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Cues and Signals

Used For Orientation and Communication in the

Superorganismic Meliponine Bees (Hymenoptera,

APIDAE, Meliponini)

a dissertation by

Dirk Louis P. Schorkopf

University of Vienna
February 2010

Cover illustration (if provided with this copy) shows the surface of a *Trigona spinipes* nest. Illustration under the title was kindly presented by Martina Schorkopf.

Dedicated to all my teachers

not all of them professors
not all of them human beings
Thanking them for their guidance
by providing signals and cues
in so many ways

καὶ τὸ μὲν οὖν σαφὲς οὔτις ἀνὴρ ἴδεν οὐδέ τις ἔσται εἰδὼς ἀμφὶ θεῶν τε καὶ ἅσσα λέγω περὶ πάντων· εἰ γὰρ καὶ τὰ μάλιστα τύχοι τετελεσμένον εἰπών, αὐτὸς ὅμως οὐκ οἶδε· δόκος δ' ἐπὶ πᾶσι τέτυκται.

ΞΕΝΟΦΑΝΗΣ

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* Cited from: Sextus Empiricus ("adversos mathematicos), cited in Hermmann, D. (1957): Die Fragmente der Vorsokratiker. Hamburg: Rowohlt.

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Preface

"Besides the dissertation committee nobody will read a thesis anyway"
This is what I constantly hear from close or distant friends in research – some of them supervising thesis-writing students themselves.
Although I fear that there is some truth in the saying, I nevertheless believe that a thesis represents a good opportunity to revisit the work done and published during several long years of total dedication to a subject of scientific interest. For the readers, however few, I briefly list what can be expected in this thesis.

Chapter I is a general introduction and provides some selected background information relevant to the research interests of this thesis. It also provides a list of a few important definitions as well as the main questions asked. All readers already familiar with the field of research of this thesis may skip this chapter, except those who wish to have some idea about the particular concepts I had in mind when working for and writing up the thesis.
Chapters II and **III** consist of papers already published. For those who already know the content, some notes and the appendix may provide more information.
Chapter IV presents a paper currently under revision.

Chapter V summarizes and comments coauthored papers which appeared while I was working for my thesis and are strongly related to it.
Chapter VI contains some unpublished data and observations considering chapters II to IV.
Chapter VII finally presents the answers to the questions asked at the beginning (chapter I), some general conclusions and a summary, followed by the references.

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„Der beste Dank ist, wenn alles gut geht"[1]
[„Best acknowledgement of gratitude
is achieved when everything
turns out well"]

This thesis would not have been realized without the help and support by a numerous number of people worldwide.

Main financial support came from a FWF[2] project (P17530) granted to my thesis supervisor Friedrich G. Barth. Professor Barth recruited me for the latter project in the end of 2004, presented me unsolicited with a brand new GC-FID on my birthday in 2005 and renewed my contract no less than 3 times

[1] From: Haruki Murakami (2006) Kafka am Strand. München: Random House. [German translation by Ursula Gräfe of "Umibe No Kafuka" published in 2002 by Shinchosha, Tokyo]
[2] „Förderung Wissenschaftlicher Forschung", Austria

before the project had to finally end in the beginning of 2008. Rarely somebody was able to drive me to the boarders of my resources and abilities as he did. He also critically read this thesis and significantly ($P_{\text{chi-square test}} < 0.001$) improved the style and logical thread of my hitherto written manuscripts. Without him I wouldn't be where I am now.

My truly loving and understanding wife supported me in so many ways and provided the vitamins, catalysts and motivation needed to survive many difficult and challenging events such as unemployment and a marathon.

My chummy colleagues Michael Hrncir, Stefan Jarau and Veronika Schmidt contributed greatly to the success of my career. Michael  would not even desert me when I needed him to work with not less than six hives simultaneously (when I write simultaneously, I really mean simultaneously). Stefan introduced me to the, sometimes  quite mythical world of chemical ecology and generously shared his knowledge. Veronika was our most accomplished organization  talent, did share knowledge, humorous tales and food. Austria gained one of its best scientific journalists, but lost one of ist most promising bee researchers.

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[3] Firstly "Neurobiology division of the Institute of Zoology", followed by "Department of Neurobiology and Behavioural Sciences", then "Department of Neurobiology and Cognition Research"; today called "Department of Neurobiology".

generously entrusted me their GC-MS in Ribeirão Preto (muito obrigado por a confianca!).

Ronaldo Zucchi always welcomed us to stay in his department and to use his facilities. A person whom we could always rely on and whom we prize. I dare say: no Austrian bee researcher in Ribeirão Preto in the last decade without his help...

To realize the experiments at Piracicaba I thank Roberto Antonio Zucchi for introducing me to José Maurício S. Bento, who made his laboratory facilities available to us. I am very grateful for the opportunities of research at the ESALQ in Piracicaba.

Sidnei Mateus and Weyder Santana introduced me to several species of meliponine bees and also showed me how to handle them. Many colonies important for this thesis were only available due to S. Mateus' generosity and commitment.

True Brazilian Hospitality was granted by Fabio Nascimento and his wife Ivelize during my stay at Aracaju. In addition I want to thank Fabio for constantly helping me to realize my ambitious research plans whilst staying at the University Federal de Sergipe and for patiently waiting for the results to be published (by my honour – they will!). Without the bee keeper Erinaldo's generous offer to borrow 12 of his most precious colonies, however, I would not have gone far. I had some of the most wonderful insights to the behaviour of meliponine bees whilst working with his colonies.

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João Maria Franco de Camargo and S. R. de Menezes Pedro verified the identity of many specimens. J.M.F. Camargo in addition shared unpublished data important for this thesis and provided me with important "first hand" biogeographical and taxonomic information apart from some original reprints containing several of his precious illustrations. I will certainly miss our conversations on the field and in his laboratory. I feel elated for having been able to share some sparks of his immense knowledge on tropical bees and ecology. When asking him (like seemingly F.G. Barth, and possibly many others before): "Professor Camargo, why don't you print a book with commented illustrations? [after some silence I added:] Please! I would be the first to by it!" He replied (hiding a smile in his face) "I had bad experience with book prints... You see: Every dot and line I draw is there for a good reason. Unfortunately, there is obviously no editor who would accept to realize an accurate copy of my illustrations and I would never accept publication if one of these lines or dots was missing". If only I could speak to Albrecht Dürer to persuade him!

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(picture shows ***Melipona quadrifasciata*** *collecting nectar)*

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CHAPTER I

GENERAL INTRODUCTION

Orientation and communication

Orientation and communication are indispensable requirements for most, if not all, living creatures. Accordingly, these abilities can be found from the unicellular protists to single cells of an organism up to the most elaborate organisations of many individuals acting as evolutionary units which are currently believed to be represented by the so called "superorganisms"[4] (Wilson 2000; Hölldobler and Wilson 2009).

Higher organisational levels

Although some ideas about evolutionarily effective entities on higher organisational levels had already existed for about 100 years (e.g. Wheeler 1911), it seems to have been in the 1960s only that some

[4] Superorganism (as defined in Wilson 2000): "Any society, such as the colony of an eusocial insect species, possessing features of organization analogous to the physiological properties of a single organism. The insect colony, for example, is divided into reproductive castes (analogous to gonads) and worker castes (analogous to somatic tissue); it may exchange nutrients by trophallaxis (analogous to the circulatory system), and so forth."

researchers were able to provide the satisfactory scientific tools and models (e.g. Hamilton 1964) which enabled biologists to testify for or against the existence of higher level mechanisms of selection. Generally, the urge to understand the existence of the so called "complex social societies" increased, especially when the theories of evolution by Darwin and Wallace (Darwin and Wallace 1858, Darwin 1859, Wallace 1870) became available. However, the latter's theories of evolution encountered difficulties in explaining the existence of individuals in a population which abstain from reproducing themselves in support of other members, such as the workers in ant or honey bee colonies. This is why the kinship theory of Hamilton (1964 and thereafter), which also was drafted to explain the evolution of social behaviour of hymenopteran societies (Hamilton 1964) was so much welcomed by science. The honey bees (*Apis mellifera*), which have interested mankind since ancient times because of their valued products (honey and wax), turned out to be the most prominent study subject for research questions regarding the study of insect societies and of hymenopterans in particular. They are easy to handle and their frequent presence in human settlements all around the world has guaranteed their leading status in social insect research.

Proximate questions

Why and how is it possible that thousands of individuals collaborate to survive while most other living creatures seem to largely achieve survival in solitude? This is a question which has bothered thinkers dating back at least to Aristotle and is a question of importance in this thesis. We can approach this riddle by proximate or ultimate reasoning and theory construction. For a 'full understanding' it would be most satisfactory if both types of reasoning were unveiled. Yet the present thesis can only borrow theories from others to explain ultimate causes. I will focus on several proximate questions and try to find adequate answers to the *how,* in an often neglected but nevertheless interesting and ancient superorganismic taxon, the Meliponini.

Later (chapter II-V) I will present some published data together with a few, still unpublished observations and data, some of which I am preparing for publication. Almost all of them will deal with the worker caste in meliponine bees and their ways of communication and orientation which obviously affect the superorganismic organization of their colonies.

A few facts about bees in general

The honey bee (*Apis mellifera*) represents only one among over sixteen thousand described bee species around the world (Michener 2000). Even biologists are often astonished when confronted with the fact that more bee than bird species dwell on earth. Another fact about bees is equally poorly known: More than 90% of all bee species are so called "solitary" species: species where females do not found colonies but care for their offspring all by themselves.

Bees

But what exactly are bees? They belong, together with ants and wasps, to the aculeate Hymenoptera, in which group the females have adapted their ovipositor to serve as a sting-like structure. Bees can be distinguished from all other Aculeata because of both morphological and physiological characteristics. I will only mention a few of the most characteristic traits of bees, which are better described elsewhere (Michener 2000). (i) With the exception of few non-flying Mutillidae, branched, often plumose, hairs can only be found in bees among the Hymenoptera. Some argue that such hairs were an important innovation to facilitate pollen collection, while Michener (Michener 2000) thinks they evolved rather as protection against dehydration. (ii) Another

distinguishing feature of bees are their "basitarsi" (a distal section of their legs), which are broader than the subsequent tarsal segments in the hind legs. (iii) Other additional features are quite frequent in bees, but neither unique nor omnipresent in them: a) pollen collection and pollen feeding in adult females combined with the lack of active prey capture b) the proboscis (the nectar sucking mouthparts) is often long and can thus often be easily seen during feeding or even after feeding when folded up.

The bees (Apiformes) are believed to derive from a single spheciform (Sphecidae) species (Michener 1944, Michener 2000). Michener (Michener 2000) recognizes seven bee families with more than 400 genera. Although sociality has evolved several times in the bees, the biggest and most complex colonies (degree of sociality and the number of individuals per nest) did evolve in the family APIDAE. Meliponine bees (Meliponini), as well as honey bees (Apini) and the bumblebees (Bombini) belong to this family (Michener 2000).

Meliponini

Meliponini, presently most often called "stingless bees" (e.g. Michener 2000, Ruttner 2003, Roubik 2006), but better simply called "meliponines" (see

reasons given below[5]) represent a less well known taxonomic group of superorganismic species (Camargo and Pedro, 1992; Michener, 2000). Their highly eusocially organized colonies consist of a few hundred to more than one hundred thousand individuals (Michener 1974; but see Wille 1983[6]).

Body size

The smallest body size found in meliponine worker bees was reported to be approximately 1.7mm (Pedro and Camargo 2009). The largest meliponine workers seem not to exceed a body length of 14 or 15

[5] I tend to avoid the term "stingless bees" and to use "meliponines", Meliponini, or similar vocabulary instead because of the following six reasons: 1) Although the sting apparatus is considerably reduced in Meliponini, the queens and workers are not truly stingless (Michener 2000; Abdalla and Cruz-Landim 2001) 2) Bee species other than those among the Meliponini are known to have reduced stings as well (e.g. *Dioxys* Lepeltetier & Serville 1825; *Ensliniana* Alfken 1938; or *Alocandra* Michener 1986). 3)Truly stingless bees are the male bees in all of the 16 000 bee species occurring worldwide (Michener 2000). 4) "Stingless bees" suggests that these are not capable to aggressively defend themselves which is not true for many meliponine species (see chapter II). 5) Most people which have never ever heard of meliponines before actually believe "stingless bees" to represent a stingless breed of *Apis mellifera* (Schorkopf, personal observations in American, European and African countries). 6) I prefer to describe or call something by a "neutral" name (a name which does not describe any feature of a species) or a character it possesses rather than something it does not appear to possess.

[6] Wille (1983) doubted the large numbers mentioned by Michener (1974). However, it seems that very populated nests (>100 000) actually do exist, even if rare (J.M.F. Camargo, personal communication: Remembering some of his personal observations in the Amazon basin, he mentioned a few *Trigona* nests inspected by himself which easily exceeded 100 000 individuals due to the vast number and size of brood-containing combs alone).

mm (Michener 2000, Camargo and Pedro 2008) and thus do not exceed the body size of *Apis mellifera,* the most common honey bee species (Ruttner, 2003). The appearence of Meliponini can be similar to honey bees (especially in the meliponine genus *Melipona*), wasps or flies (at least to the untrained eye).

Pollinators

Meliponines belong to the most important pollinators in tropical rain forests and are also valued study objects for those seeking a broad insight into tropical ecology (Roubik, 1989). The many species of meliponines (see below) differ considerably from each other in regard to their biology (e.g. Michener 1974; Wille 1983; Roubik 1989; Nogueira-Neto 1997; Michener 2000, Roubik 2006).

Castes

Three morphological castes (as defined by Oster and Wilson 1978) can be distinguished: 1) the workers, 2) the queen 3) the males. Few published studies, often of preliminary character (Bassindale 1955; Kerr and Santos Neto 1956, Hebling et al. 1964; Darchen 1969; Sommeijer 1983, 1984; Simões and Bego 1991; Kolmes and Sommeijer 1992, Giannini 1997) exist on the differentiation of so called ethological castes (typically age polyethism in social bees[7]) within a meliponine colony. Young worker bees are generally

[7] Age polyethism = A type of polyethism in which individuals pass through different forms of worker task specialization as they grow older (Wilson 1971)

allocated to intranidal (inside the nest) duties, while older bees generally also switch to nest defence or extranidal duties, such as foraging.

Meliponine species

Worldwide, 506 living meliponine species names are considered to be valid by the most recent reviews (Eardley 2004, Camargo and Pedro 2007, Rasmussen 2008). Most researchers believe that some new meliponine species will be described in the future. Hence, the total number of described biological species is likely to rise.

Tropics and subtropics

Unlike other eusocial Corbiculata (Bombini, Apini), Meliponini occur exclusively in the tropics and subtropics (Michener 2000, Noll 2002) although some species seem to come close to temperate zones in South America. In the New World Meliponines are found from Alamos, Sonora (27°N) to Montevideo, Uruguay (34°S) (Camargo and Pedro 2007). The highest altitudinal record for a meliponine is presently held by *Geotrigona tellurica*, found at 4000m in the Bolivian Andes (Camargo and Moure 1996).

A considerable number of the meliponine species and genera are endemic to the Neotropics, where the speciation of Meliponini seems to have reached its peak. The rareness of other superorganismically organized bees in the past in this region is one possible reason why the speciation and radiation has led to such a high number of neotropical species.

Thirty two living meliponine genera are currently recognized to occur exclusively in the Neotropis (Camargo and Pedro 2007).

Taxonomy

Important information on the taxonomy of Meliponini of the neotropical regions can be found in the following works: Camargo and Pedro 2007 (all genera); Schwarz 1932, 1948 (*Melipona*, *Trigona*, *Paratrigona*, *Schwarziana*, *Parapartamona*, *Cephalotrigona*, *Oxytrigona*, *Scaura* and *Mourella*); Ducke 1916, 1925 (Brazilian Meliponini); Camargo 1980 (genus *Partamona*); Camargo and Moure 1994 (genera *Paratrigona* and *Aparatrigona*); Camargo 1996 (genus *Camargoia*); Camargo and Moure 1996 (genus *Geotrigona*); Ayala 1999 (Mexican Meliponini); Camargo and Pedro 2003, 2004, 2005 (genera *Partamona*, *Ptilotrigona* and *Dolichotrigona*); and Marchi and Melo 2006 (for Brazilian *Lestrimelitta*). African Meliponini were recently reviewed by Eardley (2004), while Rasmussen (2008) reviewed both the Asian and Australian Meliponini. A source which includes all of the world's meliponine supraspecific taxa, as well as those of all other bees, has been provided by Michener (2000) who also points to some unresolved problems regarding meliponine taxonomy and their placement within the Apoidea.

Defence and competition in Meliponini

Competition

Competition for resources is a major driving force of evolution. While usually only intraspecific and interspecific competition can be distinguished, superorganismically organized species, such as ants and meliponines, also have to deal with the intra- and inter-superorganismical competitive levels, which in meliponines equals intra- and intercolonial competition. This is because in meliponines a superorganism is represented by a colony.

Cooperation

Competition represents one of the two major mechanisms of social behaviour. The other major mechanism is cooperation, which is evident within the meliponine superorganism (intrasuper-organismical or intracolonial cooperation), yet rarely reported between naturally occurring colonies (intercolonial or intersuperorganismical cooperation). The latter may be found for brief periods between mother and daughter colonies (Nogueira-Neto 1954, Nogueira-Neto 1970, Sakagami 1982, Wille 1983, Inoue et al. 1984, Engels and Imperatriz-Fonseca 1990, Nogueira-Neto1997, van Veen and Sommeijer 2000, Roubik 2006). This is believed to be the period between the start of the establishment of a new daughter colony and the moment when the daughter colony can exist independently. Independence is achieved when the daughter colony raises enough workers to

accomplish two tasks very important for the survival of an emerging colony: foraging and defence.

Defence

A meliponine nest holds rich treasures of food (honey and pollen) in addition to the core of their reproductive court and offspring. These must be defended from many animal species and even man. Hence, meliponine nests are considered to be so called "fortress holders" where resources are concentrated at a particular location, secured and defended from competitors and predators. The Meliponini have evolved a rich diversity of defence mechanisms. One of the most intriguing peculiarities, however, is the lack of stinging behaviour, for which most bees and wasps are famed.

Defence, however, does not only occur in close proximity to the nest but must also sometimes be shown during foraging. Some species of Meliponini even aggressively attack foragers of other colonies or species to monopolize resources (Hubbel and Johnson 1974; Slaa 2003). A few species have even evolved special techniques to actively attack other meliponine nests and to rob them and their food provisions. These, sometimes called "robber bees", are cleptoparasites which have made the robbing of other nests their living (Michener 1974; Wille 1983; Roubik 1989; Michener 2000). In the course of

evolution these bees have even lost the morphological and behavioural ability to collect food from flowers and obligatorily depend on other bees to establish food provisions which they can access.

Communication, orientation and a few other terms of special interest to this thesis

As mentioned before, communication represents an ability of paramount importance to organisms. Several features of the orientation capabilities of superorganisms can only be explained by the existence of highly efficient interindividual communication. Because communication and orientation are terms of major importance to this thesis it may be useful to briefly define these and other terms of similar importance to the present work in order to avoid misunderstandings.

Communication

In this thesis, communication is understood[8] as the process in which the signaller (an individual or a

[8] My current understanding and synthesis of terms relevent to communication are mostly based on opinions and thoughts in the following publications: Lorenz 1939; Tembrock 1971; Wilson 1971, 2000; Hailmann 1977; Dawkins and Krebs, 1978; Green and Marler 1979; Lloyd 1983; Lewis 1984; Markl 1985; Lindauer 1990; Dusenbery 1992; Hasson 1994; Hauser 1996; Bradbury and Vehrencamp 1998; Seeley 1998; Greenfield 2002; Maynard Smith and Harper 2003; Wyatt 2003; Alcock 2005; Danchin et al. 2008; Hailman 2008; Winans and Bassler 2008.

collective) provides or sends out a signal to induce a change in a receiver (an individual or a collective[9]) to increase[10] the signaller's inclusive fitness. The process remains evolutionarily stable if – on a whole – the receiver also joins in inclusive fitness. An example for communication in meliponines is the emission of pheromones inducing defensive behaviour by an attacked or threatened meliponine individual or collective (chapter II). The pheromone emission can lead to the successful prevention of nest intrusion or to the overpowering of attackers by attentive nestmates able to receive and interpret the signal (the pheromones inducing defensive behaviour) and immediately supporting the defence of the attacked colony. In case of the Meliponini, both attacked and attacking individuals can thereby increase their inclusive fitness, even if both die whilst doing so, as long as their mother colony survives and produces offspring on the long run (Hamilton 1964, Wilson 2000). In contrast, if there is neither a sender nor receiver, the intruder is likely to succeed in entering the nest and the colony can loose much of its hoarded resources or brood which will lead to a considerable indirect fitness loss.

[9] Wich may be the sender itself.

[10] the action thereby bearing the realistic potency of increasing the signaller's inclusive fitness (direct or indirect fitness).

Signals and cues

Both signals and cues are stimuli which can be perceived by the sensors of a receiver. Both types of stimuli contain information processed by the receiver in a way ultimately leading to a change in its physiology and behaviour. However, the probability of such a change must be relevant to the evolution of the biological species by improving the inclusive fitness in an evolutionarily relevant proportion of individuals. Whereas typical cues represent any informative variable (e.g. temperature values or sunlight), which can also occur or exist independently from potential receivers, signals are bound to the aim of a sender and shaped by the qualities and abilities of the sender and receiver (strongly influenced by natural history in biological systems). To clarify the signal status of a stimulus it is essential to identify and explain the aim and potential gain in the inclusive fitness of the sender (by provoking a change in the receiver). In contrast, one must only clarify the aims of the receiver when calling a stimulus a cue. For example animals will frequently cause vibrations, air- or water particle movements or similar during locomotion. Such "disturbances" due to locomotion by individuals in the environment can be detected and utilized as cues by a receiver (e.g. a predator). The moving individual did not aim at changing anyone's behaviour or physiology when incidentally causing the disturbances produced during locomotion. Thus,

we can confidently call informative variables as cues in all those cases where no communicative aim can be assigned.

Orientation[11]

Orientation is the spatial[12] alignment of an organism achieved by using extra- or intra-individual stimuli as a reference, such as light, magnetic fields or chemical gradients. Every living organism is believed to possess some means of orientation. Piloting and navigation, where stimuli are used as reference points for guided movement are good examples for orientation capacities. Orientation is also important for communication mechanisms between organisms, such as the orientation of gametes and sexual partners along a pheromone gradient. The recognition and interpretation of reference stimuli are either inherited or learnt.

Recruitment (after Wilson 2000)

Recruitment is a special form of assembly (the calling together of the members of a society for any communal activity) by which members of a society are directed to some point in space where work is required.

[11] Following the publications by: Merkel 1980, Hölldobler and Wilson 1991, Lehrer 1997, Wilson 2000

[12] In addition we should not forget to consider the temporal alignment.

Main questions of this thesis

Which cues and signals are used by the meliponine superorganism for efficient communication and orientation on the intra – and intersuperorganismical level?

The present thesis is about important aspects of foraging and defence which represent two major "everyday" challenges of organisms and consequently also superorganisms for survival. More specifically I asked the following questions:

(i) *Defense behaviour*

Which signals and cues are used during defence behaviour? Are pheromones used for effective communication on the intra- and intersuperorganismic level and do bees use simple optical cues to find threatening targets positioned at some distance to their nest (chapter II and chapter VI)? I approached these questions by choosing two well known meliponine species (*Trigona spinipes* and *Scaptotrigona* aff. *depilis*) and asked the following details: A) Which glands are involved in the elicitation of aggressive and defensive behaviour? B) Which substances are contained in these glands? C) Which behaviour do the secretions of theses glands evoke when applied close to the nest and at the food source, respectively? D)Do the bees react differently to

secretions of bees from other conspecific nests or from the other (sympatric) species? E) To which extent can mere physical manipulations elicit defensive behaviour? F) Do meliponines use the "brightness" of objects (as compared to the background environment) as a major cue for the localization of threatening objects? G) Does the importance of such a cue alter with time or distance from the nest?

(ii) *Coordination and orientation via pheromones* Which signals and cues are involved in the activation, coordination and orientation of meliponine foragers and forager groups towards a specific resource? Are pheromones used similarly to what has been found in other superorganismic societies, such as in ants and termites? Again I further approached these questions by using two of the most studied, so called "pheromone trail laying" meliponine species (*T. spinipes* and *S.*aff. *depilis*). H) In *T. spinipes* I studied whether saliva is deposited by trail laying foragers when landing for scent marking between nest and food source. I) Does the saliva contain any attractive substance or blend of substances representing the pheromone, which induces trail following behaviour in *T. spinipes*?. J) What are the active

component(s)? K) Which is their actual glandular origin? L) Is the synthetic form of the assumed pheromone effective in behavioural experiments? M) Does it have the same effect compared to that of the natural saliva?

