptor in the leaf-cutting ant *Atta vollenweideri”*
7th *Meeting of the German Neuroscience Society*, Göttingen, Germany (**poster**)

Ruchty M., Romani R., Kübler L. S., Roces F. and Kleineidam C. J. (2007):
“Thermal orientation behavior and sensory physiology in the leaf-cutting ant *Atta vollenweideri”*
8th *Congress of the International Society for Neuroethology*, Vancouver, Canada (**poster**)

Ruchty M. and Kleineidam C. J. (2008):
“Infrared sensing in leaf-cutting ants”
19th *Neuro-DoWo*, Saarbrücken, Germany (**talk**)

Kleineidam C. J. and **Ruchty M.** (2008):
“Infrared sensing in leaf-cutting ants (*Atta vollenweideri*)”
XXIII *International Congress of Entomology*, Durban, South Africa (**talk**)

Ruchty M., Roces F., Kübler L. S. and Kleineidam C. J. (2008):
“Thermo-sensitive sensilla coeloconica and their adaptation to infrared reception in leaf-cutting ants”
6th *Forum of European Neuroscience*, Geneva, Switzerland (**poster**)

Ruchty M., Roces F. and Kleineidam C. J. (2009):
“Infrared sensing in ants”
8th *Meeting of the German Neuroscience Society*, Göttingen, Germany (**poster**)

Ruchty M., Roces F. and Kleineidam C. J. (2009):
“Adaptations of thermo-sensitive sensilla to infrared reception in ants”
39th *Neuroscience meeting*, Chicago, USA (**poster**)