(iii) *Pheromone paths*

How relevant are pheromone paths (which are laid down between a highly profitable target and the nest) for the orientation and efficient concentration of worker forces in scent trail laying meliponine species (chapter III, IV, V and VI)? Once more, I used *T. spinipes* and *S. aff. depilis* to explore in more detail: N) Are one or both species able to successfully recruit nestmates to food sources without pheromone paths leading towards them? O) If so, why do flying foragers invest any efforts in elaborating substrate bound pheromone paths? P) What happens if several food sources with or without scent path leading to them are offered simultaneously? Q) Would any newcomer bees arrive at the food sources without a pheromone path? R) Does another scent trail laying meliponine (*Scaptotrigona postica*) behave similar to the hitherto tested species?

"Tandis que, assis sur le rocher, j'étois occupé à déterminer l'inclinaison de l'aiguille aimantée, je me trouvai les mains couvertes d'une esèce d'abeilles velues, un peu plus petites que l'abeille mellifique du nord de l'Europe. Ces insectes font leurs nids dans la terre. Ils volent rarement; et, d'après la lenteur de leurs mouvemens, je les aurois crus engourdis par le foid des montagnes. Le peuple, dans ces r'egions, les appelle de petit anges, angelitos, parce qu'ils ne piquent que très-rarement. Ce sont sans doute des apiaires du groupe des Melipones. Quoi qu'en aient dit plusieurs voyageurs, il n'est pas vrai que ces abeilles, propres au Nouveau-Continent, soient dépourvues de toute arme offensive. Elles ont l'aiguillon plus foible, et elles s'en servent plus rarement. Lorsqu'on n'est pas encore bien rassuré sur la douceur de ces angelitos, on ne peut se défendre de quelque crainte. J'avou que, souvent, pendant les observations astronomiques, j'ai été sur le point de laisser tomber les instrumens, quand je me sentois les mains et le visage couverts de ces abeilles velues. Nos guides assuroient que ces insectes ne se mettoient en défense que lorsqu'on les irritoit en les prenant par les pattes. Je n'ai pas été tenté de faire cet essai sur moi-même."

From: Alexandre de Humboldt [Alexander von Humboldt, added remark] (1814) Voyage aux Régions Équinoxiales Du Neouveau Continent, par Al. de Humboldt et A. Bonpland. Tome Premier, Livre IV. Paris: F. Schoell.

Agonistic behaviour in *Trigona spinipes.* The top picture shows two fighting bees from different colonies. The picture on the left corner shows one of the most frequent defensive postures in meliponine bees (the bee with the open mandibles; please also note the approaching bee indicated by the white arrow). The bottom right picture shows the cooperative attack of several individuals defending an artificial feeder against a bee from an alien nest. The red arrow indicates a bee exemplifying the V-shaped aggressive/defensive posture typical of *Trigona* bees.

CHAPTER II

IDENTIFICATION OF SIGNALS AND CUES INDUCING DEFENSIVE AND AGGRESSIVE BEHAVIOUR ON THE INTRA- AND INTERSUPERORGANISMIC LEVEL

Mandibular gland secretions of meliponine worker bees: further evidence for their role in interspecific and intraspecific defence and aggression and against their role in food source signalling

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SUMMARY

Like ants and termites some species of stingless bees (Meliponini), which are very important pollinators in the tropics, use pheromone trails to communicate the location of a food source. We present data on the communicative role of mandibular gland secretions of Meliponini that resolve a recent controversy about their importance in the laying of such trails. Volatile constituents of the mandibular glands have been erroneously thought both to elicit aggressive/defensive behaviour and to signal food source location. We studied *Trigona spinipes* and *Scaptotrigona* aff. *depilis* (‘*postica*’), two sympatric species to which this hypothesis was applied. Using extracts of carefully dissected glands instead of crude cephalic extracts we analysed the substances contained in the mandibular glands of worker bees. Major components of the extracts were 2-heptanol (both species), nonanal (*T. spinipes*), benzaldehyde and 2-tridecanone (*S.* aff. *depilis*). The effect of mandibular gland extracts and of individual components thereof on the behaviour of worker bees near their nest and at highly profitable food sources was consistent. Independent of the amount of mandibular gland extract applied, the bees overwhelmingly reacted with defensive behaviour and were never attracted to feeders scented with mandibular gland extract or any of the synthetic chemicals tested. Both bee species are capable of using mandibular gland secretions for intra- and

interspecific communication of defence and aggression and share 2-heptanol as a major pheromone compound. While confirming the role of the mandibular glands in nest defence, our experiments provide strong evidence against their role in food source signalling.

INTRODUCTION

Odours and pheromones[1] are omnipresent as signals and cues and are important carriers of information in arthropods. In social insects in particular, communication critically depends on chemical signalling, with the need for efficiency growing with the degree of sociality. The highest degree of sociality in insects, as well as in any other animal, is found in species where the members of one colony together represent a highly eusocially organized superorganism (Wilson and Sober, 1989; Seeley, 1989; Wilson and Hölldobler, 2005; Reeve and Hölldobler, 2007; Gardner and Grafen, 2009). The most well known examples of superorganisms are ants, honey bees and termites. Thanks to their study we now know that an efficient communication between colony members using pheromones and other semiochemicals[2] is indeed an essential basis for

[1] Pheromones here and elsewhere in this thesis are used as defined by Karlson and Lüscher (1959) and Wilson and Bossert (1963).
[2] Semiochemicals are chemical substances with some information value to the receiver (chemical signals or cues).

the maintenance of a superorganism. Often, the use of pheromones to recruit nestmates for collecting food or for defence is taken to illustrate the relevance of pheromones for the coordination of colony activities (Vander Meer, 1998; Wyatt, 2003). While a lot is already known about chemical communication in ants and honey bees, our knowledge is far behind and partly controversial in case of the Meliponini, the so-called stingless bees, a less well known taxonomic group of superorganismic arthropods (Camargo and Pedro, 1992; Michener, 2000). This is surprising because the meliponines are very important pollinators in tropical rain forests and also valued study objects for those seeking broad insights into tropical ecology (Roubik, 1989). In this paper we will dissect current controversy concerning the Meliponini which refers to the communicative role of their mandibular gland secretions. The mandibular glands were the first glands proposed to play an important role in meliponine communication (Lindauer and Kerr, 1958). Likewise they were reported to be important in the communication of a great number of other Hymenoptera, although sufficiently detailed studies are available for very few species. The many species of meliponines (>400 circumtropical species) differ vastly in foraging habits and defensive 'strategies' and bees of the same or different sympatric species frequently compete for resources (Schwarz, 1932; Johnson and Hubbell, 1974; Roubik, 1989; Nagamitsu and Inoue, 1997; Eltz et al., 2002; Slaa, 2003; Slaa, 2006). Therefore they are

particularly suited for flower ecology studies and for the study of defensive and aggressive behaviour. The behavioural differences between them have so far been mainly attributed to differences in body and/or colony size and nesting behaviour (Michener, 1974; Camargo and Pedro, 1992; Roubik, 2006). Accordingly, one finds (i) intranidal and intrasuperorganismic, (ii) internidal and intersuperorganismic, and (iii) interspecific conflicts. Analogous to the situation in ants, cleptoparasitic species ('robber bees') add to the diversity of meliponine bee behaviour. In all these behaviours, efficient communication between colony members will enhance the effect of defence and aggression as well as the efficiency of foraging. It should also be advantageous for the bees to 'understand' the signals of other colonies or even other species involved in this communication, particularly in agonistic contexts (Maynard Smith and Harper, 2003). Like in other superorganismic hymenopterans, defensive communication rests on chemical signals in meliponines, and the actual defenders are the worker bees. Pheromones serving hymenopteran defensive communication are often produced in the mandibular glands. These are well developed in all meliponine species as well but differ considerably in size between different genera and castes (Nedel, 1960; de Cruz-Landim, 1967). Interestingly, even before the chemoecological study of mandibular gland function as the source of an alarm pheromone (Blum, 1966; Blum et al., 1970; Luby et al., 1973),

Lindauer and Kerr (Lindauer and Kerr, 1958) had proposed its role in the production of scent trail substances used by some meliponine species to communicate the location of a profitable food source. Since then, the 'one gland – two functions hypothesis' has developed into a putative fact (Kerr and da Cruz, 1961), accepted in nearly all papers on meliponine communication published so far. If we assume that mandibular gland secretions do indeed induce both scent trail following to distant food sources in newly recruited worker bees and defensive/aggressive behaviour in the same workers near the nest, it would be interesting to examine how the bees accomplish the appropriate behaviour in a given situation. While the existence of the scent trail is undisputed in several recently published works (reviewed by Nieh, 2004), the mandibular gland origin of the actual scent marks has now been brought into question (Jarau et al., 2004; Jarau et al., 2006; Schorkopf et al., 2007). Here, we therefore critically re-evaluate the communicative role of the mandibular gland contents with regard to their function in defence behaviour and food source localization. For the present study we chose two meliponine species previously also examined in behavioural studies by Lindauer and Kerr (Lindauer and Kerr, 1958; Lindauer and Kerr, 1960) to ask the following questions. (1) Which substances are contained in the mandibular glands of worker bees? (2) Which behaviour do the mandibular gland volatiles evoke when applied close to the nest and at

the food source, respectively? (3) Do the bees react differently to mandibular gland volatiles of bees from other conspecific nests or from other (sympatric) species? Whilst our results clearly confirm the role of mandibular gland secretions in nest defence, their role in food source signalling must now be considered highly unlikely.

MATERIALS AND METHODS

Research site

The experiments were carried out at the Ribeirão Preto Campus of the University of São Paulo (Brazil) between November 2004 and March 2006.

Trigona spinipes

We studied four nests of *T. spinipes* (Fabricius 1793; Apidae, Apinae, Meliponini) naturally established on campus grounds. *T. spinipes* builds external nests in the canopies of trees. Note that Lindauer and Kerr (Lindauer and Kerr, 1958; Lindauer and Kerr, 1960) used the species name *ruficrus* (Latreille 1804), a junior synonym of *spinipes*. Three of the colonies (Ts1, Ts4 and Ts8) were transferred from 'canopy level' (4–7 m) to 'ground level' (1.6–1.9 m), whilst the fourth colony (Ts2) was kept at 0.4 m after it had fallen to the ground due to several days of heavy rain.

Scaptotrigona postica*/*Scaptotrigona* aff. *depilis

Seven out of the nine colonies studied were brought from a local meliponary and kept in wooden boxes 1.5–1.8 m above the ground. Two other colonies naturally occurred in tree trunk hollows (nest entrances at heights of 1.5 and 1.7 m) which is typical for this species. As mentioned before, our intention was to study meliponines to which the 'one gland – two functions' hypothesis had been applied. The presumed role of the mandibular glands as a source of pheromones signalling the presence of a food source was first studied in detail for a species described as '*Trigona (Scaptotrigona) postica*' (Lindauer and Kerr, 1958; Lindauer and Kerr, 1960). However, the actual *Scaptotrigona postica* (Latreille, 1807) (Apidae, Apinae, Meliponini) does not naturally occur in the region where Lindauer and Kerr (Lindauer and Kerr, 1958) performed their, now legendary, experiments (J. M. F. Camargo, personal communication). Given these authors worked with a colony originating from the same region it seems likely that they were observing a very similar looking, common and so far undescribed species still commonly called '*postica*' in publications (Camargo and Pedro, 2007) (J. M. F. Camargo, personal communication). Unfortunately no specimens are available for reference to the work of Lindauer and Kerr (M. Lindauer, personal communication). In the present study we worked with an as yet undescribed species, which belongs to the '*Scaptotrigona depilis* group' (J. M. F. Camargo, personal communication)

and which we, therefore, call *Scaptotrigona* aff. *depilis* [species *depilis* (Moure, 1942)]. Reference specimens have been added to the collection of J. M. F. Camargo at the University of São Paulo in Ribeirão Preto.

Chemical analysis – extraction of gland material

We carefully cleaned the mandibular glands from other tissues in physiological solution and under a stereomicroscope. Special care was taken not to contaminate the mandibular gland surface with the contents of other glands *via* the bath solution. The volatile contents of the glands were extracted using pentane (HPLC grade; 200µl, 24h at room temperature), and the extracts were reduced to 180µl (*S.* aff. *depilis*) or 60µl (*T. spinipes*) with a gentle stream of nitrogen for subsequent chemical analysis. For quantitative analyses, tetradecane and nonadecane served as internal standards. The relative amount of any identified substance was calculated by a comparison of its peak area with the summed area of all peaks (except peak areas smaller than 0.3% of the internal standard). Gas chromatographs (HPGC6890A and Shimadzu GC-2010; carrier gas, hydrogen) with flame ionization detectors were used for quantitative analysis. For qualitative analyses, we used gas chromatography combined with mass spectrometry (Shimadzu GC-2010/GCMS-QP2010; carrier gas, helium). An Agilent DB-5MS column (30m x 0.25mm, 0.25µm thickness[3])

[3] The original paper (Schorkopf et al. 2009) mistakenly printed 0.25 mm thickness instead.

was used. The temperature programme started at 50°C (5 min) and increased the temperature by 10°C per min up to 310°C (holding 310°C for 15min). The compounds were identified by comparison of mass spectra of natural products with data from the literature (McLafferty and Stauffer, 1989; Francke et al., 2000) and those of authentic reference compounds.

Behavioural studies

At the nest entrance

To test whether a substance elicited attack behaviour we placed a clean black[4] cotton ball [a sock stuffed with PVC foil; methodology similar to that used by Smith and Roubik (Smith and Roubik, 1983)] measuring ~10 cm in diameter as a target at a distance of 50 cm (*S.* aff. *depilis*) or 150 cm (*T. spinipes*) from the nest entrance during the night preceding the experiment. The ball either hung on a thread from a wooden broomstick or was fixed onto it. The number of bees biting the cotton ball in the 30 s following their exposure to a test substance was taken as a measure of aggressiveness. All substances were applied either directly to the nest entrance structure (*T. spinipes*) or presented on a filter paper (about 2cm x 3cm) fixed in front and some milllimetres below the nest entrance (*S.* aff. *depilis*). In this way the free passage of the bees was not

[4] Bees prefer to attack dark compared to light objects (see chapter VIA).

disturbed. The test substances were applied in turn, always following the application of a control substance (10µl of the solvent pentane). The time interval between the application of the control and test substance was less than 2 min. This included 30 s of observation of the bees' behaviour following the application of the control substance. The first mandibular gland extract or substance to be tested on each day was chosen at random (note, following the application of pentane as a control substance). The substances tested for *S.* aff. *depilis* and *T. spinipes* are listed in Figs. 1 and 2, respectively. In *T. spinipes* we also tested the labial gland substance octyl octanoate, which is known to be a trail pheromone (Schorkopf et al., 2007). In addition to the controls in which the pure solvent was applied, we included another control in which 10µl of atmospheric air was blown towards the nest entrance. At the feeding site

Bees leaving the feeder due to mandibular gland secretions
For *S.* aff. *depilis* we tested the effect of mandibular gland extracts and of individual synthetic chemical constituents thereof (Fig. 3) on bees visiting a feeder. Following Jarau and colleagues (Jarau et al., 2004), bees were trained to visit a small plastic dish offering a 50% w/w sugar solution at some distance from the nest (15–60 m). The gland extracts and different substances were applied on a filter paper (1cm x 1cm) fixed 1.5 cm above the feeder dish with a pin; 10µl of the tested substance was slowly dropped onto the filter paper. During the subsequent 10 s we

counted the bees leaving the feeder. The percentage of bees leaving the feeder after application of a test substance was evaluated statistically. The substances were applied in turn but always following the application of 10µl pentane as a control substance. The time interval between application of the control and subsequent application of the test substance was less than 30 s (including 10 s of behavioural observation after application of the control). The first substance to be tested on each day was chosen at random (note, following application of pentane as control). Only those bees that had never been tested on any of the substances (except pentane) at the feeding dish before were included in the statistical analysis. The procedures used to study *T. spinipes* were similar to those used for *S.* aff. *depilis*. To increase the sample size (number of simultaneously tested bees), however, we changed a few aspects of the experiment and setup. We first trained more than 20 bees to visit a feeder [described by Jarau and colleagues (Jarau et al., 2000) except for the following difference: we used an unused 35mm plastic film box to contain the 50% w/w sugar solution instead of a glass vial]. Before testing the gland extract volatiles we replaced the plastic film box containing the sugar solution with an empty one (unused, of the same brand). The latter had slits and holes cut into the plastic to allow a better diffusion of test substances (applied on a filter paper inside the vial) into the air outside the vial, thereby exposing the foragers to

them. The other procedures followed those for *S.* aff. *depilis*. The chemical species tested are listed in Fig. 3.

Choice test with feeders scented with mandibular gland extracts

We also tested the effect of the mandibular gland extracts in a simple choice test as described by Jarau and colleagues (Jarau et al., 2004). Our feeders were watch glasses with droplets of 50% (w/w) unscented sucrose solution at a distance from the nest of 48.7±36.7m (mean ± s.d., sometimes more than 70 m). The tested substances (observation period, 20 min) were 0.1 bee equivalents of mandibular gland extract taken from individuals of the same or different nests (same species), and pentane and the hypopharyngeal gland extracts as controls. We also tested 1.0, 0.1, 0.01 and 0.001 bee equivalents of the mandibular gland extract in the same way in *S.* aff. *depilis* to see how the bees' choice was affected by a decrease in the amount applied. Nieh and colleagues (Nieh et al., 2003) tested mandibular gland extracts in *Trigona hyalinata* and found them to be both repellent and attractive for bees at a foraging site; while the mandibular gland extracts in their study had a repellent effect in the first 7 min after application, this surprisingly changed to the opposite effect after the 8th minute. Jarau and colleagues (Jarau et al., 2004) later suspected that the attractiveness of the extracts used by Nieh and colleagues (Nieh et al., 2003) originated from contamination with extracts of other cephalic glands (labial glands). We tested this

assumption by choice tests using deliberately 'contaminated' mandibular gland extracts. To this end the mandibular gland was picked at its base and extracted without previous careful cleaning from other tissues ('contaminated' mandibular gland extracts). Given the above-mentioned observations by Nieh and colleagues (Nieh et al., 2003), the effect of the mandibular gland extracts on the bees' choice behaviour was tested for different time periods following mandibular gland extract application. We studied the bees' choice behaviour (1) immediately after application of the contaminated mandibular gland extract (*S.* aff. *depilis* only) and (2) 10min after its application (both species). Consequently the choice feeder setup was either presented to the bees immediately (as in 1) or 10 min after the application of the tested substances. The same choice tests were repeated with uncontaminated mandibular gland extracts. If contamination by other glands does not affect the attractiveness of mandibular gland extracts, the behaviour (attraction, avoidance, indifference) of the bees should not differ at identical test time points in the two tests (using uncontaminated or contaminated mandibular gland extracts). Solvent presented alone served as a control.

Tests to see whether mandibular gland secretions induce trail following in newcomers (S. aff. depilis)

When newcomers follow a conspecific scent trail to a food source they sometimes land on some of the scent marks of the trail before reaching the actual

food source. It is unlikely to begin with that newcomers would first follow a scent trail of mandibular gland secretions and then avoid and flee from the same secretions at the actual food source location. However, one could argue that the bees use their mandibular gland secretions to build up the scent trail not quite to the food source itself and react differently to the same secretions when actually reaching the food source. To test this assumption we checked whether mandibular gland extracts could elicit trail-following behaviour in newcomer bees of *S.* aff. *depilis.* We laid artificial scent trails towards artificial food sources (50% w/w, unscented sucrose solution) that consisted of droplets of mandibular gland extract or its solvent (as a control) applied to the substrate in the direction of the test feeders. We adopted the trail following assay used previously (Schorkopf et al., 2007) with minor changes; two artificial scent trails (T1 and T2; length, 5m each) were laid, beginning at a branching point 21–35 m (median, 30m) away from the nest and ending at one of the two test feeders. Bee numbers following T1 were statistically compared with those following T2. Between the two feeders stood a recruitment feeder (15 foraging bees), again 5 m away from the branching point and also from each of the two test feeders. Tested scents were either mandibular gland extracts (same nests) or equal amounts of the solvent pentane. The amount of the solution forming the trails increased as they neared the respective feeders, where scent concentrations were highest. The

amount of test substance increased (beginning from the branching point, 0 m) in the following order (bee equivalents dissolved in pentane): 0.0 (0 m), 0.05 (1 m), 0.1 (2 m), 0.2 (3 m), 0.3 (4 m) and 0.9 (at the feeder, 5 m). All the newcomers recruited during the 20 min experimental period landing on any of the artificial scent marks were marked with a colour, removed from the experiment and included in the statistical analysis. We also observed whether any bee circulated, inspected or otherwise followed the artificial scent trail in front of the actual food source. Every bee included in the statistics was used only once, avoiding pseudoreplication or learning effects.

Statistics

For normally distributed data of equal variance, we used the oneway ANOVA to test for the significance of differences in the percentage or number of bees that had landed on either of the two feeders presented for their choice. Tukey tests were applied for the pairwise multiple comparisons. Non-parametric statistics were applied (1) when the general requirements for ANOVA were not met and (2) when testing for differences among the experiments on the reaction of foraging bees to mandibular gland volatiles. The Kruskal–Wallis *H*-test was applied instead, followed by the Student–Keuls test for pairwise multiple comparisons. Wilcoxon signed-rank tests were applied when testing for differences between the responses to a specific chemical species and the preceding control substance (pentane). The Mann–Whitney rank sum test was applied to test for significant differences in the bioassay

which checked whether the mandibular gland secretions induce trail-following behaviour in newcomer bees.

RESULTS

Mandibular gland volatiles

Mandibular gland extracts in both *T. spinipes* and *S.* aff. *depilis* contained a variety of volatile substances (Table 1). In all cases the majority of these substances were moderately to highly volatile (volatility higher than that of 2-tridecanol). 2-heptanol was the only major compound (more than 10% of the sum of all detected peak areas) occurring in both species. *T. spinipes* had only one other major component (nonanal), while *S.* aff. *depilis* showed two additional ones (benzaldehyde and 2-tridecanone). According to Table 1, the chemical composition of mandibular gland volatiles of *T. spinipes* differs substantially from that of *S.* aff. *depilis.* The most striking difference is the amount of volatiles contained in each individual pair of mandibular glands: the sum of all volatile peak areas (as compared with the same amount of standard substances) was about 7–30 times higher in *S.* aff. *depilis* than in *T. spinipes.* The amount of the major component 2-heptanol was even greater (10–40 times). These findings correlate with the much larger size of the mandibular glands in *S.* aff. *depilis*[5].

[5] illustrations were included in the appendix.

Table 1. Volatile compounds so far identified in mandibular gland extracts (GC/MS) of *Trigona spinipes* and *Scaptotrigona* aff. *depilis*, arranged according to substance class. Quantification of single compounds relative to the sum of all detected peak areas:+++, >10%,++, >1%,+, <1%. § Enantiomeric composition not determined in this study [determined for *Scaptotrigona* in Engels et al. (Engels et al., 1990)]. §§ double bond position not determined.

	Trigona spinipes	*Scaptotrigona* aff. *depilis*
Alcohols		
2-Heptanol §	+++	+++
2-Octanol §	++	
2-Nonanol §	+	+
1-Nonanol	++	
2-Undecanol §		+
2-Tridecanol §	++	++
2-Pentadecanol §	+	++
2-Heptadecanol §		++
Docosenol §§	+	
Hydrocarbons		
Dodecene §§	+	
Dodecene §§	+	
Dodecene §§	+	
Tetradecene §§	++	
Tetradecane	+	+
Pentadecane	+	
Hexadecene §§	++	
Hexadecane	+	+
Octadecene §§	++	
Octadecane	+	+
Heneicosene §§	+	
Tricosene §§	+	
Pentacosene §§	+	
Terpenes		
Citral / Geranial	+	
Farnesol	++	

Table 1 (continued).

	Trigona spinipes	*Scaptotrigona* aff. *depilis*
Ketones		
2-Heptanone		++
2-Nonanone		+
(Z6)-Undecen-2-one ?		+
2-Undecanone		++
2-Dodecanone		+
2-Tridecenone		+
2-Tridecanone	+	+ + +
2-Pentadecanone		++
2-Heptadecanone		++
Esters		
Undetermined Butyrate	+	
Pentyl hexanoate?		+
2-Pentyl hexanoate ?		+
Hexyl hexanoate		+
2-Heptyl hexanoate ?		+
2-Heptyl hexenoate E2?		+
Undetermined Hexanoate		+
Aldehydes		
Nonanal	+ + +	
Docosenal §§	++	
Docosenal §§	++	
Aromatic compounds		
2-Phenylethanol	++	+
Benzaldehyde	+	+ + +
Phenylacetaldehyde	++	
Methyl Benzoate	+	
Lactones		
γ - Decalactone?		+

Behaviour

Bees attack a target upon exposure to mandibular gland extracts at the nest entrance All colonies of both species attacked the black cotton ball 50cm (*S.* aff. *depilis*) or 150cm (*T. spinipes*) in front of the nest, when 0.1 bee equivalents of volatiles of the mandibular glands were released at the nest entrance structure (Figs. 1 and 2), irrespective of the colony or species the mandibular gland was taken from. In contrast to this finding, bees rarely attacked the same target when air, labial gland extract, hypopharyngeal gland extract or the solvent pentane had been released in the same way. A highly significant difference ($P<0.001$), therefore, resulted when comparing these substances with the mandibular gland extracts. The fact that any bee attacked at all upon the release of one of the control substances is an artefact of the test procedure: even without injecting a substance into the nest entrance the movements and vibrations caused by the experimenter can cause an attack response (see appendix).

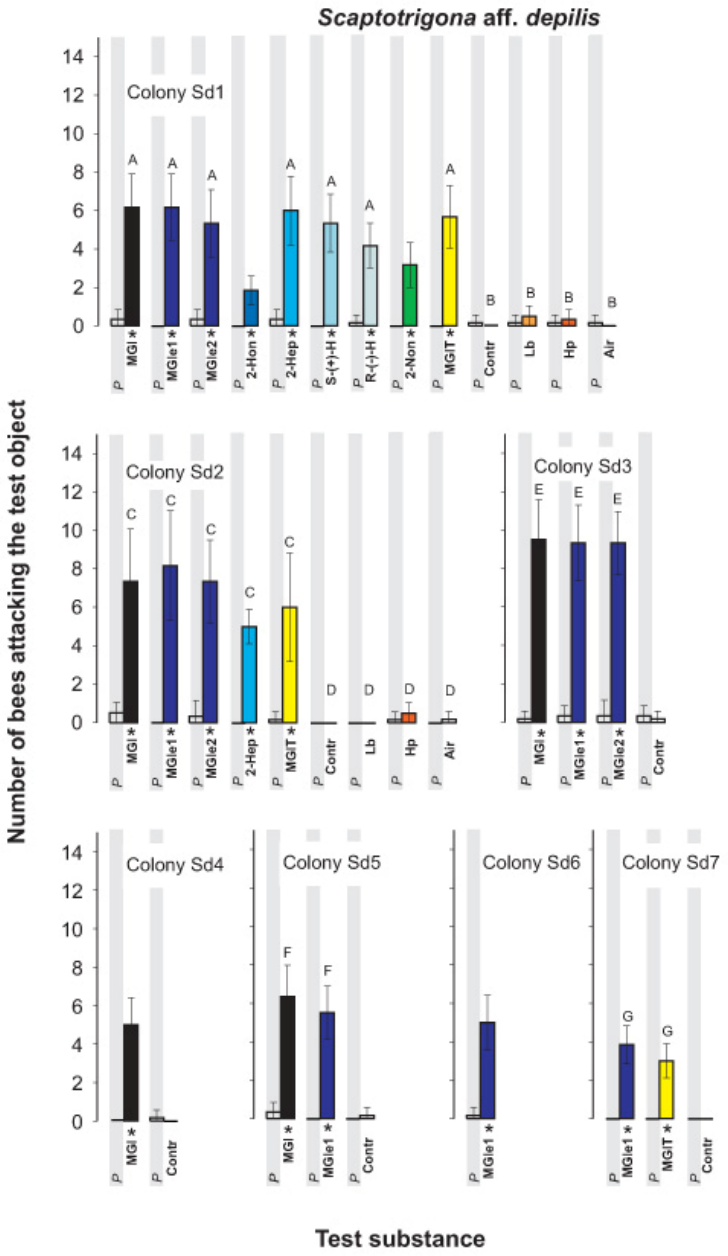
Scaptotrigona aff. depilis
Colony Sd1
Colony Sd2
Colony Sd3
Colony Sd4
Colony Sd5
Colony Sd6
Colony Sd7
Number of bees attacking the test object
Test substance
MGl *
MGle1 *
MGle2 *
2-Hon *
2-Hep *
S-(+)-Hl *
R-(-)-Hl *
2-Non *
MGlT *
Contr
Lb
Hp
Air
MGle1 *
MGle2 *
MGlT *

Figure 1. Number of workers of *Scaptotrigona* aff. *depilis* attacking a black cotton ball at a distance of 50 cm from the nest within 30 s of release of the test substances at the nest entrance. Test substances: (i) 0.1 bee equivalents of the mandibular gland extracts of nestmates (MGl) or conspecific bees of other colonies (MGle1, MGle2); (ii) corresponding amounts of 2-heptanone (2-Hon), 2-heptanol (racemate; 2-Hep), S(+)2- heptanol [S-(+)-H], R(-)2-heptanol [R-(–)-H], 2-nonanol (racemate; 2-Non), solvent (as control), labial glands (Lb), hypopharyngeal glands (Hp) and air. Also tested: 0.1 bee equivalent of *T. spinipes* mandibular glands (MGlT). P, additional control with pentane preceding each of the above-mentioned substances. *Significant difference (α=0.05) in bee numbers between P and the substance tested subsequently. Every test was repeated 6 times in each of the seven colonies. Columns with the same letter at the top (except the preceding controls, P, which were not included in the ANOVA) represent values that do not differ significantly from each other (α=0.05) (means ± s.d.).

All the synthetic volatiles naturally occurring in the mandibular glands and used in the bioassays caused biting and attack, albeit to different degrees. Among individual substances, 2- heptanol elicited the strongest response, but the difference between its effect and that of other compounds (Figs. 1 and 2) was not always significant. Interestingly, the two enantiomers of this alcohol elicited similar responses. Although we always observed a slightly higher attack rate with S(+)-2-heptanol than with R(-)-2-heptanol, the difference between the responses was not significant.

Bees abandon the feeder when exposed to mandibular gland extracts

Bees of both species abandoned the feeder at a high rate when exposed to 0.1 bee equivalents of mandibular gland volatiles (Fig. 3). This was also observed when using mandibular glands taken from conspecific workers of other nests or even from bees belonging to the other of the two species. When the same amount of pure solvent or of labial gland extract was applied, only a few foragers left the feeder. Their number did not differ significantly (Mann–Whitney *U*-test; *S.* aff. *depilis*: N=6 trials, P>0.8; *T. spinipes*: N=6 trials, P>0.6) from that found when no substance was applied at all and air was ejected instead. Consequently, the difference in the median values among all these treatment groups (N=6 trials) was highly significant in both species (Kruskal–Wallis analysis of variance on ranks: *S.* aff.

depilis: H=48.56, d.f.=9, P<10–6; *T. spinipes:* H=26.97, d.f.=5, P<10–4). The pairwise comparisons between the effect of mandibular gland extracts and air, solvent or labial gland extracts showed highly significant differences as well (P<0.001).

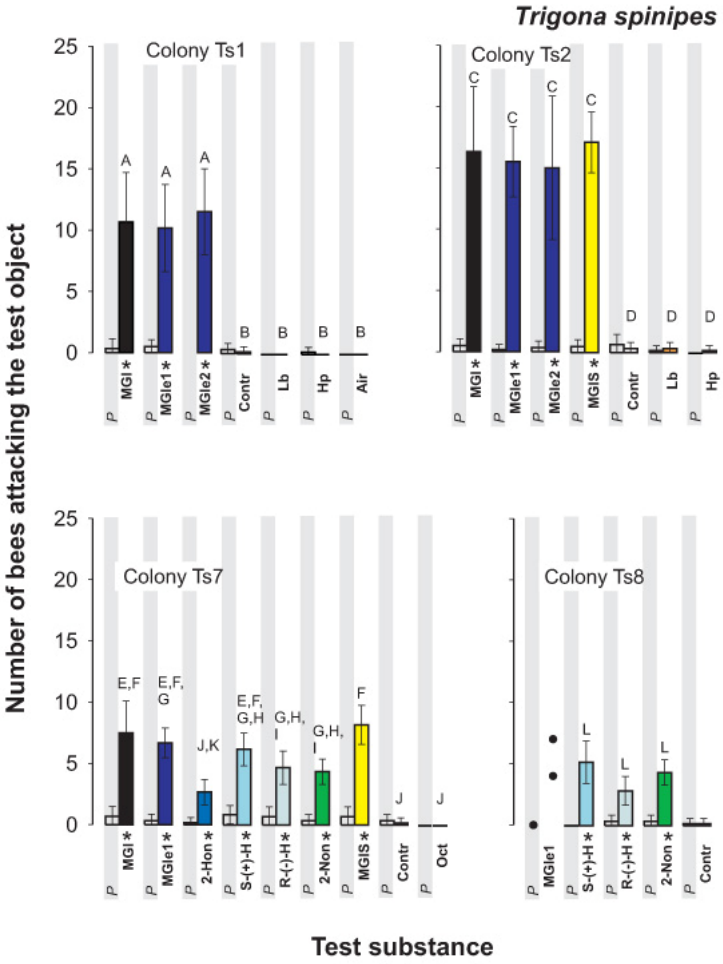

Figure 2. Number of workers of *T. spinipes* attacking a black cotton ball at a distance of 150 cm from the nest within 30 s following the release of the test substance at the nest entrance. Test substances: (i) 0.1 bee equivalents each of the mandibular gland extracts of nestmates or conspecific bees of other colonies; (ii) corresponding amounts of S(+)2-

heptanol, R(-)2-heptanol, 2-nonanol (racemate), solvent (as control), labial and hypopharyngeal glands, air and octyl octanoate (Oct). Also tested: 0.1 bee equivalents of *Scaptotrigona* mandibular glands (MGlS) and a corresponding amount of 2-heptanone. *Significant difference (α=0.05) in bee numbers between P and the substance tested subsequently. Every test was repeated 6 times in each of the seven colonies (except colony Ts8 where MGle1 and its preceding control were both only tested twice). Data for four different nests are shown (colonies Ts1, Ts2, Ts7 and Ts8). Columns with the same letter at the top (except the preceding controls, P, which were not included in the analysis of variance) represent values that do not differ significantly from each other (α=0.05) (means ± s.d.). For other abbreviations see legend of Fig. 1.

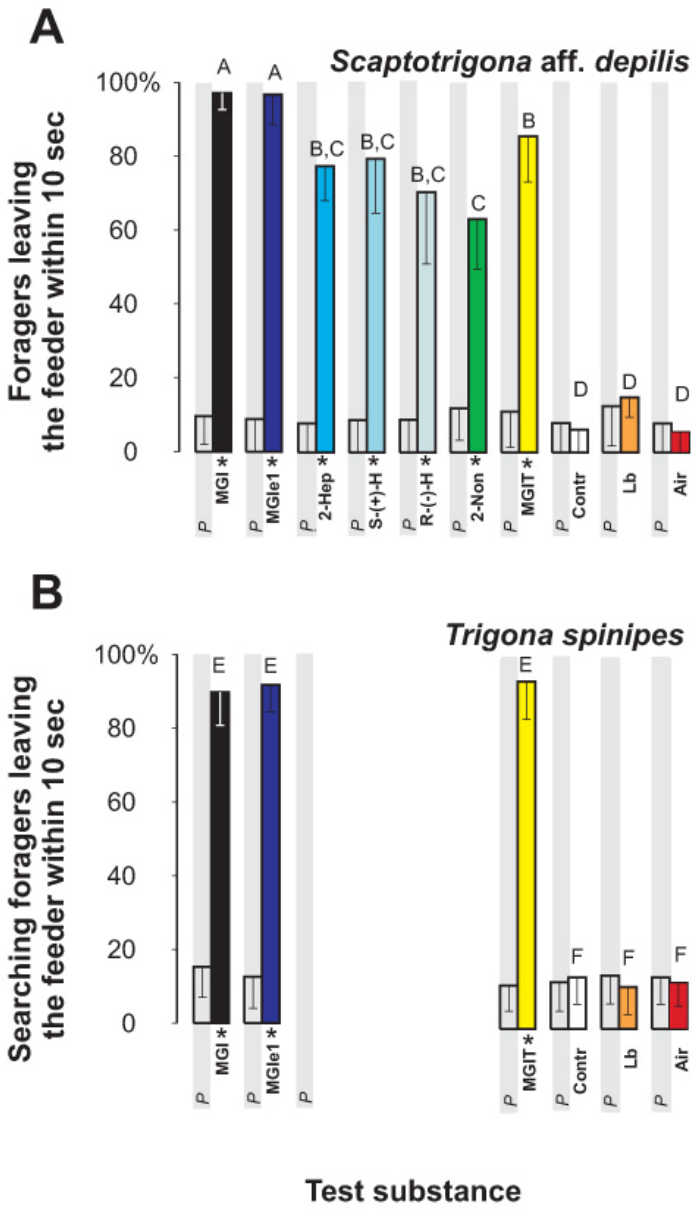
A
Scaptotrigona aff. depilis
Foragers leaving the feeder within 10 sec
100%
80
60
40
20
0
A
A
B,C
B,C
B,C
C
B
D
D
D
P
MGI *
MGIe1 *
2-Hep *
S-(+)-H *
R-(-)-H *
2-Non *
MGIT *
Contr
Lb
Air
B
Trigona spinipes
Searching foragers leaving the feeder within 10 sec
E
E
E
F
F
F
MGI *
MGIe1 *
MGIT *
Contr
Lb
Air
Test substance

Figure 3. Percentage of foragers leaving the feeder within 10 s following the application of scent (open bars) or control substance (solvent pentane); data are medians (bars; six trials each) and the corresponding 95% confidence intervals (negative direction). Substances were tested on feeding foragers of *S.* aff. *depilis* (A) and food-searching foragers of *T. spinipes* (B): 0.1 bee equivalents of mandibular gland extracts from individuals of either the same or other nests, and from individuals of the sympatric species *T. spinipes* or *S.* aff. *depilis*. 2-heptanol (racemate and pure enantiomers) and 2-nonanol (racemate) were only applied in case of *Scaptotrigona*. Control: equal amounts of solvent (pentane), labial gland extract of the same nest or air. Pentane (solvent) was applied preceding (time interval <30 s) each application of the above-mentioned chemicals. *Significant difference (α=0.05) in bee numbers between P and the substance tested subsequently. For other abbreviations see legend of Fig.1.

The effect of mandibular gland extract was not nest specific for either *T. spinipes* (N=6 trials) or *S.* aff. *depilis* (N=6 trials; Kruskal–Wallis: $P>0.05$). According to the pairwise comparisons, *S.* aff. *depilis* reacted significantly less ($P<0.05$) to mandibular gland extracts taken from *T. spinipes* than to its own, whereas *T. spinipes* reacted in the same way to both mandibular extracts ($P>0.05$). No preference for feeders or trails scented with mandibular gland extracts in choice tests *Scaptotrigona* aff. *depilis* newcomer bees never landed on or followed artificial trails in front of the artificial feeders when the trails consisted of mandibular gland extracts or equal amounts of the solvent pentane only. Hardly any bees landed on a feeder at the end of either of the two artificial scent trails. The statistical difference between newcomer numbers arriving at the end of either of the artificial scent trails (T1, T2) was highly insignificant (Mann–Whitney: $P>0.4$; N=5). Neither *Trigona* nor *Scaptotrigona* foragers preferred feeders scented with pure mandibular gland extracts to feeders to which only solvent had been applied (Figs. 4 and 5).

Scaptotrigona bees even avoided feeders scented with mandibular gland extract. When decreasing the amount of mandibular gland extract applied to the feeder, its obvious repellent effect decreased as well (Fig.5A).

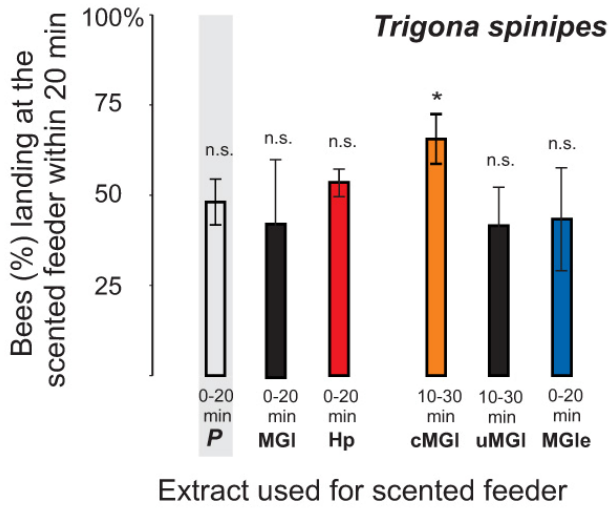

Figure 4.. Percentage of recruited newcomers of *T. spinipes* landing on the feeder scented with one of the test substances in choice tests with the pure solvent (pentane) in the second feeder. Test substances: (i) extracts (0.1 bee equivalents) of hypopharyngeal glands, mandibular glands of the same colony and of conspecific bees of a foreign colony; (ii) pure solvent (pentane) serving as control, and contaminated (cMGl) or uncontaminated (uMGl) extracts of the mandibular gland. The feeders scented with cMGl or uMGl were presented after a post-application delay of 10 min (see Materials and methods). Each test was repeated at least 6 times. *Significant difference ($P<0.05$) between the choice tests shown; n.s., no significant difference ($P>0.05$). For other abbreviations see legend of Fig. 1.

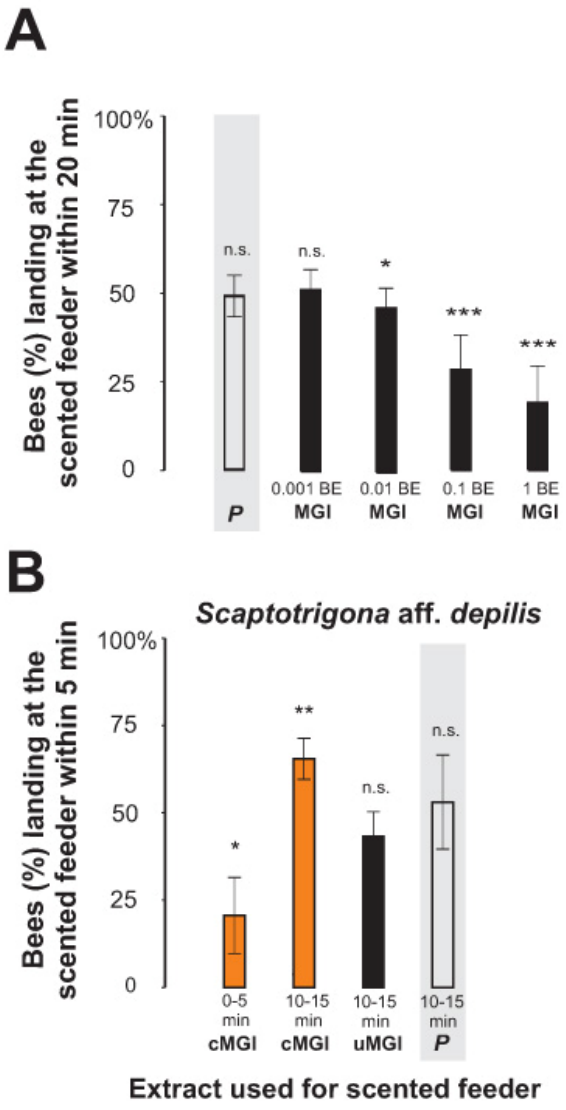

Figure 5. Percentage of *S.* aff. *depilis* bees in choice tests. Bees had to choose between a feeder treated with test substance (in pentane solvent) or a control feeder treated with solvent (equal amount of pentane). (A) Effect of different amounts of

mandibular gland extract. The test substance feeder was scented with 0.001, 0.01, 0.1 or 1 bee equivalents (BE) of mandibular gland extract or pentane for control. (B) Choice tests for the effect of labial gland contamination of mandibular gland extracts. The test substance feeder was scented with 1 bee equivalent of 'contaminated' (cMGl) or 'uncontaminated' (uMGl) mandibular gland extract or pentane for control. n.s., no significant difference; asterisks indicate significant difference (*P<0.05, **P<0.01, ***P<0.001) between scented and unscented (solvent) feeder. For other abbreviations see legend of Fig. 1.

When applying as little as 0.001 bee equivalents, neither a repellent nor an attractive effect was seen (ANOVA, $F_{1,10}$, P>0.6). With 1.0 bee equivalent of mandibular extract often no bee landed on either of the choice feeders (distance from each other, 20 cm) during the first experimental minute. Bees approaching the setup then flew agitatedly in circles and up and down. Obviously the amount of behaviourally active volatiles applied was sufficient to cause an 'agitating' effect within a radius of at least 0.5 m around the feeder. When we applied 1.0 bee equivalent of gland extracts 'contaminated' with labial gland at the test feeder, *Scaptotrigona* bees still avoided landing on it (Kruskal–Wallis, P<0.05, N=34 bees) during the first 5min after application (Fig. 5B). Interestingly, the opposite effect was observed 10 min after application of the same extract. The bees then preferred the 'contaminated' mandibular gland extract (ANOVA, $F_{1,10}$, P<0.005, N=103 bees) and the number of individuals landing on both feeders per

time unit also increased significantly (mean difference at 0–5 and 10–15min=69, ANOVA, $F_{1,10}$, $P<0.001$). The change from a repellent to an attractive effect was never seen when using 'uncontaminated' (without salivary gland) mandibular gland extract. Instead, even after 10min a repellent effect was close to being significant (ANOVA, $F_{1,10}$, $P>0.06$). In contrast to the case in *S.* aff. *depilis* we did not observe a statistically significant repellent effect during mandibular gland extract choice tests in *T. spinipes* (Figs. 4 and 5). However, the ('uncontaminated') mandibular gland extracts never acted as an attractant in any choice test in *T. spinipes* either. But why didn't it repel the bees? The explanation is as follows: choice tests in *T. spinipes* lasted for 20 min. The repellent effect evidently is very strong according to the percentage of bees that abandon the feeder during the first seconds following the application of the mandibular gland extract in the setup described above (see Fig. 3). We conclude that the amount of effective mandibular gland compounds causing avoidance and repellent effects quickly decreases with time. This conclusion is supported by the mandibular gland analysis: both major compounds of the mandibular gland secretions (2-heptanol and nonanal) in *T. spinipes* are highly volatile. Their evaporation is expected to be considerable within the first 20 min. In contrast, octyl octanoate, the most abundant substance of the salivary glands (Schorkopf et al., 2007), which is likely to have caused the attractive effect in the 10–30

min period after the application of the contaminated mandibular gland extracts, is much less volatile. We assume that octyl octanoate therefore is still present after 10 min.

DISCUSSION

Signals eliciting defensive behaviours are thought to significantly contribute to the inclusive fitness in group living or colonial animals (Maynard Smith and Harper, 2003). It therefore is not surprising that pheromones inducing defensive/aggressive behaviours are almost always present in eusocially organized insect societies (Wyatt, 2003). Trail pheromones, on the other hand, which induce trail following of recruits to food sources far away from the nest, are less frequently found but still common in several taxa of termites and hymenopterans. Among flying workers of the Hymenoptera, however, they seem to be found only in the Meliponini. Meliponines also differ from the other hymenopterans in regard to their sting apparatus, which is atrophied in both workers and queens (Abdalla and Cruz-Landim, 2001). In general, meliponines actively defend their nest by biting their offenders. We can only speculate on why the Meliponini have given up their stings as defensive organs. Neither do we know intermediate stages of sting reduction nor the evolutionary forces (e.g. robbers, predators) at work in early meliponines. Ants, phorid flies and cleptoparasitic insects may

have represented very frequent and harmful enemies in the evolutionary past, as they do today (Nogueira-Neto, 1997). If indeed true, stings possibly were not as efficient in the defence against these intruders as other mechanisms like biting or sticky resin deposition near the nest entrance. Considering the lack of a functional sting it would seem useful to have the glands secreting the defensive substances near to the mandibles, the only effective mechanical weapon of meliponine worker bees. The present paper indeed demonstrates semiochemicals in the secretions of the meliponine mandibular glands and their use for colony defence and aggressive communication at food sources. An additional function of the mandibular gland secretions as a repellent/deterrent against predators or resource competitors like ants seems possible but has not been shown yet. One case where a repellent function could turn out to be highly relevant is the food competition observed at honeydew resources. *T. spinipes* collects honeydew from hemipteran species such as *Aethalion reticulatum* Linnaeus 1767, a behaviour we frequently observed during our research. Sympatrically occurring ant species of the genus *Camponotus* (Castro, 1975) intensely forage honeydew from the same species during the day as well as during the night. However, when *T. spinipes* discovers a profitable *A. reticulatum* site it somehow manages to oust the ants, which will only return to the site after dawn when the bees have already left (Castro, 1975) (J. M. F. Camargo, personal

communication). During agonistic encounters *T. spinipes* frequently assumes an aggressive posture with open and ready-to-bite mandibles. We hypothesize that mandibular secretions are released during such encounters. Another hint of a deterrent function of mandibular gland secretions in some meliponines comes from a recent study in Asian meliponines. Ants chose to feed on meliponine individuals 'washed' in hexane or chloroform in preference to unwashed individuals (Lehmberg et al., 2008). Although the authors of this study suggest that plant terpenes on the bees' cuticles are responsible for the deterrent effects, the compounds actually responsible were not identified. The existence of a powerful chemical defence originating from the mandibular gland against vertebrate predators has already been shown for the meliponine genus *Oxytrigona* (Roubik et al., 1987). Some of the substances of *S.* aff. *depilis* and *T. spinipes* listed in the present paper (Table 1) have already been reported to be effective repellents in other insects (Eisner et al., 2005). Benzaldehyde, reported as repellent in ants (Eisner et al., 1978; Eisner et al., 2005) as well as in honey bees (Townsend, 1963; Crane, 1990), is one candidate for a substance with a specific ecoethological significance in *Scaptotrigona*. Similarly 2-heptanone and 2-heptanol are known as 'alarm' or repellent substances in many hymenopterans, including honey bees (Free, 1987; Wongsiri et al., 2006) and several ant species (Vander Meer et al., 1998). A recently accepted patent (US

patent number 6,071,973) by Vander Meer and colleagues (Vander Meer et al., 2000) lists several ant repellents (tested ant species: *Solenopsis invicta*) among which are 2- heptanone, 2-heptanol, 2-octanol, 2-nonanone and 2-nonanol. The latter five are also found in the mandibular gland secretions analysed in the present study (Table 1).

Defence rather than scent path marking

According to our data the mandibular glands of meliponine worker bees produce semiochemicals that elicit defensive and aggressive behaviour but not trail following to food sources. In this regard two points seem to be relevant. (1) The majority of the main volatile components (Table 1) found in the mandibular glands of the species studied in the present work quickly diffuse into the air. This favours prompt communication serving defensive or aggressive actions. High volatility appears unsuitable, however, for scent trails leading to a food source: the recruitment of newcomers takes some time and most of the highly volatile substances are likely to have already largely evaporated. (2) In both *T. spinipes* and *S.* aff. *depilis* mandibular gland volatiles elicit defensive/aggressive action both close to the nest and at the food source (Figs. 1–3). At the food source the bees were never attracted to mandibular gland extract or to individual chemical components contained in it, irrespective of the concentration applied (Figs. 4 and 5). The same was true for artificial scent paths tested in *S.* aff. *depilis.*

For many ants and some termites, details of the glandular sources of trail and alarm pheromones already exist (Kaib, 1999; Kaib, 2000; Wyatt, 2003). In the ants both communicative systems appear to have evolved several times and independently in different taxonomic groups (Hölldobler and Wilson, 1990; Billen, 2006). Different glands play an important role in the two functions. In most studied ant species communicative volatiles secreted from the mandibular glands apparently support defensive or aggressive actions, often even on the trail to or at food sources (Maschwitz, 1964; Leuthold and Schlunegger, 1973; Hölldobler and Wilson, 1990). In contrast we know of no study conclusively showing that the mandibular glands of worker ants play a substantial role in trail laying to food or nest sites.

Pheromones and allomones[6] eliciting aggression and defence

The experiments showing that mandibular gland secretions release aggressive or defensive behaviour not only among nestmates but also among conspecifics of different colonies or even individuals of different species imply that the bouquet of volatiles of the mandibular gland secretions contains both pheromones and allelochemicals.

[6] Allomones are interspecific chemical signals.

2-heptanol and 2-heptanone

2-heptanol and the corresponding ketone 2-heptanone are interpreted as key pheromone or allelochemical[7] substances of meliponine mandibular glands, eliciting defensive or aggressive behaviour in worker bees. 2-heptanol was the only substance identified as a major component in the mandibular glands of bees from all colonies of both species studied. We therefore suggest that it serves as both an intraspecific and an interspecific key 'defence' allomone. Several authors have already attributed an 'alarm' pheromone function to 2-heptanol and its ketone both in meliponines [M. S. Blum and W. E. Kerr, unpublished, cited in Kerr (Kerr, 1969); M. S. Blum, W. E. Kerr, F. Padovani and R. E. Doolittle, unpublished, cited in Blum and Brand (Blum and Brand, 1972)] (Luby et al., 1973; Weaver et al., 1975; Keeping et al., 1982; Smith and Roubik, 1983; Johnson et al., 1985) and in other hymenopterans (Free, 1987; Vander Meer et al., 1998). Kerr and colleagues (Kerr et al., 1981) described an increase in the number of departing worker bees in *T. spinipes* by 20–30% and found *S.* aff. *depilis* to be 'disorganized' at their nest entrance after a small cotton ball treated with 2-heptanol was put in the nest entrance. Note that M. S. Blum and W. E. Kerr [unpublished, cited by Kerr (Kerr, 1969); no quantitative data shown] had previously observed

[7] Chemical substances acting between species (see Whittaker and Feeny, 1971). Note that the originally published paper (Schorkopf et al. 2009) mistakenly wrote "allochemicals" instead.

an attack response when using 2-heptanone at the nest entrance. However, in 1981 Kerr and colleagues (Kerr et al., 1981) were still convinced that 2-heptanol was highly attractive to foragers of *T. spinipes*. Engels and colleagues (Engels et al., 1987) observed a strong alarm response in *S.* aff. *depilis* inside the hive when placing a disc scented with 2-heptanol into the brood or storage area. Similarly, according to Cruz-López and colleagues (Cruz-López et al., 2007), 2-heptanone releases defence behaviour in worker bees of *Oxytrigona mediorufa* using the experimental protocol of Smith and Roubik (Smith and Roubik, 1983). Barrera-Gordillo and colleagues (Barrera-Gordillo, 2005) (R. Barrera-Gordillo and L. Cruz-López, unpublished data) obtained similar results with 2- heptanol and other compounds in *Scaptotrigona mexicana.* The composition of the mandibular gland 2-heptanol regarding the relative share of its two enantiomers is still unknown. However, both meliponine species studied by us reacted to both enantiomers (the difference between the attack responses was not significant). In cephalic secretions of *S.* aff. *depilis* [referred to as *S. postica* Latreille 1811 by Engels and colleagues (Engels et al., 1990)] only the S(+) enantiomer of 2-heptanol was found (Engels et al., 1990). The same applies to all other 2-alcohols of the cephalic secretions analysed by these authors. We conclude that both species do react to both enantiomers even if indeed only one of them [S(+)] is actually secreted by the mandibular glands.

Octyl octanoate: no 'mandibular gland substance'

Our analyses of numerous mandibular glands in *T. spinipes* did not provide evidence for the presence of octyl octanoate. Kerr and colleagues (Kerr et al., 1981) assumed this ester to be one of the main components of the mandibular glands and suggested its role in defence behaviour. However, a significant role of octyl octanoate was indeed demonstrated for the communication of a profitable food source in *T. spinipes*, as was its occurrence in the salivary glands (e.g. cephalic labial glands) of the same species (Schorkopf et al., 2007). The same could be true for *T. silvestriana* of Central America and Mexico, where Johnson and colleagues (Johnson et al., 1985) found similar components in head extracts and possibly mistakenly attributed octyl octanoate to the mandibular glands. When testing octyl octanoate on a filter paper 25 cm upwind of the nest entrance of *T. silvestriana* these authors observed 'erratic flights' of worker bees at the nest entrance which they interpreted as a 'weaker [alarm] response' compared with the strong responses elicited by 2-heptanol and 2-nonanol, other compounds of the cephalic extract. The same effect of octyl octanoate was never observed during our experiments with *T. spinipes*, nor could it be elicited by the salivary gland extracts, which contain octyl octanoate (Schorkopf et al., 2007). Johnson and colleagues (Johnson et al., 1985) were among the first to assume that octyl octanoate is used for scent trail laying (to a food source) in *T.*

spinipes when discussing the paper by Kerr and colleagues (Kerr et al., 1981).

A similar paradigm change: the female-attracting scent trails of male bumble bees

More than 30 years ago a similar change of paradigm was necessary regarding bumble bees. Their mandibular glands were mistakenly believed to produce scent trail substances used by male bumble bees to attract conspecific females (Haas, 1952). According to a review (Blum and Brand, 1972) even 20 years later the scientific community was convinced of the 'mandibular gland hypothesis'. Due to the studies of Kullenberg and colleagues (Kullenberg, 1973; Kullenberg et al., 1973) and those following them, we now know that it is actually the labial glands that produce volatiles attracting females. In fact it may have been the misinterpretation of evidence by Haas (Haas, 1952) that misled Lindauer and Kerr (Lindauer and Kerr, 1958), who referred to this work when proposing that mandibular gland secretions are used for marking a scent trail by meliponines.

Intranidal and internidal communication on the intraspecific and interspecific level

According to our data at least some meliponine species are capable of exchanging aggressive signals between individuals not only of the same (intranidal communication) but also of different conspecific nests (internidal communication). In addition these

species can chemically communicate aggression to a sympatric species (interspecific communication) of a different genus both at the nest (Figs. 1 and 2) and at a food source (Fig. 3). Johnson (Johnson, 1980) had already shown that in *Trigona fulviventris* flight and defensive postures followed the application of synthetic mandibular gland components (a mixture of 2-heptanol, 2-nonanol, 2-nonanone, 2- tridecanol, 2-pentadecanone and 2-heptadecanone, then believed to represent trail-marking compounds) of a competing meliponine {*Scaptotrigona* [*Trigona* in Johnson (Johnson, 1980)] *pectoralis*}. Such communication abilities are in agreement with our findings showing that both *S.* aff. *depilis* and *T. spinipes* bees use 2-heptanol as a major pheromone compound of their mandibular gland secretions. According to the present study the reaction of *S.* aff. *depilis* to the same amount of mandibular gland extract (0.1 bee equivalents) of the sympatric species *T. spinipes* is less pronounced than that to extract of its own glands (Fig. 3). The reasons may be as follows: (1) some volatiles necessary to induce an identical response in *S.* aff. *depilis* are missing in *T. spinipes* glands; (2) no behaviourally relevant volatile is missing but the amount of semiochemicals is too small in *T. spinipes* to elicit an identical[8] response in

[8]The originally published version (Schorkopf et al. 2009) misleadingly printed "to elicit a response" instead of "to elicit an identical response" which contradicts the previous statement above ("...*S.* aff. *depilis* to the same amount of mandibular gland extract (0.1 bee equivalents) of the sympatric species *T. spinipes* is less pronounced than that to extract of its own glands (Fig. 3).". Indeed, the bees *do*

S. aff. *depilis.* With the data at hand we can only argue for reason 2: 2-heptanol is found in much larger absolute amounts in *S.* aff. *depilis* than in *T. spinipes*. By rejecting the 'one gland – two functions' hypothesis (Lindauer and Kerr, 1958; Kerr and Cruz, 1961) we do not deny the possibility that mandibular gland secretions are involved in behaviours other than defence and aggression. However, our new data do argue against their function as scent trail markings, which has often been postulated but was never convincingly proven in any species of the Meliponini. It seems Lindauer and Kerr (Lindauer and Kerr, 1958; Lindauer and Kerr, 1960) themselves already communicated an argument against their mandibular gland hypothesis when writing (Lindauer and Kerr, 1960): 'With certain species of Meliponini, the capture [of newcomers landing on the tested feeder; added remark] must be made very carefully: the bees must not be seized with forceps or touched with any other object, for they then secrete through the mouth a liquid with a characteristic scent, which frightens off both marked bees and newcomers, so that no more bees land on the feeding table; a closer investigation of this phenomenon is still needed'. Such an investigation had not been carried out until the present study.

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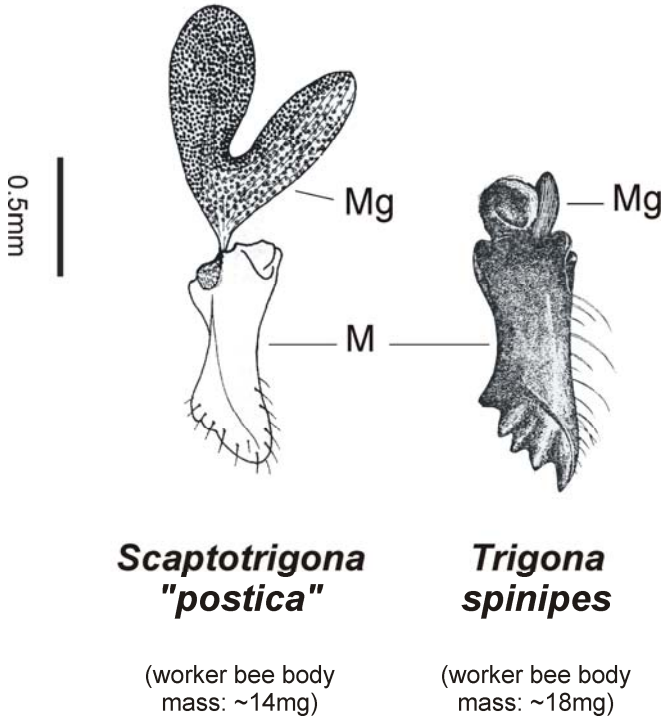
respond to extracts of mandibular glands in both species, but to a different degree.

ACKNOWLEDGEMENTS

We dedicate this paper to the late Martin Lindauer and Warwick Estevam Kerr, the pioneers of meliponine communication research, which now has reached its 50th anniversary. Financial support came from the Austrian Science Fund FWF to F.G.B. (P17530). The input of Robert Twele and Wittko Francke, University of Hamburg, Germany, in the structure assignment of volatiles is gratefully acknowledged. We thank João M. F. Camargo, Geusa de Freitas and Jairo de Souza (University of São Paulo) for their help in finding appropriate bee colonies. J. M. F. Camargo and S. R. de Menezes Pedro helped to verify the identity of the species used for this study and provided us with important biogeographical and taxonomic information about them. Izabel C. C. Turatti and Norberto P. Lopes (University of São Paulo) generously offered access to their chromatographic equipment. We thank two anonymous reviewers for their comments and suggestions. Last but not least the continued hospitality of Ronaldo Zucchi in Ribeirão Preto is greatly appreciated. The present research complies with the current Brazilian environmental laws, SISBIO 65469826, Nr. 15200/1.

APPENDIX CHAPTER II



Appendix C.1 Figure 1. Mandibular glands (Mg) attached to the mandibles (M) of *Scaptotrigona "postica*[9]*"* (original drawing by Oscar Nedel, see Nedel 1960) and *Trigona spinipes* (original drawing by João Maria Franco de Camargo; see Cruz-Landim 1967[10]).

[9] Due to the reasons given in the methods section of chapter II, there are doubts whether the bees dissected by Oscar Nedel, who got his material from Martin Lindauer (his thesis supervisor at that time) and Warwick E. Kerr, were indeed *S. postica* or just were bees looking very similar to this species.

[10] The drawing of the *T. spinipes* glands by J.M.F. Camargo, was unfortunately indicated as a different species in the figure legends in Cruz-Landim 1967; the latter was kindly confirmed by the illustrator himself (J.M.F. Camargo, personal communication).

A traditional rattle manufactured by members of the Kuikuro (Xingú tribe; Mato Grosso, Brazil). The brownish dark wax of meliponine nests is frequently used to seal and to fix loose parts of tools and instruments, such as seen above (note both ends of the calabash). The length of the rattle (a gift from Solange Bispo dos Santos) is about 27 cm.

CHAPTER III

A PHEROMONE TO COORDINATE THE FORAGING AND EXPLOITATION OF HIGHLY PROFITABLE FOOD SOURCES

Spitting out information: *Trigona* bees deposit saliva to signal resource locations

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SUMMARY

Stingless bees of the species *Trigona spinipes* (Fabricius 1793) use their saliva to lay scent trails communicating the location of profitable food sources. Extracts of the cephalic labial glands of the salivary system (not the mandibular glands, however) contain a large amount (approx. 74%) of octyl octanoate. This ester is also found on the scent-marked substrates at the feeding site. We demonstrate octyl octanoate to be a single compound pheromone which induces full trail following behaviour. The identification of the trail pheromone in this widely distributed bee makes it an ideal organism for studying the mechanism of trail following in a day flying insect.

INTRODUCTION

Pheromone trails play an essential role in the exploitation of food resources in populous and eusocially organized insect species (Hölldobler & Wilson 1990; Pasteels & Bordereau 1998; Wyatt 2003). The communication of highly profitable resources is particularly advantageous when these are limited and several species are competing for them (Johnson & Hubbell 1974; Schaffer et al. 1984; Nagamitsu & Inoue 1997; Slaa et al. 1997). So far, in-depth studies

of pheromones used to mark trails showing the way towards a food source have almost been exclusively carried out in insect species with non-flying foragers. Here, we examine the pheromone used by a stingless bee (*Trigona spinipes*) whose foragers are known to lay scent trails guiding nestmates to a food source (Lindauer & Kerr 1960; Nieh et al. 2004). The genus *Trigona* includes some of the most populous species among the stingless bees (Michener 1974; Wille 1983) and species with highly efficient mechanisms of food source communication (Lindauer&Kerr 1960; Kerr 1963; Jarau et al. 2003; Nieh et al. 2004), including the known or assumed use of pheromones. However, only little is known about the glandular origin of trail pheromones and their chemical composition in stingless bees. Foragers of *T. spinipes* do protract their glossae to rub it on the substrate during presumed scent trail marking (Nieh et al. 2004). Our aims were to find out whether (i) *T. spinipes* indeed deposits saliva when landing for scent marking and (ii) the saliva contains any attractive substance or blend of substances representing the pheromone, which induces trail following behaviour. After having found evidence for the presence of such a pheromone we wanted to identify (iii) the active component(s) and (iv) their actual glandular origin. For this purpose, we tested the effectiveness of the synthetic form of the assumed pheromone in behavioural experiments and compared it with that of the natural saliva.

MATERIAL AND METHODS

The experiments were carried out on the Ribeirão Preto Campus of the University of São Paulo (Brazil) between November 2004 and March 2006. We studied seven nests of *T. spinipes* (Fabricius 1793; APIDAE, Meliponini). Photographs of field studies are given in the appendix of this chapter.

Trail following bioassays

The artificial scent trails (T1, T2; 10 m long; figure 1) were laid at distances of 60–180 m from the nest, mimicking the natural scent laying pattern (Nieh et al. 2004). Feeders contained highly profitable (50% w/w), unscented sugar solution. The amounts of either labial gland extract or octyl octanoate (pentane solution) used for the artificially laid scent trails, increased with the distance (values in metres given in brackets) to the branching point (Bp, figure 1) in the following order (bee equivalents dissolved in pentane): 0.0 (at the Bp, 0 m), 0.05 (1 m), 0.1 (3 m), 0.15 (5 m), 0.2 (7 m), 0.3 (9 m) and 0.9 (at the feeder, 10 m). For control trails, the same amounts of solvent were applied. The distance of the recruitment feeder (RF) from the feeders of trails T1 (open circle) and T2 (filled circle) and the Bp was 10 m. In *T. spinipes*, the scent trails become less effective after 20 min (Nieh et al. 2004). We, therefore, renewed the artificial scent trails every 20 min. Any bee landing on the feeder of T1 or T2 was captured. When the newcomer (unmarked) bees landed on the feeder, there were no

other individuals present. In this way, we avoided potential effects of local enhancement (Slaa et al. 2003). All the newcomers were marked with colour and included in the statistical analysis. Thus, every bee included in the statistics was only used once, avoiding pseudoreplication. Fifteen colour marked foragers were allowed to forage at RF to ensure the recruitment of newcomer bees. Any other bee landing on RF was removed from the experiment.

Statistics

For normally distributed data of equal variance, we used the one-way ANOVA to test for significant differences in the percentages of bees that had landed on either of the two tested feeders. Tukey tests were applied for the pairwise multiple comparisons.

Chemical analysis

Approximately 90% of the supposed scent trail markings in *T. spinipes* (Nieh et al. 2004) are deposited within 1m from the food source (in most cases more than 50% at the feeder itself). We therefore analysed the scent marks left by foragers in the immediate vicinity of the feeder, which was a clean Petri dish (diameter, 14–18 cm) supporting the actual feeding dish. Scent-marking bees always had stopped feeding before leaving a scent mark on the Petri dish (extending their glossae again). Therefore, we were sure that the cause of saliva discharge on the Petri dish was scent marking and not food uptake. After the experiment, during which 15 recruiting foragers had scent marked ad libitum for 40 min, the feeder was removed and the Petri dish rinsed with 10 ml of the solvent (Pentane, HPLC-grade). The resulting solution was treated in the same way as the gland extracts. For the extraction (24 h at room temperature) of the glands, which were carefully cleaned from other tissues, we used pentane (HPLC-grade) as well. The extracts were reduced to 60 µl. Internal standard substances (tetradecane and nonadecane) were used to quantify the amount of the detected substances. The percentage of octyl octanoate was calculated by comparing its peak area with the sum of all peaks (except peak areas less than 0.3% relative to the internal standard). Gas chromatographs (HP-5890, HP-GC6890A, Shimadzu GC-2010; carrier gas: hydrogen) with flame ionization detectors were used

for quantitative analysis. For qualitative analyses, we used gas chromatography combined with mass spectrometry (Shimadzu GC-2010/GCMS-QP2010; Fisions Instruments GC 8000 series/MD 800; carrier gas: helium). The column was an Agilent DB-5MS column (30 m x 0.25 mm, 0.25 μm thickness), the temperature programme started at 50°C (5 min) and increased temperature by 10°C min^{-1} up to 310°C (15 min). Compounds were identified by the comparison of mass spectra with literature data (Francke et al. 2000) and authentic reference substances. Octyl octanoate was obtained from Sigma-Aldrich (3050 Spruce Street, Saint Louis, MO 63103 US; Product Number W281107).

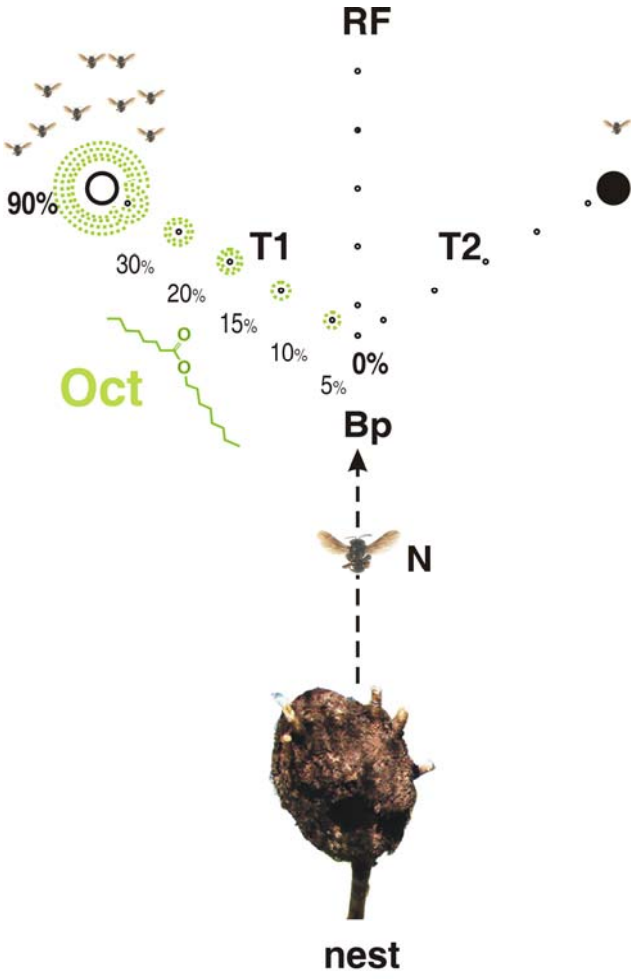


Figure 1 (colour version[1]): Trail following bioassays: two artificial scent trails (T1, T2) were laid (directions of T1 and T2 were switched in consecutive experiments), beginning at Bp, at some distance from the nest. Scents deposited along

[1] Slightly modified from published version

T1 and T2 were either the solvent pentane, pentane extracts of labial glands or synthetic octyl octanoate (dissolved in pentane). The amounts of the solutions forming the trails increased towards the respective feeders, where scent concentration was highest (see bee equivalents given in %). In the experimental set-up illustrated, T1 was scented with either octyl octanoate (dots; Oct) or labial gland extract (naturally containing Oct) leading to the feeder of T1 (open circle), while T2 was scented with equal amounts of pure pentane (no dots) leading to the feeder of T2 (filled circle). Significantly, more newcomers landed on the feeder at the end of T1 than on that of T2. Fifteen bees feeding at the recruitment feeder (RF) ensured the continued recruitment of newcomers (N) from the nest.

RESULTS

Pentane extracts of both the scent-marked substrate and the saliva contained octyl octanoate (saliva: 2.63 µg +/- 0.45 s.e.m.; n = 21 foragers from N = 6 nests), an ester, which turned out to be highly attractive for foragers searching for food (figure 2).

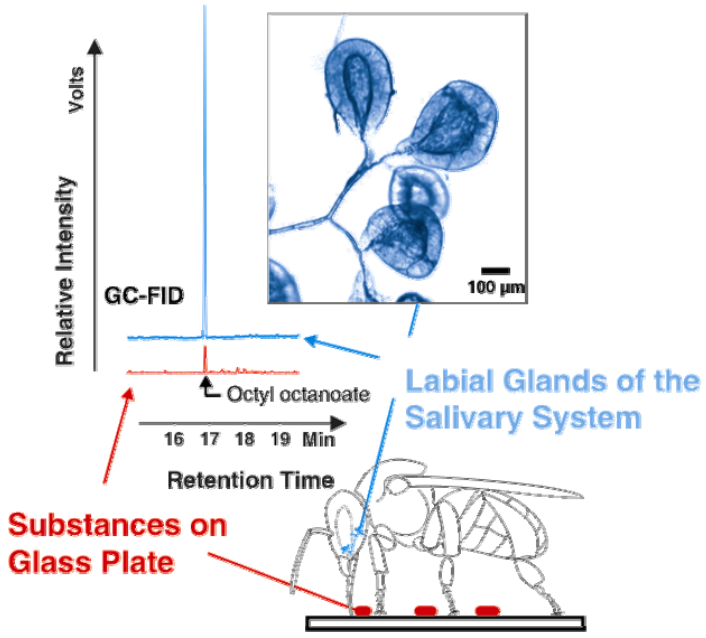


Figure 2 (colour version). Substances inducing trail following: the recruiting forager deposits saliva from its labial glands on substrates outside the nest. Gas chromatography revealed that extracts of both the labial glands and the substances on the glass plate contain a substantial proportion of octyl octanoate (labial glands: 73.8% +/- 2.6 s.e.m, n=30, N=7; substances on glass plate: 24.1% +/- 6.8 s.e.m, n = 6, N = 3).

We found octyl octanoate only in the labial gland parts of the salivary system, not however in either the mandibular or hypopharyngeal glands. In bioassays, synthetic octyl octanoate was used to lay scent trails whose attractiveness was simultaneously tested against another artificial trail consisting of a pentane extract of labial glands or of pure pentane (figure 1). To provoke enough newcomer bees (i.e. bees who had never fed at an artificial feeder before) to visit the artificial feeding site in search for food, we installed RF (figure 1). Clearly, newcomers chose to follow the trails scented with octyl octanoate (89.5% +/- 2.7 s.e.m. out of a total of n=1164 foragers) and labial gland extract (90.3% +/- 2.3 s.e.m. out of a total of n=913 foragers), respectively, and neglected the trail made of an equivalent amount of solvent (ANOVA, octyl octanoate trail versus solvent trail: $F_{1,10}$, $p<0.001$; ANOVA, labial gland extract trail versus solvent trail: $F_{1,12}$, $p<0.001$). As expected from these results, there was no preference when the bees had to choose between following an octyl octanoate trail and a labial gland extract trail (ANOVA: $F_{1,10}$, $p>0.81$; n=300). Likewise, the synthetic compound and the natural extract proved to be equally attractive when presented subsequently. We conclude that octyl octanoate represents the trail pheromone in *T. spinipes*.

DISCUSSION

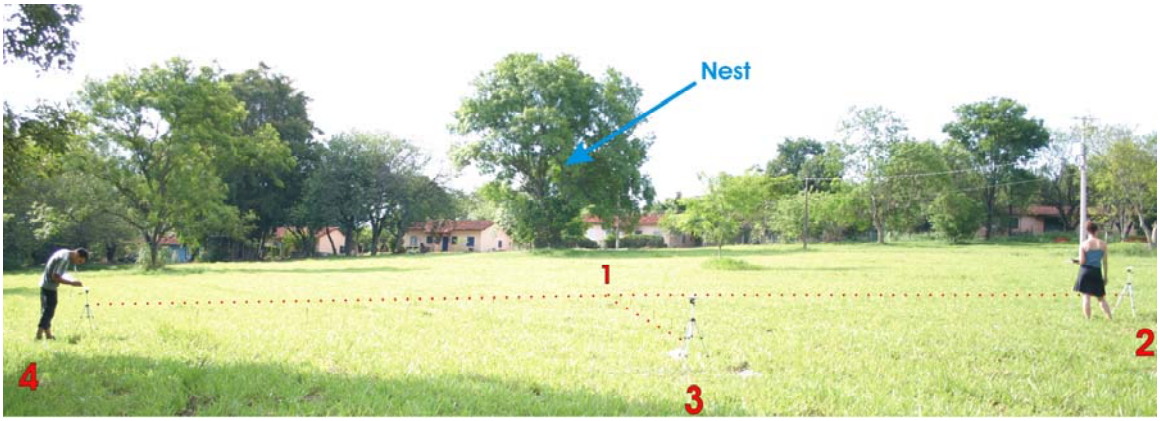
More than 25 years ago, Kerr et al. (1981) pioneered meliponine scent trail communication biology by testing the effect of 2-heptanol (found in cephalic extracts of worker bees) when laid out to form an artificial scent trail in *Trigona* bees. Unfortunately, the results of the only one experiment done can easily be interpreted as providing evidence against 2-heptanol being a food source trail pheromone. The main reasons for this were already discussed earlier (Jarau et al. 2004). Our findings together with those of Jarau et al. (2004, 2006) demonstrate that it is the labial glands which produce trail pheromones in scent path laying stingless bees. Extracts of the labial glands are also highly attractive to newcomer bees in *Trigona recursa*, who follow artificial scent trails made of labial gland extracts as well (Jarau et al. 2004). While we present evidence for a single compound pheromone in *T. spinipes*, in *T. recursa* a blend of compounds not yet fully identified is necessary to induce the full intensity of trail following behaviour (Jarau et al. 2006). A particularly important component of this blend is hexyl decanoate, another ester of similar volatility as octyl octanoate. Obviously, different species of stingless bees use different pheromone compounds for communicating food source locations even when closely related to each other phylogenetically. This makes sense in situations when several species of stingless bees compete for the same resource and use similar

foraging strategies and ways of communicating. Both species, *T. recursa* and *T. spinipes*, indeed occur sympatrically and both feed on nectar. By showing that octyl octanoate represents the trail pheromone or at least its most significant component in *T. spinipes*, a widely distributed species of South America, we not only found a very promising model organism to experimentally study the significance of scent trails for the exploitation of food sources in stingless bees, but also a species to investigate the behavioural and neurobiological mechanisms of three-dimensional trail following in a day-flying insect.

ACKNOWLEDGEMENTS

Financial support came from the Austrian Science Fund FWF to F.G.B (P17530) and the German 'Fonds der Chemischen Industrie' to W.F. We are indebted to Izabel C. C. Turatti and Norberto P. Lopes for generously offering us their chromatographic equipment, and to Linde Morawetz for assistance with numerous trail experiments. We thank the two anonymous reviewers for their valuable comments. We also thank Sidnei Mateus, Geusa de Freitas and Jairo de Souza for their help in finding appropriate bee colonies.

APPENDIX CHAPTER III

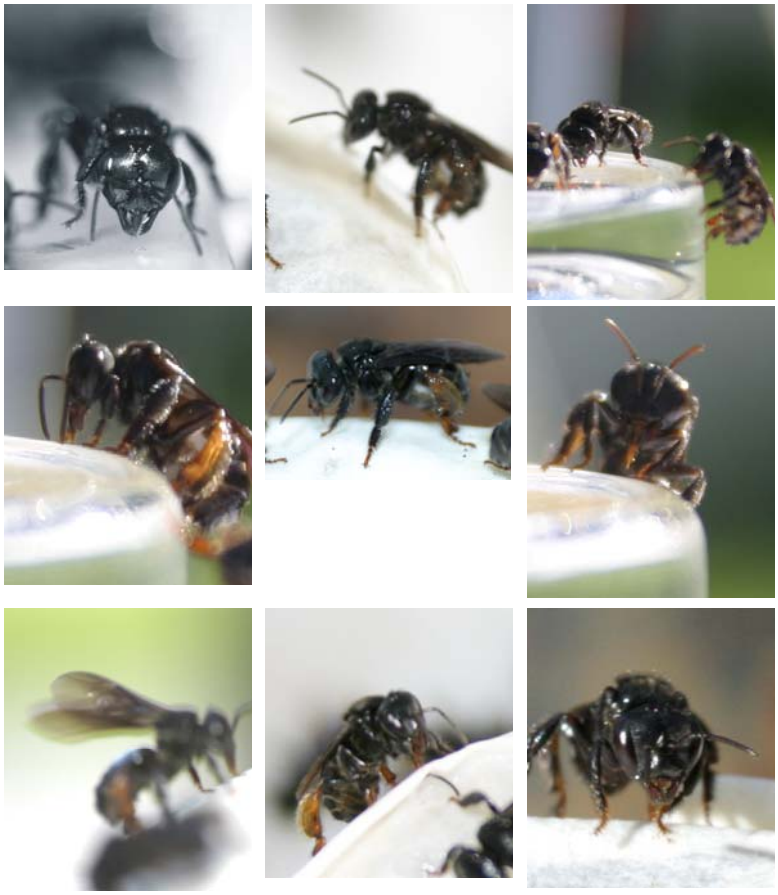


Appendix c.III figure 1. Photograph taken during a trail experiment with *Trigona spinipes* (location of colony Nr.5 in the tree indicated by the blue arrow). The artificial trails (each 10 m long) led from the branching point (1), towards one of the two artificially scented feeders (2, 4). To ensure the arrival of newcomer bees the unscented recruitment feeder (3) was installed between feeders 2 and 4.

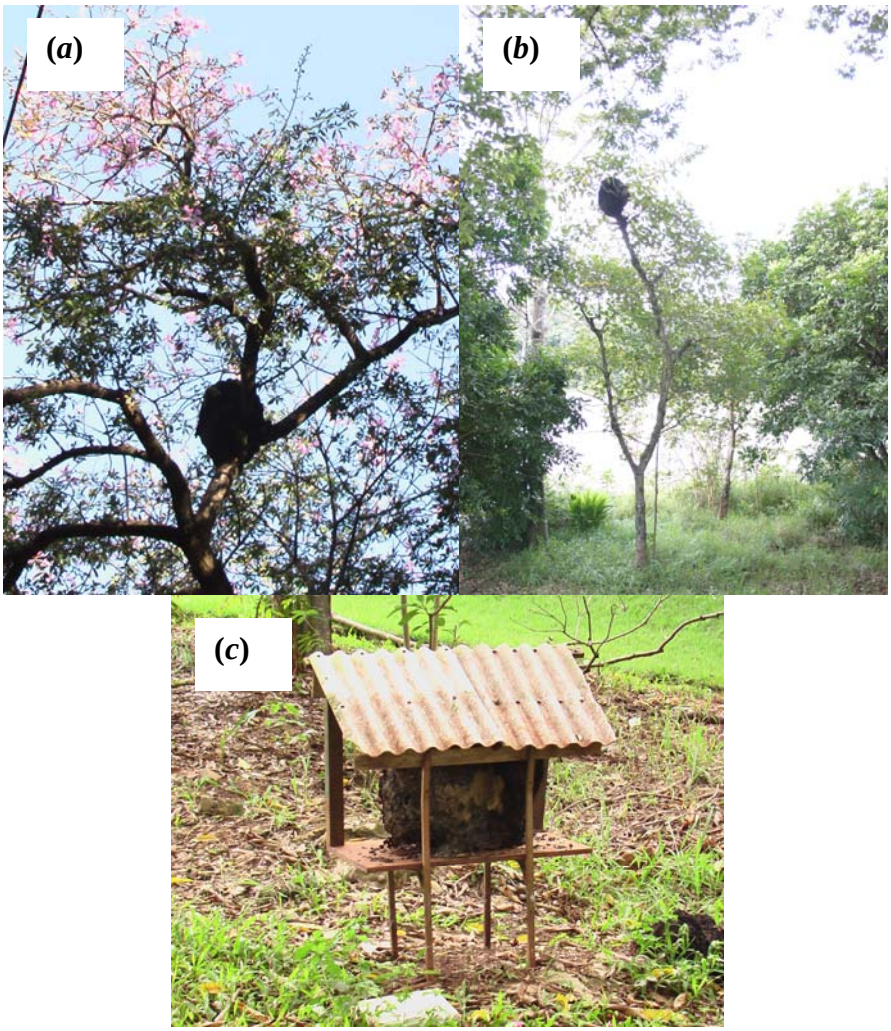
Appendix c.III figure 2. Newcomer bees (*T. spinipes*) feeding at an artificial feeder.

(*a*) **(*b*)**

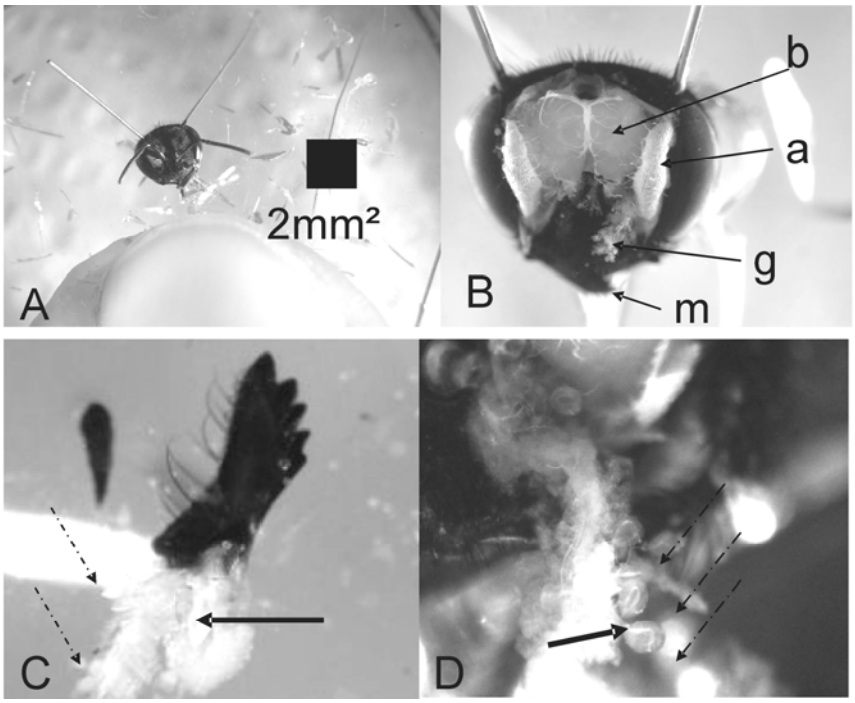
Appendix c.III figure 3. Examples of substrates (leaves of a mango tree, about 27 cm in length) changing their attractiveness (no bees seen in a) for food searching newcomer bees, due to artificial scent marking with either octyl octanoate or salivary gland extract (b).



Appendix c.III figure 4. Scent marking foragers (*Trigona spinipes*) depositing the trail pheromone (octyl octanoate) containing saliva on to the feeder or on to substrates in the nearest vicinity of it.



Appendix c.III figure 5. Examples of colonies of *Trigona spinipes* occurring on the University Campus (USP, Ribeirão Preto). Naturally established colonies (a, b) are usually found high up in the trees. We also worked with colonies kept on the ground (c).


2mm²
A
b
a
g
m
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D

Appendix c.III figure 6. (A) The head of a *Trigona spinipes* worker bee in comparison to a human finger tip. (B)After the first dissection steps in saline solution one is able to see a part of the glands belonging to the salivary system (g) between the air sacks (a) and the brain (b). Due to the small size of the mandibular glands[2] which are connected to the mandibles (m), these are not yet visible. (C) For illustrative reasons, one of the mandibles was cut free from the head capsule. As one can see, the mandibular glands (black arrow) are much smaller than the mandibles to which they are attached. To get clean mandibular gland content, I had to clean these glands of other tissues, such as that of other gland material (labial glands, dashed line arrows) and muscular[3] tissue (white arrow). (D) Parts of the pheromone trail pheromone containing labial glands (dashed line arrows) often got stuck in the mass of hypopharyngeal glands (black arrow), which had to be removed before the extraction of either glandular material.

[2] In other meliponines, e.g. of the genus *Scaptotrigona,* these glands are very large and thus obvious at the same stage of the dissection.
[3] The muscles attached to the mandibles are comparatively large in *T. spinipes.* I frequently could not make out any mandibular glands in the latter species unless I cut some muscular mass away.

"Far more important than being lucky is the ability to recognize a lucky moment as such"

Thomas Dyer Seeley

(interview notes taken from memory
during his visit in Vienna 2004)

Sights from the USP Piracicaba Campus (bottom) and the nearby airport (top) 50 years ago (black and white) and today (colour). Black and white pictures are copied from Lindauer and Kerr (1958, with permission). N.C. de Noronha Jr. kindly provided the colour picture of the water tower.

Pheromone path laying (top to bottom) and following (top) individuals of *Scaptotrigona* aff. *depilis* to a highly rewarding food source. Scent marking bees preferred to walk on or near the edges of substrates (in this case leaves of a coffee plant *Coffea arabica*) whilst rubbing their glossae against them.

CHAPTER IV

ADJUSTING THE VIEW ABOUT THE ROLE AND IMPORTANCE OF PHEROMONE PATHS IN MELIPONINES *OR* REVISITING A 50 YEAR OLD EXPERIMENT

Substrate Bound Pheromone Paths in Meliponine Bees: Helpful But Not Obligatory for Recruitment Success

(submitted to the Journal of Experimental Biology on the 20th of September 2009)

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Abstract
Different from single spot pheromone sources chemically marking the location of resources or sexual partners, substrate bound pheromone paths assisting orientation are rarely found among *flying* organisms. Yet, they do exist in meliponine bees (APIDAE, Apinae, Meliponini), commonly known as stingless bees, a group of important pollinators in tropical forests. Worker bees of several Neotropical meliponine species, especially in the genus *Scaptotrigona* Moure 1942, deposit pheromone paths on substrates between highly profitable resources and their nest. Different from results and claims in the past we found that these pheromone paths are not an indispensable condition for successful recruitment but rather a means to further increase the success of recruiters in persuading their nestmates to forage food at a particular location. Our results are relevant for a speciation theory in scent path laying meliponine bees, such as *Scaptotrigona.* In addition the finding that pheromone path laying bees are able to recruit to food locations even across barriers like a large body of water affects tropical pollination ecology and theories on the evolution of resource communication in insect societies with a flying worker caste.

INTRODUCTION

In many superorganismically organized insect societies (Wilson and Sober, 1989; Seeley, 1989; Wilson and Hölldobler, 2005; Reeve and Hölldobler, 2007; Gardner and Grafen, 2009) pheromone trails are eminent orientation cues for individuals recruited to profitable resources. The phenomenon is best studied in the wingless foragers of highly populated ant species which lay pheromone paths along the way towards food sources while running (Hangartner, 1969; Hölldobler and Wilson, 1990; Traniello and Robson, 1995; Vander Meer et al., 1998; Wyatt, 2003; Billen, 2006). Among superorganismic species with a flying worker caste similar pheromone paths are only found in some species of the bee tribe Meliponini which are among the most important pollinators in tropical forests. Thus foragers of some *Scaptotrigona* species use pheromone paths to guide inexperienced nestmates to a food source as was first described by Lindauer and Kerr (Lindauer and Kerr, 1958, Lindauer and Kerr, 1960; Lindauer, 1975). Foragers which had detected a highly profitable food source alighted on solid substrates to leave scent marks on their way back from the food location to the nest; bees recruited in the nest were then able to follow these marks to the food. Until now it has been believed that in *Scaptotrigona* and other pheromone - path laying meliponines the pheromone path is an obligatory necessity for successful recruitment (Lindauer and Kerr, 1958, Lindauer and Kerr, 1960; Kerr and Esch, 1965; Frisch, 1965; Frisch, 1967; Esch, 1967; Kerr, 1969; Blum et al., 1970; Wilson, 1971; Blum and Brand, 1972; Michener, 1974; Lindauer, 1975; Kerr et al., 1981; Nieh et al., 2003; Nieh et al., 2004; Nieh, 2004; Alcock, 2005). This view originated from results obtained

by experiments of Lindauer and Kerr 50 years ago (Lindauer and Kerr 1958, Lindauer and Kerr, 1960; Lindauer, 1975). Their study still provides the most convincing evidence for the need of scent paths in those meliponine species which do use scent path communication. Because bees are not able to lay pheromone paths on water surfaces Lindauer and Kerr trained *Scaptotrigona "postica"* (possibly *S.* aff. *depilis*, see Methods) bees to fly across a lake. Despite large numbers of experienced bees constantly foraging at the highly profitable artificial food source, inexperienced individuals were never observed to be successfully recruited from their nest (located on the opposite side of the lake) unless a leaf bedecked rope (length > 70 m) was installed that crossed the lake above the water surface and provided the substrate for the scent marks.

Kerr (1969) later pointed to the importance of the pheromone paths for meliponine evolution: For *Scaptotrigona* every river or stream was suggested to act as a geographical barrier by inhibiting proper scent path construction and therefore promoting speciation. In line with this assumption Kerr (Kerr, 1969) explained the high number and density of different species of *Scaptotrigona* found throughout the Neotropics.

Another well known meliponine species for which pheromone trails were described is *Trigona spinipes* Fabricius 1793 (Lindauer and Kerr, 1958, Lindauer and Kerr 1960; Kerr, 1969; Kerr et al., 1981; Nieh et al., 2004).

Following several of our own previous observations where *T. spinipes*-foragers seemed able to successfully recruit nestmates even without pheromone paths (Schorkopf, unpublished data), we speculated that *T. spinipes* does not depend on pheromone paths laid out between the feeder and the nest to efficiently recruit

inexperienced bees. These observations motivated us to take a closer look at the relevance of the scent path in *T. spinipes* and to compare and contrast it with the importance of scent paths for recruitment communication in *Scaptotrigona.* Specifically, we asked the following questions: i) Are one or both species (*S.* aff. *depilis, T. spinipes*) tested for scent- path laying (listed in Nieh, 2004; Barth et al., 2008) able to successfully recruit nestmates to food sources *without* pheromone paths leading towards them? ii) If so, why do flying foragers invest any efforts in elaborating substrate bound pheromone paths? iii) What happens if several food sources with or without scent path leading to them are offered simultaneously? Would any newcomer bees arrive at the food sources without pheromone path? iv) What are the implications of the answers to questions i) to iii) for the theory of evolution and speciation of meliponine bees? Hence, this paper does not seek to question the importance of scent marks left at the food source but rather to examine the importance of the substrate bound pheromone path leading towards it.

METHODS

Pheromone path

The term pheromone path in this paper describes a succession of pheromone marks laid down on solid substrates as a series of chemical sign posts at more or less frequent spatial intervals for some notable distance by one or several individuals in order to enable or to support the efficient navigation of other individuals *towards* targets in space. A pheromone path does not include the final destination per se, to which it leads and which may be marked in addition.

Experimental procedures

Several of our experimental procedures 2008 (see also Appendix for additional illustrations) followed those applied by Lindauer and Kerr 50 years ago (Lindauer and Kerr, 1958; Lindauer and Kerr, 1960). To best build on their pioneering studies we did our experiments at the same lake on the Piracicaba University Campus (Lindauer and Kerr, 1958) in São Paulo State, Brazil in February. We used three colonies of *Scaptotrigona* aff. *depilis* (species *depilis* Moure 1942) and one colony of *Trigona spinipes* (Fabricius 1793). Scent trails have been described for both genera (Barth et al., 2008). If not mentioned otherwise, only one colony was allowed to forage during an experiment at a time. While we were able to train *Trigona spinipes* bees of a colony naturally nesting on one of the trees on the Piracicaba Campus (~150 m away from the lake) we had to transfer all *S.* aff. *depilis* colonies from the Ribeirão Preto Campus (USP, São Paulo State) to Piracicaba because no *Scaptotrigona* colonies occurred in the area of Piracicaba Campus. Thus, all the *Scaptotrigona* bees mentioned in the results of this study exclusively belonged to the transferred colonies. These colonies were all located at the same spot on the lake shore close to the Department of Engineering.

The main question of this study was whether foragers were able to successfully recruit (sensu Wilson, 2000) nestmates without the help of a pheromone path. Recruitment, as defined by Wilson (Wilson, 2000), is a special form of assembly (the calling together of the members of a society for any communal activity) by which members of a society are directed to some point in space where work is required.

All bees landing on one of the experimental feeders were marked with permanent colour (acrylic paint) which is well studied for recruitment studies in both tested species (e.g. Nieh et al., 2004, Schmidt et al., 2006) and remains evident on the forager's notum (thorax) for the rest of its

live (Schorkopf, unpublished observations). In this way inexperienced bees (newcomers) could be distinguished from experienced bees and all bees could be classified by colony and experiment and by different feeders in cases where more than one feeder was used simultaneously. In addition to colour marking, we frequently watched out for swarms of presumed freshly recruited *Scaptotrigona* bees flying around the nest entrance, henceforth called "recruit groups". This is because Lindauer and Kerr (Lindauer and Kerr, 1958) had described such assemblies (of up to "hundreds" of recruits) awaiting further signals from recruiting bees assisting them to find their way to the respective food source location. When looking out for recruit groups, we frequently also verified whether the marked bees of the actual recruitment experiment were indeed exclusively flying from or to the actually tested colony, which they always did, as expected.

The feeders rested on tripods about 1 m above ground level and always contained unscented sucrose solutions. During the experiment we used a highly rewarding 1:1 (weight to weight, w/w) sucrose solution. For training purposes less concentrated solutions (6% - 18 % w/w) were used to minimize or avoid recruitment previous to the experiments proper. To get an idea about the recruitment rate at conventional "above ground" situations at the Piracicaba lake surroundings and weather conditions, our first proper recruitment experiments with *Scaptotrigona* aff. *depilis* and *Trigona spinipes* (TS1, SD1; Tab. 1) were performed under conditions similar to those prevailing for other recruitment experiments conducted in the past (reviewed in Nieh 2004 and Barth et al. 2008). We also followed Lindauer and Kerr's (Lindauer and Kerr, 1958; Lindauer and Kerr, 1960) original sequence of recruitment experiments as closely as possible.

The bees are unable to lay pheromone trails on the water surface. To exclude the possibility that they were flying along the much longer way around the lake, rather than

across it, we observed the flight direction of foragers which consistently took the shortcut across the lake between nest and feeder. We often had to use binoculars to increase the effective range of our observations which was especially useful in case of *Scaptotrigona* aff. *depilis* which hardly measures more than 0.7 cm in length. Fifty years ago (Lindauer and Kerr, 1958) the presence of the same lake at Piracicaba between nest and feeder led to a complete cessation of the recruitment of newcomer bees. To make the laying of pheromone paths above the lake possible, in another set of experiments we extended a nylon rope (diameter: about 1 cm) about 0.8 to 1. 5 m above the water surface between a feeder and the nest (one end of the rope fixed to a tree trunk about 1.8 m above ground, its other end to a tractor; Fig. 1). We knotted strips of white cloth (about 15 – 25 cm broad and 50 cm long) onto the rope about every 2 m to provide additional scent marking area. By relocating the tractor along the shore we could change the position of the rope. In this way, any potential bias towards one feeder during the experiments using two feeders simultaneously at different locations (same lake side) could be controlled as well as any potential bias caused by the mere existence of the rope. *Scaptotrigona* aff. *depilis* (often still called *S. postica;* Camargo and Pedro, 2007, see discussion below) foragers are indeed able to lay scent paths on land that fully extend between food sources and their nest (Schorkopf and Morawetz, unpublished data), similar to that described by Lindauer and Kerr (Lindauer and Kerr, 1958; Lindauer and Kerr, 1960; Lindauer, 1975).

The genus *Scaptotrigona* Moure 1942 (Moure, 1942) still contains several species and subspecies of uncertain taxonomic status (including several undescribed species; Camargo and Pedro, personal communication). Several *Scaptotrigona* species actually look very similar and are therefore very difficult to identify at the species level. Fifty years ago the situation was even worse and it seems justified to question the correctness (Camargo and Pedro,

2007) of some of the species names given in Lindauer and Kerr's seminal works (Lindauer and Kerr, 1958; Lindauer and Kerr, 1960). In case of *Scaptotrigona* no species name was provided in the publication of 1958 (Lindauer and Kerr, 1958). In 1960 the same authors referred to "*Trigona (Scaptotrigona) postica"* (Lindauer and Kerr, 1960). However, the actual *Scaptotrigona postica* (Latreille 1807; APIDAE, Apinae, Meliponini) does not naturally exist in the region where Lindauer and Kerr (Lindauer and Kerr, 1958) performed their experiments (J.M.F. Camargo, unpublished data). Provided these authors were working with a colony originating from the same region it seems likely that they were observing a frequent species very similar in appearance. So far this species remains undescribed and is still commonly referred to as "*postica"* (Camargo and Pedro, 2007; J.M.F. Camargo, personal communication). Unfortunately, no specimens are available for reference to the work of Lindauer and Kerr (M. Lindauer, personal communication). In the present study we worked with this as yet undescribed species of the "*Scaptotrigona depilis* group" (J.M.F. Camargo, unpublished data) which we call *Scaptotrigona* aff. *depilis* (species *depilis* Moure 1942). Reference specimens have been added to the collection of J.M.F. Camargo at the University of São Paulo in Ribeirão Preto.

To mark a substrate between food source and nest with trail pheromone, flying foragers of both *T. spinipes* and *S.* aff. *depilis* land on the respective substrate and then contact it with their glossa before taking off to continue flight. This behaviour has been described in more detail for *T. spinipes* (Nieh et al., 2004) and *T. recursa* (Jarau et al., 2004). To find out more about the frequency of pheromone mark deposition on the rope (unfortunately not quantified in the experiment by Lindauer and Kerr, 1958; Lindauer and Kerr,1960) we noted when and where a bee alighted for scent mark deposition on a section of the rope 4 m beyond (referred to as -4m) and 38 m in front of the feeder in the direction to the nest.

During the experiment only a limited number of marked bees (= "experienced bees", Tab. 1) were allowed to continuously forage at a feeder. All other bees were captured and kept in plastic tubes until the end of the experiment when they were marked as experienced foragers and released.

Significance of pheromone paths

In order to find out why the bees lay pheromone paths at all we asked whether newcomer bees would arrive at feeders with no pheromone path in the simultaneous presence of another feeder with pheromone path. In these experiments foragers were allowed to visit one of two feeders only. Importantly, the rope above the lake allowing the deposition of scent marks led to one of the two feeders only.

Note on the "likelihood" of meliponine bees to land on artificial feeders "by chance"

One could argue that some recruited bees in this study may have landed on the feeders not by having followed any recruitment signals of companions but simply by having been attracted by the feeders for other reasons. Different from the likelihood that *experienced* foragers land on an unscented and *unvisited* artificial feeder (feeder type described in Jarau et al., 2000) the likelihood of *inexperienced* bees to land is extremely low. Obviously unscented artificial feeders of the type described in Jarau et al. (Jarau et al., 2000) do not provide enough cues for inexperienced bees to recognize them as potential food sources. Indeed, newcomer bees were never seen to land at unscented artificial feeders unless these were positioned close to the next or close to another feeder (several cm to a few m) to which other bees were heavily recruiting (Schorkopf, unpublished data and observations). Even in these situations (which do not apply to the present study) a small likelihood of visits would not significantly affect the statistical outcome of this study (see Results). Finally, according to our experience gathered in Piracicaba no inexperienced bees ever landed on unscented feeders during the many training procedures or preparations for the actual recruitment

experiments unless experienced bees provided them with the respective cues and signals. These observations were later confirmed by those made at the control feeders in the two respective experiments with *Scaptotrigona* aff. *depilis* (SD5 and SD 6; Tab. 1).

RESULTS

We had speculated that *T. spinipes* does not depend on pheromone paths laid out between the feeder and the nest to successfully recruit inexperienced bees. Under conditions in which the deposition of a pheromone path was impossible *T. spinipes* was indeed still able to recruit newcomers. In three experiments (N = 3; Tab. 1) a total of 158 inexperienced bees reached the feeder after having flown a distance of about 100 m above the lake. In striking contrast to our expectations, derived from the work of Lindauer and Kerr (Lindauer and Kerr, 1958), the recruitment of inexperienced bees (a total of 318) also continued in every *Scaptotrigona* aff. *depilis* colony tested[1] under conditions excluding the deposition of a pheromone path (experiments SD2, SD3, SD5, SD7 and SD8; Tab. 1).

[1] Note that no bees (neither experienced, nor inexperienced) were ever observed to land on the control feeder (same dimensions and content but no foraging bees) positioned at the same distance, but in the opposite direction from the nest during any experiment with *S. postica* (no water barrier between nest and control feeder).

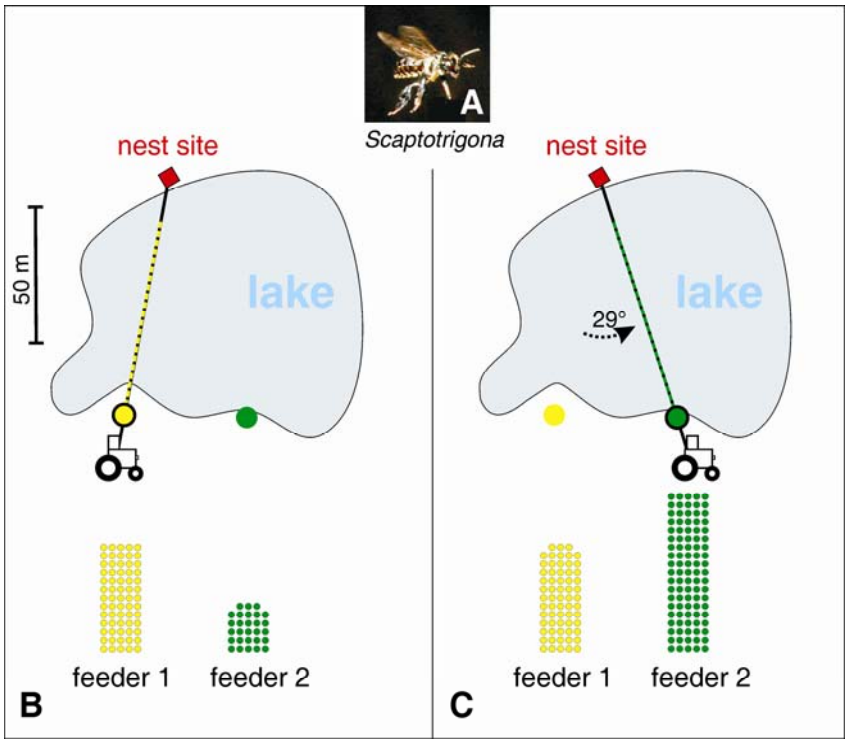


Figure 1. Experiments demonstrating an increase in the effectiveness of the recruitment of nestmates by foragers of *Scaptotrigona* aff. *depilis* (A) by the presence of a pheromone path. (B) The pheromone path across the lake between feeder and nest was made possible by a rope extended between a tree at the nest site and a tractor next to one of two experimental feeders. Simultaneously, equal numbers of foragers were allowed to forage at feeders 1 and 2. Bees foraging at feeder 1 were not allowed to forage at feeder 2 and vice versa. (C) Following the situation shown in B where the rope ended close to feeder 1 (yellow), the rope was shifted to feeder 2 (green). Each small circle (colour corresponding to that of the feeder) represents one newcomer.

Table 1. Recruitment success of continuously foraging bees (recruiting bees) shown for experimental situations in which pheromone path laying was either possible (experiments above the ground or with a rope installed above the lake) or not (no rope installed above the lake). Numbers of inexperienced bees (= recruited bees which have no experience at all with artificial feeders) as well as experienced bees (rerecruited and reactivated bees with previous experience with artificial feeders during other experiments or training sessions) are given.

Experiment	Species	Pheromone path / rope installed? (+ rope if yes)	Recruitment success	Number of bees allowed to forage at artificial feeder (recruiting bees)	Duration of experiment (min)	Number of inexperienced bees/ experienced bees landing on feeder
TS1	*T. spinipes*	**Yes** (no rope)	Yes	73	41	**103** / --
TS2		**No** (no rope)	Yes	133	71	**138** / 57
TS3		**No** (no rope)	Yes	40	30	**6** / 42
TS4		**No** (no rope)	Yes	36	22	**14** / 4
SD1	*S. aff. depilis*	**Yes** (no rope)	Yes	56	40	**89** / 16
SD2		**No** (no rope)	Yes	56	48	**86** / 30
SD3		**No** (no rope)	Yes	56	47	**70** / 22
SD4		**Yes** (+ rope)	Yes	56	44	**51** / 8
SD5		**No** (+ rope)	No / Yes	0	53	**0** / 6
		No (no rope)	Yes	59	53	**71** / 14
SD6		**Yes** (+ rope)	Yes	56	53	**31** / 1
		No (no rope)	No	0	53	**0** / 0
SD7		**Yes** (+ rope)	Yes	56	25	**65** / 22
		No (no rope)	Yes	56	25	**28** / 4
SD8		**Yes** (+ rope)	Yes	5	29	**95** / 0
		No (no rope)	Yes	5	29	**63** / 0

Table 1 (continued). Local conditions: For experiments in which bees flew above the lake the minimum flight distance above the water is shown in addition to the total distance between feeder and nest. Experiments above the ground are indicated by a bold **G**. Main wind directions (relative to the axis feeder-nest = 0°; wind directly blowing from nest to feeder = 180°) given if measured during the experiment (div. = wind direction constantly changing).

Experiment	**Distance between nest and feeder** (m)	**Flight distance above lake** (m)	**Duration** of experiment (min)	Average **wind direction and speed** in m/s	Average Temp. in °C and relative humidity in %
TS1	155	**G**	41	div. / <0.1	29°C / 69%
TS2	240	98	71	0° / 0.13	28°C / 68%
TS3	240	98	30	5° / <0.1	27°C / 68%
TS4	240	98	22	230° / 0.1 /	30°C / 55%
SD1	100	**G**	40	200° / 0.1	29°C / 61%
SD2	89	85	48	div. / <0.1	31°C / 46%
SD3	89	85	47	div. / 0.24	27°C / 67%
SD4	89	85	44	div. / 0.86 /	29°C / 58%
SD5	87	83	53	170° / 0.79	29°C / 56%
	89	85	53	200° / 0.79	29°C / 56%
SD6	89	85	53	190° / 0.64	29°C / 58%
	87	83	53	210° / 0.64	29°C / 58%
SD7	87	83	25	150° / 0.41	27°C / 81%
	89	85	25	120° / 0.41	27°C / 81%
SD8	89	85	29	160° / 0.33	27°C / 79%
	87	83	29	130° / 0.33	27°C / 79%

According to our regular inspections of forager flight directions the bees did in fact cross the lake to reach the food source (as they did in the old experiments by Lindauer and Kerr 1958).

When provided with a rope above the lake between the nest and the feeder S*captotrigona* readily started to deposit scent marks on it. The number of scent marks on the rope strongly accumulated towards the feeder (Fig. 2). No bees were observed to deposit pheromone marks on the rope beyond the feeder (between 0 and -4 m). On a section of 38 m (0 m – 38 m in the direction to the nest) starting at the feeder (0 m) substantially more ($p_{\text{Chi-square-test}} < 0.001$) pheromone marks (%, Fig. 2) were deposited on the segment close to the feeder (0 – 19 m) than on the segment further away from it (19 – 38 m). In fact, 90% of all pheromone marks on the rope were deposited not more than 16 m away from the feeder.

In the following additional experiment we used two instead of only one recruitment feeder. Both recruitment feeders were positioned equidistant to the nest but in different directions and ca. 40 m from each other (Fig. 1). To rule out the possibility that the rope alone could serve as an orientation cue for newly recruited bees, we first installed the rope in the direction of the additional feeder. The trained foragers (Tab. 1), which constantly recruited newcomers in the presence of highly rewarding food sources, were only allowed to visit the feeder without the rope. Importantly, no newcomer bees arrived at the feeder to which the rope led. Successful recruitment, however, was observed again to the feeder to which the foragers were allowed to forage (despite the lack of a rope leading to it). Obviously, the unmarked rope alone did

not serve as a guideline for any newcomer bees assisting them to find their target.

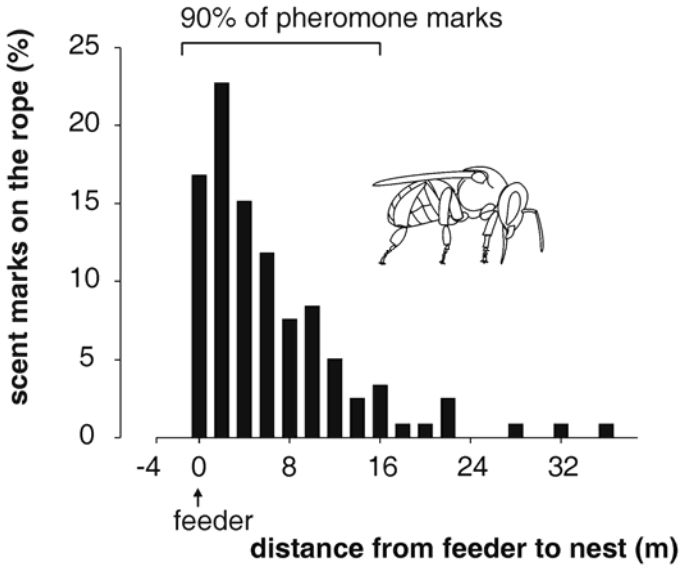


Figure 2. Percentage of marking events (%, n = 238; observation time 104 min) of foragers of *Scaptotrigona* aff. *depilis* on a section of the rope starting 4 m beyond the feeder (-4m) and extending up to 38 m (cluster length = 2 m) towards the nest.

In contrast, either the rope or the artificial feeder seemed to serve as an optical orientation cue to a few experienced bees (marked bees which in the past had already experienced one or several feeding events at artificial food sources; Tab. 1).
In subsequent experiments (SD7, SD8, Tab. 1) foragers were trained to forage at either one of both

simultaneously presented feeders (Fig. 1). Consequently both resulting groups of bees foraging at feeder 1 or 2 (Fig.1) started to recruit bees to their respective feeders. The result of these experiments was that significantly ($p_{Fisher} < 0.00001$) more inexperienced bees turned up at the feeder associated with the pheromone path (Fig. 1). Importantly, in *S.* aff. *depilis* the majority of freshly recruited inexperienced bees predominantly (> 88%; n = 172 bees; $p_{X^2} < 0.001$) approached the feeder in the company of experienced foragers, irrespective of whether a pheromone path led to the feeder they were approaching or not.

DISCUSSION

Pheromone paths: not indispensible for recruitment

Ever since the pioneering study on meliponine communication by Lindauer and Kerr (Lindauer and Kerr,1958) it has been considered a fact (Alcock, 2005) that inexperienced bees of scent path laying meliponine species rely on a pheromone path to find the food source advertised by their foraging nestmates. Most unexpectedly, however, both *T. spinipes* and *Scaptotrigona* aff. *depilis* foragers continued to successfully recruit inexperienced bees (newcomers) even when prevented from laying pheromone paths between the resource and their nest.

According to our observations foragers readily lay pheromone paths along their route back to their nest after having discovered a profitable food source provided there is an appropriate substrate to do so. The latter was not available in several experiments where the bees had to fly above a lake. Lindauer and Kerr (Lindauer and Kerr,1958; Lindauer and Kerr, 1960) already reported that

bees continued trying to deposit a pheromone trail by scent marking the water surface. One bee (bee nr. 106) actually drowned whilst trying. Our own observations of scent marking support these observations, although the bees never seemed to make such sacrifices as described by Lindauer and Kerr (Lindauer and Kerr,1958; Lindauer and Kerr, 1960). With the rope installed, the bees almost instantly used it to build up a pheromone path.
Scent marking on such ropes also occurs in *T. spinipes* (Nieh et al., 2004). According to Nieh et al. (Nieh et al. 2004) the pheromone marks, which consist of saliva from the labial glands (Schorkopf et al., 2007; Schorkopf et al., 2009), are not equally distributed along the rope in *T. spinipes.* Instead their number increases substantially towards the feeder. The same holds for *S.* aff. *depilis* (Fig. 2). However, the stretch of rope starting at the feeder which contained 90% of the scent marks in *S.* aff. *depilis* measured about 16 m whereas in *T. spinipes* almost all mark depositions were observed within 1 m from the feeder.

Why the effort of pheromone path laying?
Because the bees successfully recruited newcomers even without the aid of elaborate pheromone paths, the question is why they invest so much obvious effort (time, energy and saliva from the labial glands) to produce them in the first place. By depositing scent marks the bees take the additional risk of signal interception by competitors ("eavesdropping"), predators and parasites, as shown in some ant species (Wilson, 1965; Hölldobler and Wilson, 1990).
The results of our recruitment experiments using two feeders simultaneously answer the question. Recruitment to the feeder at the end of the pheromone path was

significantly stronger than to the feeder without a pheromone path (Fig. 1). Theoretically, the rope alone (without scent marks) might have served as a visual landmark for the inexperienced recruits. However, newcomer bees have never been observed following an unmarked rope or alightening on the unvisited feeder at the end of the same (see also Lindauer and Kerr, 1958; Lindauer and Kerr, 1960). Obviously, pheromone paths significantly support the orientation of inexperienced bees towards a food source advertised by a forager in the nest. Our finding, that recruitment to the feeder does not depend on a scent path or on the mere presence of the rope per se but still persists to a considerable extent without them, supports our conclusion that in scent path laying meliponine worker bees pheromone paths are not an obligatory necessity for successful recruitment. Remind that our experiments do not argue against the presence of scent marks left by foragers at the food source itself even in the absence of the rope.

Consequences for foraging strategies and speciation

An important consequence of the bees' ability to successfully recruit without pheromone paths is a gain of independence from substrates for pheromone application. This can be advantageous in flooded areas or terrain similarly opposing proper pheromone path laying. In the light of the new findings Kerr's hypothesis of speciation (Kerr, 1969) in the genus *Scaptotrigona* (see Introduction) must be rejected. Large rivers like the river Amazon or large canyons and mountain ridges still form barriers of potential relevance for speciation. However, such barriers would work independent of the availability of pheromone paths.

Societies with a flying worker force

The majority of social terrestrial animals does not possess a flying worker force (Wilson, 1971; Choe and Crespi, 1997; Wilson, 2000, Camazine et al., 2001; Alcock, 2005; Costa, 2006). The added numbers of hymenopteran species possessing a flying worker caste, however, is considerable (several hundreds) with the most prominent representatives being the polistine (Polistinii) and vespine (Vespinii) wasps and the meliponine (Meliponini) and apine (Apini) bees. In the best studied insect society, that of the honey bee (*Apis mellifera*) like in most other societies with a flying worker cast, pheromone paths to food sources have not been reported yet, although they are a common characteristic of non-flying worker caste societies, such as in ants and termites. Hence, Meliponini seem to offer a unique opportunity among flying foragers to study orientation and navigation mechanisms along chemical sign posts.

The effectiveness of pheromone paths typical of so many ant (Hölldobler and Wilson, 1990; Hölldobler and Wilson, 2009; Traniello and Robson, 1995) and termite species (Kaib, 1999; Kaib, 2000; Wyatt, 2003) relies on a relatively steady and even distribution of pheromone marks. The key to explain the difference to the pheromone paths of the meliponines may be the difference in demands associated with navigation and orientation on the ground and in the air, respectively. While non-flying recruiters are able to lay pheromone paths as they walk anyway, flying foragers using pheromone paths as recruitment mechanism must invest time to land for scent marking and also have to cope with an enhanced risk of predation when doing so. It therefore seems more profitable to lay regular and prominent pheromone trails in insects walking on solid substrate than in insects flying to a food

source. “Random flight” in search of a particular odour previously communicated by a forager is another navigational guide meliponines are able to use (Lindauer and Kerr, 1958; Biesmeijer, 1997; Aguilar Mongue, 2004; Nieh et al., 2000; Schorkopf et al., in preparation). However, in the present study the food was unscented so that this possibility is ruled out. Consequently, the establishment of just a few chemical sign posts (target marking; see e.g. Schmidt et al., 2003; Sánchez et al., 2004), and to follow experienced foragers visually and possibly chemically (piloting, guiding flights and local enhancement; see e.g. Lindauer and Kerr, 1958; Slaa et al., 2003; Aguilar et al., 2005), are sufficiently efficient navigational aids for flying foragers searching for targets advertised by their nestmates.

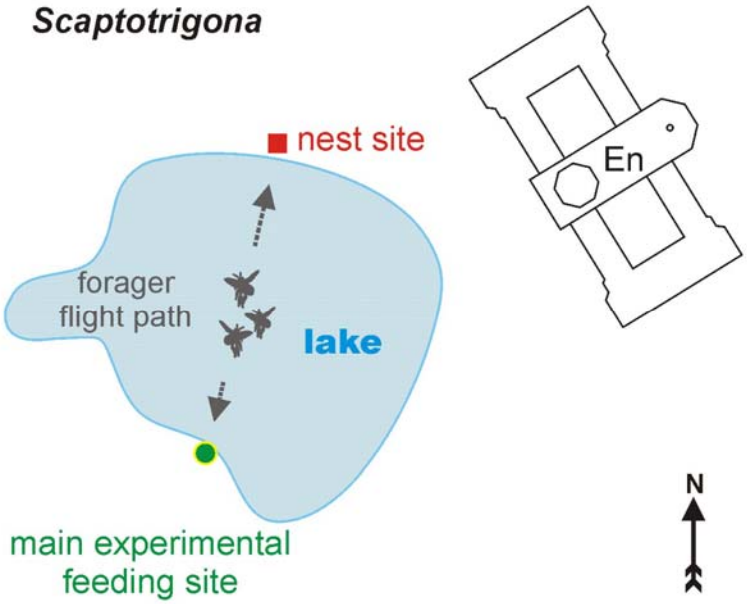
ACKNOWLEDGEMENTS

We dedicate this publication to the memory of two outstanding scientists who contributed seminal work to the study of meliponine bees: Martin Lindauer (1918 – 2008) who opened up so many paths of bee research still worth to be followed; and João Maria Franco de Camargo (1941 – 2009), an exceptional taxonomist and illustrator. We are very grateful to the Headmasters’ Office of the University of São Paulo (Piracicaba Campus) for authorising our work at the lake in front of the engineering building. N.C. Noronha, A.G.C. Signoretti, H.R. dos Santos and J.C.R. Castilho helped us to realize the physically demanding rope-above-the-lake experiments. Supported by the Austrian Science Fund FWF (project P17530 to F.G.B.). The present research complies with the current Brazilian environmental laws, SISBIO 65469826, Nr. 15200/1.

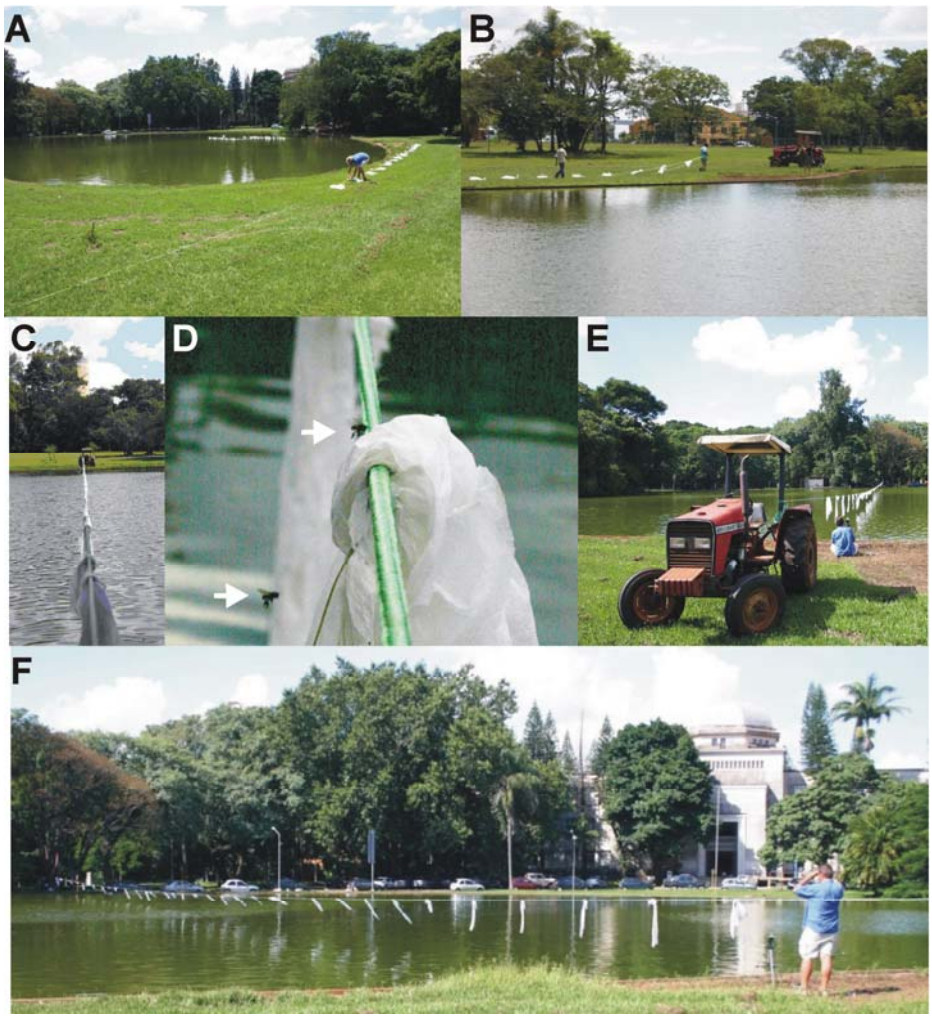
APPENDIX CHAPTER IV

A) B)

Appendix figure 1. A) Example of a typical *Scaptotrigona* aff. *depilis* nest entrance during heavy recruitment activity. **B)** Recruited and recruiting *S.* aff. *depilis* bees at an artificial feeder.



Appendix figure 2. Picture taken during a recruitment experiment with *Scaptotrigona* aff. *depilis* and the corresponding schematic view of the situation during experiments with no pheromone path between the nest and rewarding feeder. The nest was located on the lakeside in front of the Engineering building (En).



Appendix figure 3. Pictures taken during the installation of the rope between the nest and the feeding site. We attached pieces of cloth (**A**) to the rope before fixing the rope to the tractor (**B**). Finally (**C**) the rope was suspended above the lake. This enabled the bees to lay out a pheromone path (**D**, arrows indicate scent marking bees) which we were able to observe (**E, F**). With the help of the tractor it later was possible to shift the rope to other locations on the lake shore.

Appendix figure 4. A picture by Martin Lindauer (printed with permission) during his experiments with Warwick E. Kerr at Piracicaba with *Scaptotrigona sp.* in the late 1950ies showing the leave bedecked rope which they extended across the lake enabling the foraging bees to lay down a pheromone path from the feeder (in front) to the nest (in the back, possibly in the shade at the far end of the rope).

Three *Nannotrigona* bees guarding their nest entrance.

CHAPTER V

ADDITIONAL ASPECTS OF MELIPONINE COMMUNICATION AND ORIENTATION ADDRESSED IN COAUTHOR PAPERS

Preface

In the following chapter I will summarize five papers I had the pleasure to coauthor while working for my PhD thesis. All these five papers (A-E) are strongly related to my previously presented work of which the first authors are co-authors as well.

A Schmidt, V. M., Schorkopf, D. L. P., Hrncir, M., Zucchi, R. and Barth, F. G. (2006) Collective foraging in a stingless bee: Dependence on food profitability and sequence of discovery. Anim. Behav. 72, 1309-1317.

B Schmidt, V. M., Hrncir, M. , Schorkopf, D. L. P., Mateus, S., Zucchi, R. and Barth, F. G. (2008) Food profitability affects the intranidal recruitment behaviour in the stingless bee *Nannotrigona testaceicornis*. Apidologie 39, 260-272.

C Hrncir, M., Schmidt, V. M., Schorkopf, D. L. P., Jarau, S., Zucchi, R. and Barth, F. G. (2006) Vibrating the food receivers: a direct way of signal transmission in bees (*Melipona seminigra*). J. Comp. Physiol. A 192, 879-887.

D Hrncir, M. , Gravel, A.I., Schorkopf, D. L. P., Schmidt, V. M., Zucchi, R. and Barth, F. G. (2008) Thoracic vibrations in stingless bees (*Melipona seminigra*): Resonances of the thorax influence vibrations associated with flight but not those associated with sound production. J. Exp. Biol. 211, 678-685.

E Hrncir, M. , Schorkopf, D. L. P., Schmidt, V. M., Zucchi, R. and Barth, F. G. (2008) The sound field generated by tethered stingless bees (*Melipona scutellaris*): inferences on its potential as a recruitment mechanism inside the hive. J. Exp. Biol. 211, 686-698.

Recruitment in species using scent paths or lacking them

The first two papers give important insights as to how different meliponine species are able to fine tune their colonies' foraging efforts to the profitability of recently discovered food sources. Being a meliponine species which lays down pheromone paths to food sources, *Trigona recursa* is a good example for those meliponines which elaborate on extranidal and therefore field based recruitment communication by use of prominent chemical sign posts (chapter V part A). Species not establishing sophisticated pheromone paths, on the other hand, such as of the genera *Melipona* and *Nannotrigona,* seem rather to rely on intranidal signals such as thorax vibrations or jostling contacts (see chapter V part B for *Nannotrigona* and parts C-E for *Melipona*) to solicit foraging forces to profitable food sources.

Thorax vibrations

Thorax vibrations are known of being produced in many meliponine species. They can be heard by the human observer as sounds, particularly in larger meliponines such as in the genus *Melipona*. Vibrations are produced in different situations of which the best known is during the recruitment of foragers inside the nest by foragers returning from highly profitable food sites. The last three coauthor papers in this chapter (chapter V parts C, D and E) therefore will deal with important questions regarding the production of the thoracic vibrations

their transmission and potential perception in meliponine bees.

Two *Trigona recursa* nests plus their nest entrances (first five pictures; size of nest entrance: ~ 6 – 8 cm) and foragers (body lenght: ~ 6 mm) feeding from an artificial food source (pictures on the bottom; please also note the fly on top of the feeder).

CHAPTER V

A

Collective foraging in a stingless bee: dependence on food profitability and sequence of discovery

(summary of coauthor paper published in
Animal Behaviour, 2006, **72**, 1309-1317)

Veronika M. Schmidt, Dirk Louis P. Schorkopf, Michael Hrncir, Ronaldo Zucchi and Friedrich G. Barth

Summary

We examined the ability of *Trigona recursa*, a scent trail-laying stingless bee (Jarau et al. 2004), to allocate foragers to the more profitable of two food sources. Imbibing time and imbibed volume of individuals were the same at feeders containing 20% or 40% w/w (weight in weight) sugar solution (Fig. 1). However, sugar intake rate and sugar per crop load were significantly higher for the 40% solution, which was therefore more profitable. Collective foraging of two colonies was observed without interference with the recruitment process. One bee was trained to a 20% food source and another at the same time to a 40% source (Fig. 2). Recruitment to both food sources started simultaneously. In all trials the majority of recruits landed on the 40% food source. This cannot

be the result of bees comparing the two sugar concentrations because less than 1% of the recruits landed at both feeders. When we offered the 20% food source 90 min before the 40% source, the newcomers at the 40% food source never outnumbered the newcomers at the 20% source (Fig. 3). Significantly more recruits landed at the less profitable food source. This is likely to be caused by positive feedback resulting from the large number of bees that had already exploited the poor source and reinforced the scent trail. New recruits presumably selected the more intensively marked trail, neglecting the new and weakly marked one that would lead them to the richer food.

FOOD INTAKE AT 20 % AND 40 % (W/W) SUCROSE SOLUTIONS

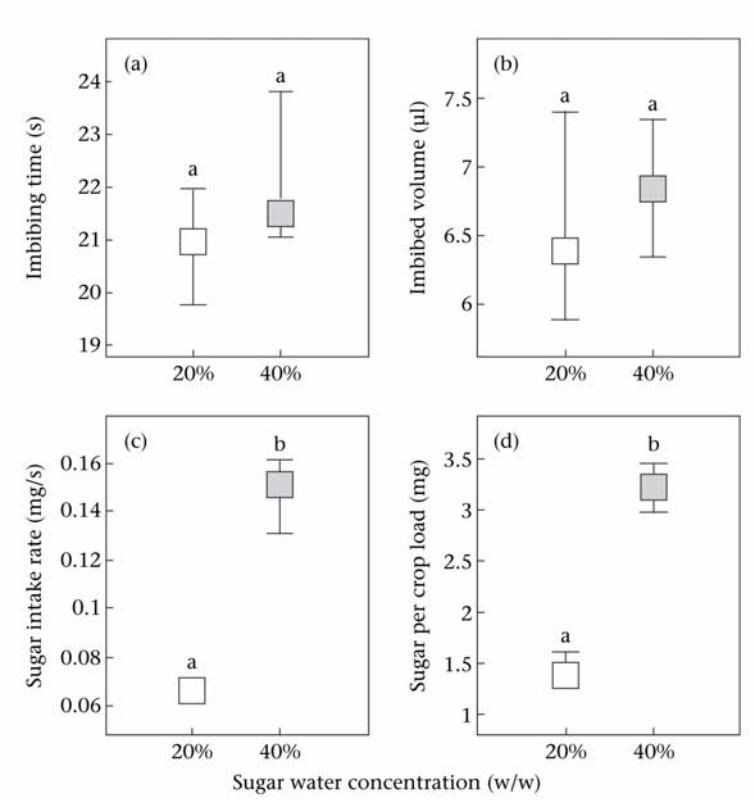


Figure 1: Food intake by *Trigona recursa* at two sugar water concentrations (weight in weight, w/w) each offered individually. (a) Time spent imbibing, (b) amount imbibed, (c) intake rate and (d) amount of sugar per crop load. Data are represented as medians, with vertical lines indicating first and third quartiles. Different letters mark significant differences (Mann-Whitney U: $P < 0.05$) between the groups.

SIMULTANEOUS DISCOVERY OF FOOD SOURCES

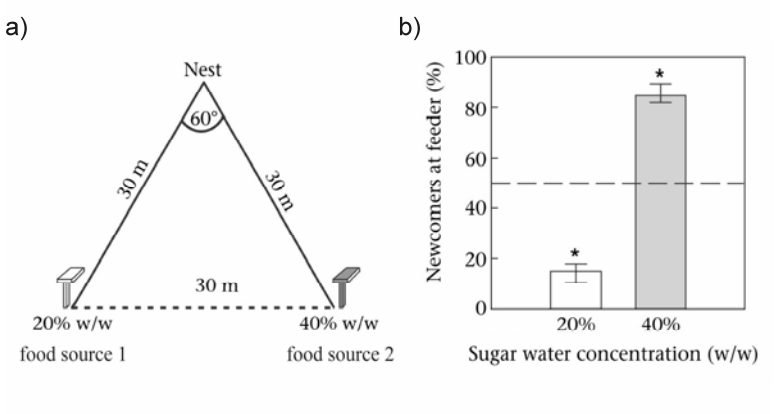


Figure 2:. Recruitment of *Trigona recursa* newcomers to simultaneously presented food sources of different profitability (20% and 40% weight in weight, w/w, concentration). a) Experimental set-up. One forager was trained to the 20% and one to the 40% (weight in weight, w/w) food source. b) Percentages of newcomers at the feeders. The bars represent medians, with the first and third quartiles indicated by vertical lines. 100% = total number of newcomers per trial (median number of newcomers at the 20% / 40% feeder = 12 / 63.5; 1st quartile = 4 / 21; 3rd quartile = 16 / 81). Dashed line indicates a random distribution and asterisks represent significant differences (Mann-Whitney U: $P < 0.05$) from it.

LATE DISCOVERY OF MORE PROFITABLE FOOD SOURCES

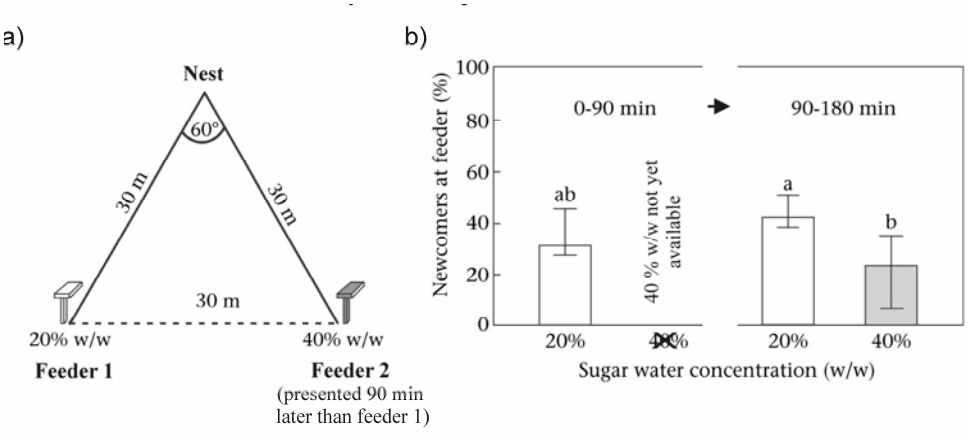


Figure 3: Recruitment of *Trigona recursa* newcomers to food sources of different profitability. The less profitable food source (20% weight in weight, w/w, concentration) is presented 90 min prior to the more profitable food source (40% w/w). a) Experimental set-up: One forager was allowed to forage from and to start recruitment to the 20% food source prior to another forager who was allowed to do the same 90 min later at the 40% food source. b) Percentages of newcomers at the feeders for different time periods after the start of the experiment (0-90 min, when only 20% sugar water solution was available; 90-180 min, when the 40% sugar water solution was presented in concurrence to the 20% sugar water solution. The bars represent medians, with the first and third quartiles indicated by vertical lines. 100% = total number of newcomers per trial (mean number of newcomers at the 20% / 40% feeder = 50.8 / 18.7 +/- 41 / 25 SD). Different letters mark significant differences (ANOVA, followed by Tukey's pairwise comparison: $P < 0.05$) between the percentages of newcomers at the feeders.

Nannotrigona testaceicornis. Top: nest entrance (diameter: ~2-3 cm). Centre: pollen collecting bees (*testaceicornis* ?). Bottom: foragers feeding from an artificial food source (forager size: ~ 4 mm; please note the artificial colour marks on most of the bees' thoraces).

CHAPTER V

B

Food profitability affects intranidal recruitment behaviour in the stingless bee *Nannotrigona testaceicornis*

(summary of coauthor paper published in Apidologie, 2008, **39**, 260-272)

Veronika M. Schmidt, Michael Hrncir, Dirk Louis P. Schorkopf, Sidnei Mateus, Ronaldo Zucchi and Friedrich G. Barth

Summary

Does the food's sugar concentration affect recruitment behaviour in the stingless bee *Nannotrigona testaceicornis*? We recorded intranidal forager behaviour while offering sugar water of constant, increasing, or decreasing concentrations (see Fig. 1 for details on food and sugar intake). Running speed was not correlated with sugar concentration but the jostling contacts/sec were (Fig. 2). Food profitability also affected the recruiter's thorax vibrations (Fig. 3): Pulse duration and duty cycle followed both concentration increases and decreases (Fig. 4). Sugar concentration also influenced the number of recruited bees (Fig. 5). In

comparison to the phylogenetically closely related *Scaptotrigona* (Schmidt et al. 2006), *Nannotrigona's* intranidal recruitment behaviour showed a more elaborate association with food profitability. This is likely to reflect differences in ecology and foraging strategies as *Nannotrigona* – in contrast to *Scaptotrigona* – does not lay scent trails to guide recruits to a food source.

SUGAR INTAKE AT DIFFERENT CONCENTRATIONS

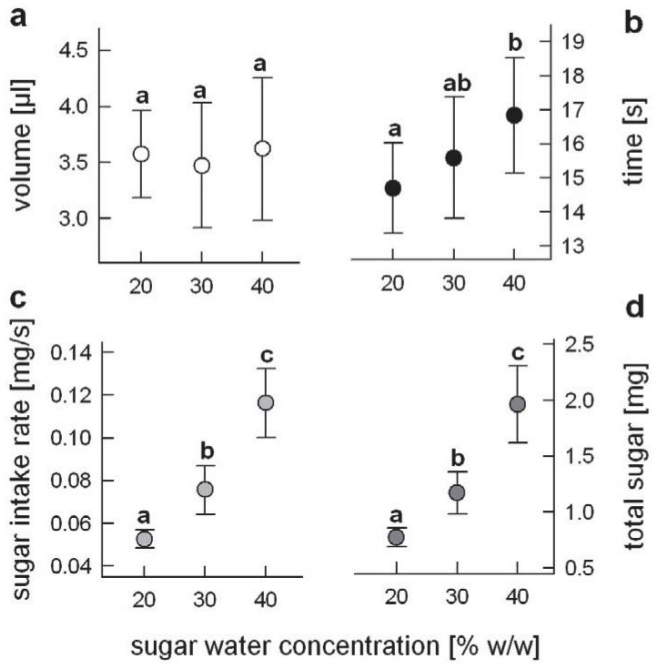


Figure 1: Food intake at three different sugar water concentrations offered subsequently in random order. (a) The imbibed volume did not differ significantly between the concentrations. (b) The imbibing time was significantly longer when 40% w/w sugar water was offered than when bees drank 20% w/w. Both (c) the sugar intake rate (mg/s) and (d) the total amount of sugar per crop load (mg) significantly increased with sugar concentration. Data represent mean ± SD. Different letters mark significant differences between the groups ($P < 0.05$).

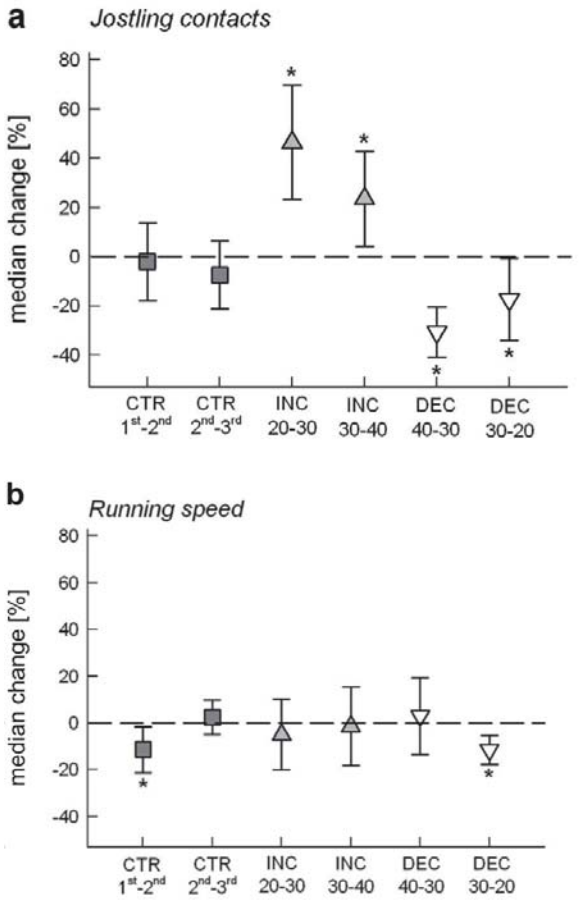


Figure 2: Median changes of jostling contacts (a) and running speed (b) of individual foragers. Control tests: Differences between first and second hour (CTR 1st–2nd) and between second and third hour (CTR 2nd–3rd) are shown. Experiments: changes of median values after each change of sugar concentration (from 20% to 30% [INC 20–30], from 30% to 40% [INC30–40], from 40% to 30% [DEC 40–30], from 30% to 20% [DEC 30–20]). Changes significantly differing from 0% (dashed line) are indicated by asterisks.

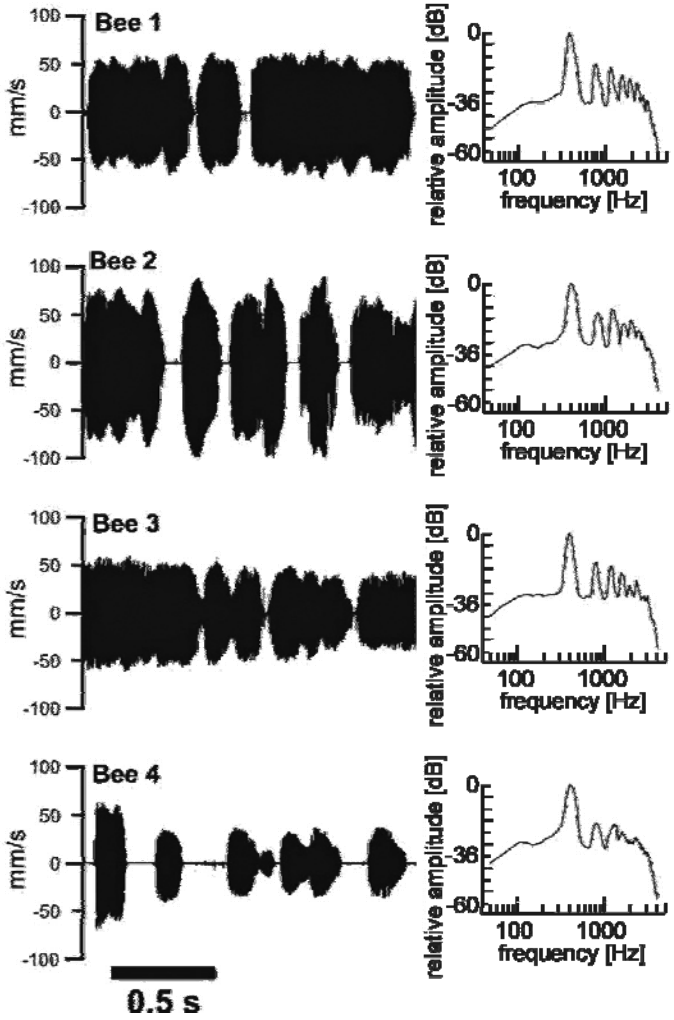


Figure 3: Variability of thorax vibrations of *Nannotrigona testaceicornis*. Sections of typical vibrational signals produced by four different individuals during the first hour of the same control experiment with 30% w/w sugar water offered. Panels on right: each signal's frequency power spectrum (FFT, 1024 pts) with its main frequency component (0 dB) and harmonics.

THE INFLUENCE OF CHANGES IN FOOD SOURCE PROFITABILITY ON THE PRODUCTION OF THORAX VIBRATIONS

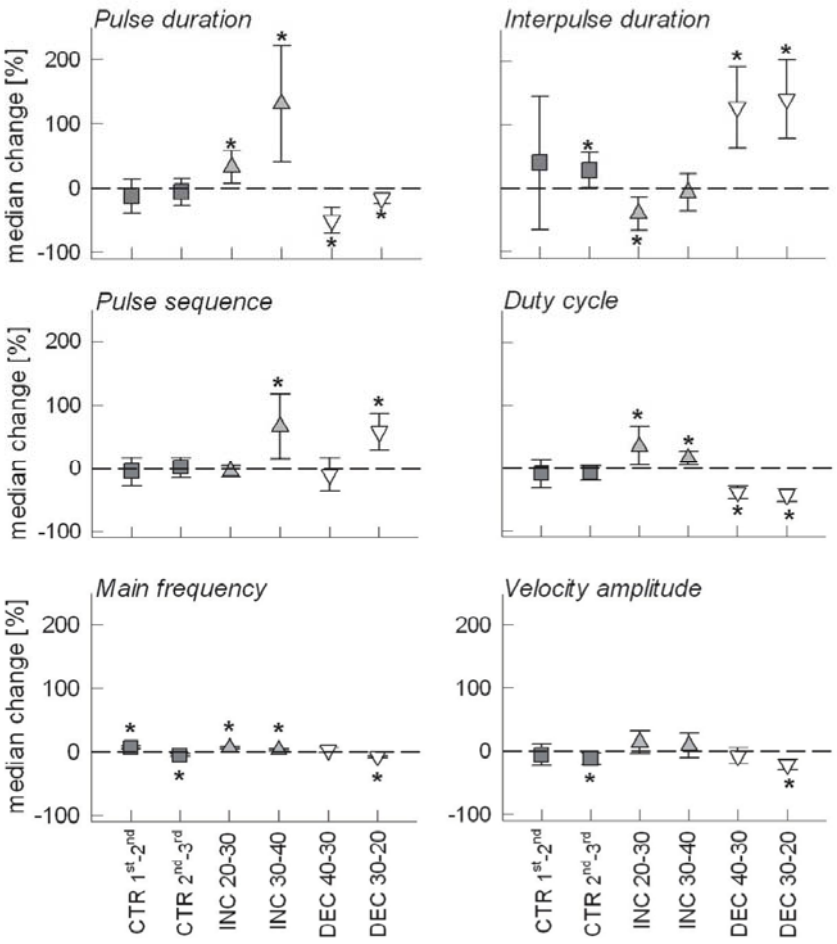


Figure 4: Changes of median values of various vibration parameters of individual foragers during control tests (CTR 1st–2nd hour, CTR 2nd–3rd hour) and after changing the sugar water concentration (increasing concentrations INC 20–30 and INC 30–40, decreasing concentrations DEC 40–30 and DEC 30–20). Values significantly differing from 0% (dashed line) are marked with asterisks.

THE INFLUENCE OF CHANGES IN FOOD PROFITABILITY ON THE RECRUITMENT SUCCES

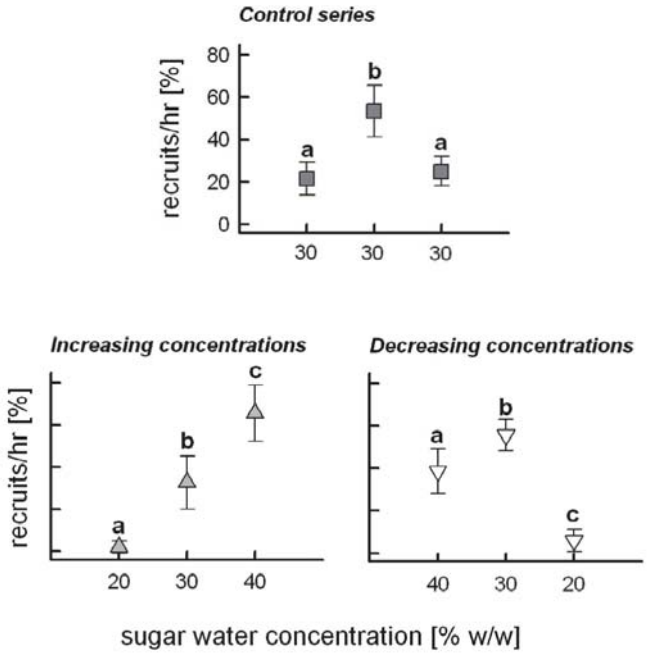
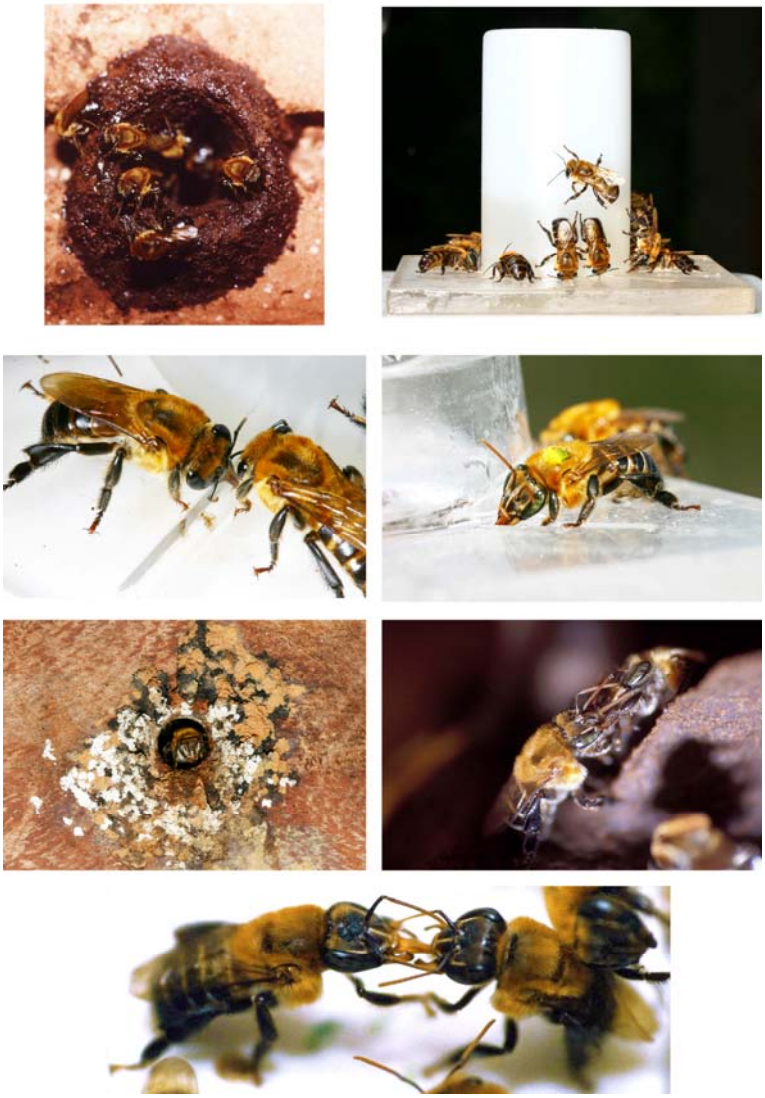


Figure 5: Recruitment to food sources of either constant (control series) or varying profitability (increasing and decreasing concentrations). 100% represents the total number of recruits per trial (3 h; min / max number of newcomers = 9 / 43). Data are mean (± SD) percentages per hour; different letters mark significant differences ($P < 0.05$).



Hive entrance (first and fifth picture), food uptake at artificial feeders (second to fourth picture) and trophallactical interactions (last two pictures) in *Melipona seminigra* (first three pictures) and *Melipona scutellaris*. The size of workers in both species is similar (body length: ~ 10-13 mm).

CHAPTER V

C

Vibrating the food receivers: a direct way of signal transmission in stingless bees (*Melipona seminigra*)

(summary of coauthor paper published in the Journal of Comparative Physiology A, 2006, **192**, 879-887)

Michael Hrncir, Veronika M. Schmidt, Dirk Louis P. Schorkopf, Stefan Jarau, Ronaldo Zucchi and Friedrich G. Barth

Summary

An element common to the recruitment communication of eusocial bees (honey bees, stingless bees and bumble bees[1]) are pulsed thorax vibrations generated by successful foragers within the nest (Hrncir et al. 2006). In stingless bees, foragers vibrate during the unloading of the collected food (Lindauer and Kerr 1958, Barth et al.

[1] Bumble bees actually do not recruit as defined by Wilson (2000; for definition see chapter I) in the strict sense and as used elsewhere in this thesis, since they do not "recruit to a point in space where work is required". However, they show important elements of recruitment behaviour (Dornhaus and Chittka 2004) and are able to mobilize and activate foragers to leave the nest (this form of activation is sometimes called "food alert" or "food alarm").

2008). In the present study on *Melipona seminigra* we demonstrate that during trophallactic contacts, the food receivers are directly vibrated by the foragers (Fig. 1). As a consequence, both the temporal structure and the main frequency component of the forager's vibrations are directly passed on to the receiver (Fig. 2, 3). The vibrations are attenuated by about 17 dB on their way from the forager's thorax (velocity amplitude of the vibrations: ~70 mm/s) to the receiver's thorax (~10 mm/s), the main amount of attenuation (~ 12 dB) occurring during transmission from the head of the forager to that of the receiver (Fig. 4). Vibrations conducted through the substrate between the forager and food receiver are comparatively small (Tab. 1) with velocity amplitudes of 0.3 mm/s. Possible ways of perception and the advantages of vibration transmission by direct contact within the recruitment context are discussed.

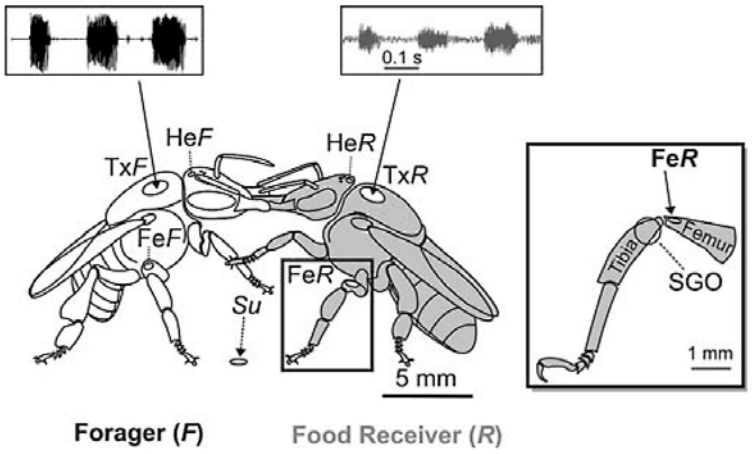


Figure 1: Foragers (F) of stingless bees generate thorax vibrations which can also be measured on food receiving bees (R) during trophallactic contacts. Vibrations were simultaneously measured with two Laser Doppler Vibrometers on the thorax (TxF, TxR), on the head (HeF, HeR), on the femur of the middle leg (FeF, FeR) of forager and receiver, and on the substrate (Su) between the bees. Inset position of receiver's middle leg during food uptake and location of subgenual organ (SGO).

VIBRATION TRANFER FROM FOOD DONATOR TO FOOD RECEIVER

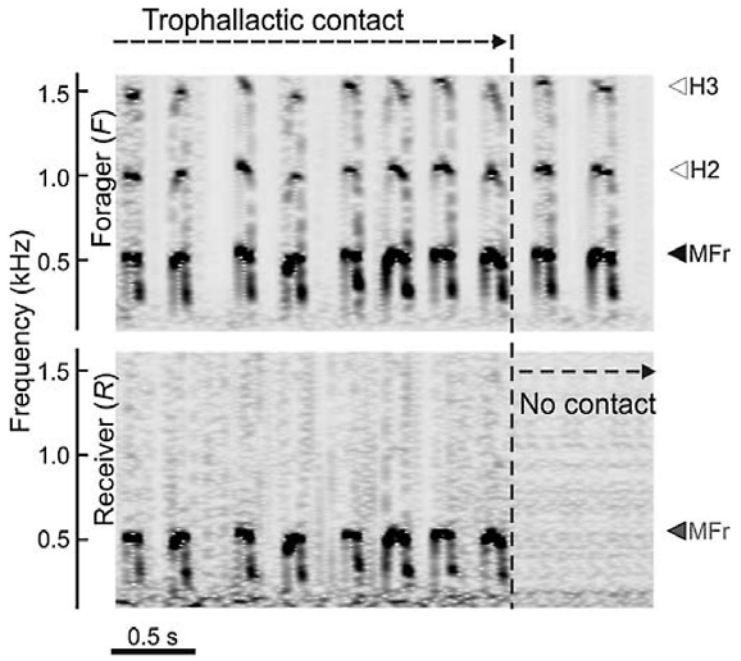


Figure 2: Simultaneous recordings of thorax vibrations generated by a forager (upper panel) and of a food receiver (lower panel) during and shortly after a trophallactic contact. Note pulsed pattern in the forager's ''vibrogram'', typical of *Melipona* bees, the main frequency component (MFr) around 500 Hz and the first two harmonics (H2, H3).

TRANSMISION ACCURACY OF VIBRATION PATTERN

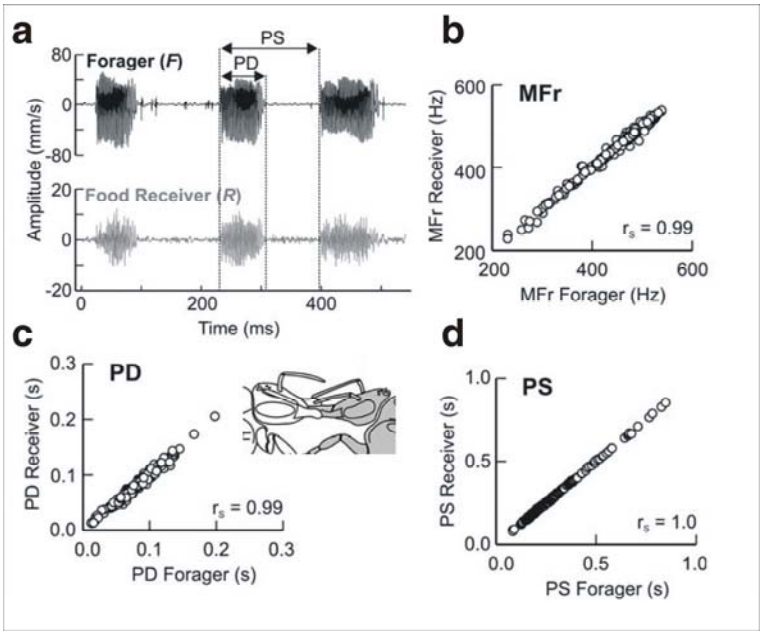


Figure 3: Vibration transmission by direct contact. **a** Typical example of simultaneous recordings from the thorax of a forager and a receiver. **b – d** Comparison of the vibrations recorded from the forager's thorax and from the receiver's thorax. Note similarity of main frequency component (MFr) and the temporal pattern of the vibrations (pulse duration PD; pulse sequence PS) (coefficient of correlation r_S ~1).

ATTENUATION OF THORAX EMITTED VIBRATIONS

Table 1: Vibration transmission during trophallaxis. Given are mean ± SD of simultaneously measured velocity amplitudes (mm/s) (one pair per line), as well as total mean ± SD of each point calculated from all data. Points of measurement were: TxF thorax of forager; HeF head of forager; FeF femur of forager; HeR head of food receiver; TxR thorax of receiver; FeR femur of receiver; Su substrate between forager and receiver.

	Forager			Food Receiver			Substrate
	Tx*F*	He*F*	Fe*F*	He*R*	Tx*R*	Fe*R*	*Su*
Direct Transmission	69.2 ± 19.1				9.75 ± 2.70		
	78.5 ± 12.7	84.4 ± 22.3					
		71.9 ± 16.8		18.9 ± 5.66			
				18.5 ± 5.03	10.1 ± 2.12		
					9.66 ± 3.91	9.96 ± 4.54	
	70.3 ± 18.8				9.40 ± 2.46		
Transmission to Substrate			82.4 ± 11.2				0.40 ± 0.28
	70.9 ± 12.5						0.34 ± 0.13
Total	**73.1 ± 22.3**	**78.6 ± 20.2**	**82.4 ± 11.2**	**18.6 ± 5.07**	**9.72 ± 3.11**	**9.60 ± 4.03**	**0.37 ± 0.18**

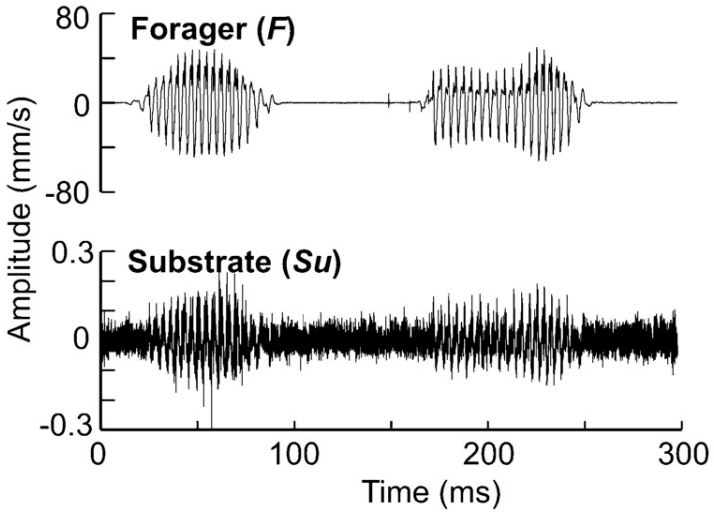


Figure 4: Vibration transmission through the substrate. Typical example of simultaneous recording from the thorax of a forager and the substrate close to the forager's leg. Note difference of velocity scales.

CHAPTER V

D

Thoracic vibrations in stingless bees (*Melipona seminigra*): resonances of the thorax influence vibrations associated with flight but not those associated with sound production

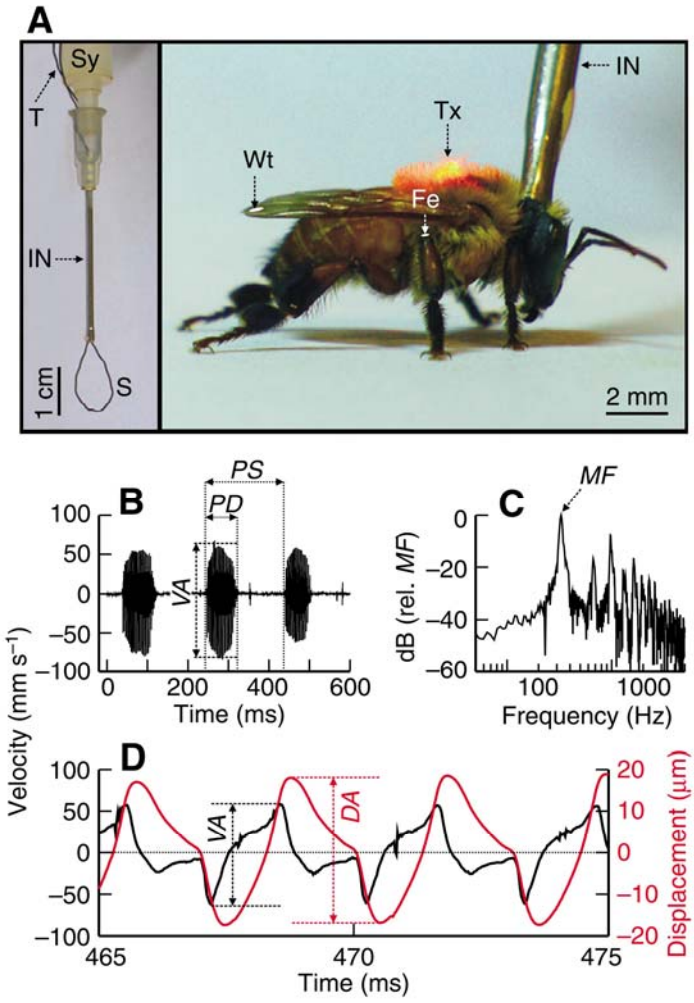
(summary of coauthor paper published in the Journal of Experimental Biology, 2008, **211**, 678-685)

Michael Hrncir, Anne-Isabelle Gravel, Dirk Louis P. Schorkopf, Veronika M. Schmidt, Stefan Jarau, Ronaldo Zucchi and Friedrich G. Barth

Summary

Bees generate thoracic vibrations with their indirect flight muscles in various behavioural contexts (Hrncir et al. 2006). The main frequency component of non-flight vibrations, during which the wings are usually folded over the abdomen, is higher than that of thoracic vibrations that drive the wing movements for flight. So far, this has been concluded from an increase in natural frequency of the oscillating system in association with the wing adduction. In the present study, we measured the thoracic oscillations in stingless bees during stationary flight and during

two types of non-flight behaviour, annoyance buzzing (Fig. 1) and forager communication, using laser vibrometry. As expected, the flight vibrations met all tested assumptions for resonant oscillations: slow build-up and decay of amplitude (Fig. 2); increased frequency following reduction of the inertial load; and decreased frequency following an increase of the mass of the oscillating system (Fig. 3, 4). Resonances, however, do not play a significant role in the generation of non-flight vibrations. The strong decrease in main frequency at the end of the pulses indicates that these were driven at a frequency higher than the natural frequency of the system. Despite significant differences regarding the main frequency components and their oscillation amplitudes, the mechanism of generation is apparently similar in annoyance buzzing and forager vibrations. Both types of nonflight vibration induced oscillations of the wings and the legs in a similar way. Since these body parts transform thoracic oscillations into airborne sounds and substrate vibrations, annoyance buzzing can also be used to study mechanisms of signal generation and transmission potentially relevant in forager communication under controlled conditions.


A
Sy
T
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1 cm
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Wt
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2 mm
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200
400
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Time (ms)
Velocity (mm s−1)
C
MF
0
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dB (rel. MF)
100
1000
Frequency (Hz)
D
VA
DA
100
50
0
−50
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20
10
0
−10
−20
Displacement (µm)
465
470
475
Time (ms)

Figure 1: Stingless bees of the genus *Melipona* generate pulsed annoyance buzzing when tethered by a sling around their neck. (A) Sling-tethering method, showing the sling (S) formed by a nylon thread (T) guided through an injection needle (IN). Sy, syringe for fixing the thread. Vibrations were measured on the thorax (Tx), the distal mesothoracic femur (Fe), and the wingtips (Wt) using a laser vibrometer. Photo showing a sling-tethered worker of *M. rufiventris*. (B) The following parameters of the pulsed vibrations were analysed: velocity amplitude (VA), duration of single pulses (PD), and pulse sequence (PS). In addition the frequency spectra (C) provided the main frequency component (MF). (D) The displacement component (red line; DA: displacement amplitude) of the vibrations was derived by integrating the vibration velocity recorded by the laser vibrometer (black line).

BUILD UP AND DECAY OF THORAX VIBRATIONS

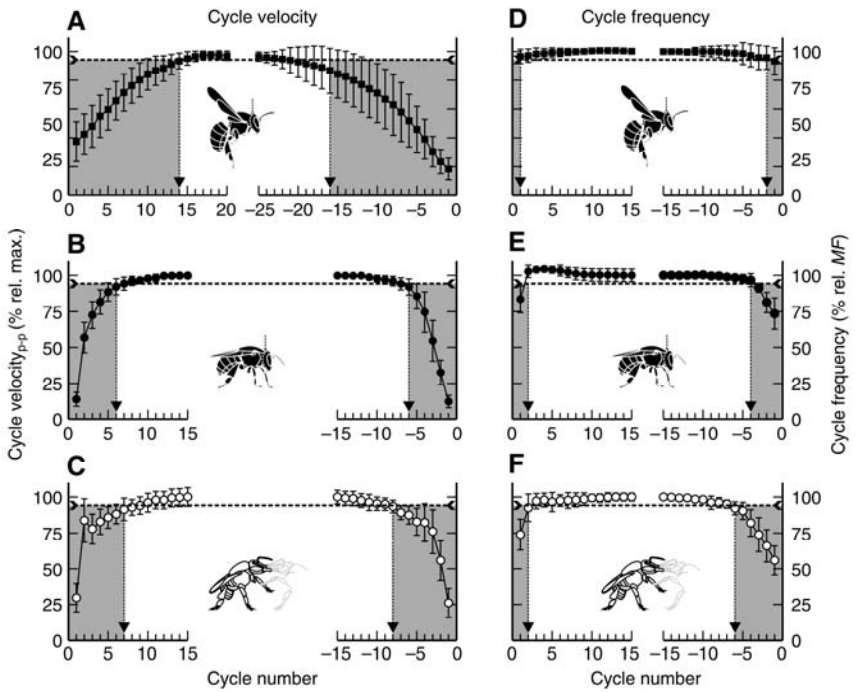

Figure 2: Build-up and decay (shaded area) of cycle velocity (A–C) and cycle frequency (D–F) of thoracic oscillations during stationary flight (A,D; filled squares, N=15), during annoyance buzzing (B,E; filled circles, N=15), and during forager vibrations (C,F; open circles, N=15). Graphs show the means ± s.d. of relative values (percent of the maximum velocity or of the main frequency, MF). Broken lines indicate 95% of maximum.

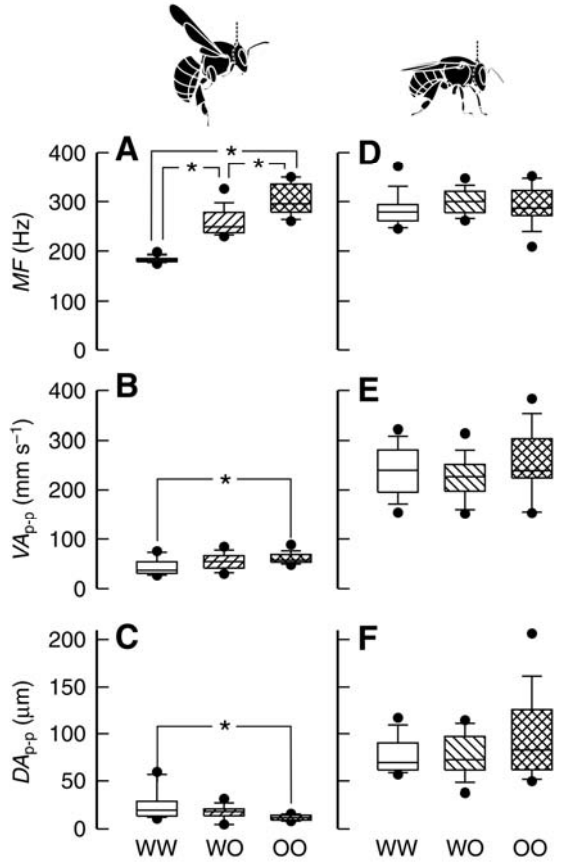

Figure 3: Effect of wing removal on the thoracic vibrations during flight (A–C), and during annoyance buzzing (D–F) of 12 sling-tethered bees. (A,D) Main frequency; (B,E) velocity amplitude; (C,F) displacement amplitude; WW, intact bees; WO, OO, bees after removal of one or both wing-pairs. Asterisks indicate significant differences between the indicated treatments (Dunn's test for pairwise comparison: $P<0.05$). See text for statistics. Box plots indicate inter-quartile range (box), the median value (horizontal line), 95% range (whiskers) and outliers of all data.

EFFECT OF MASS ON THE OSCILLATION SYSTEM

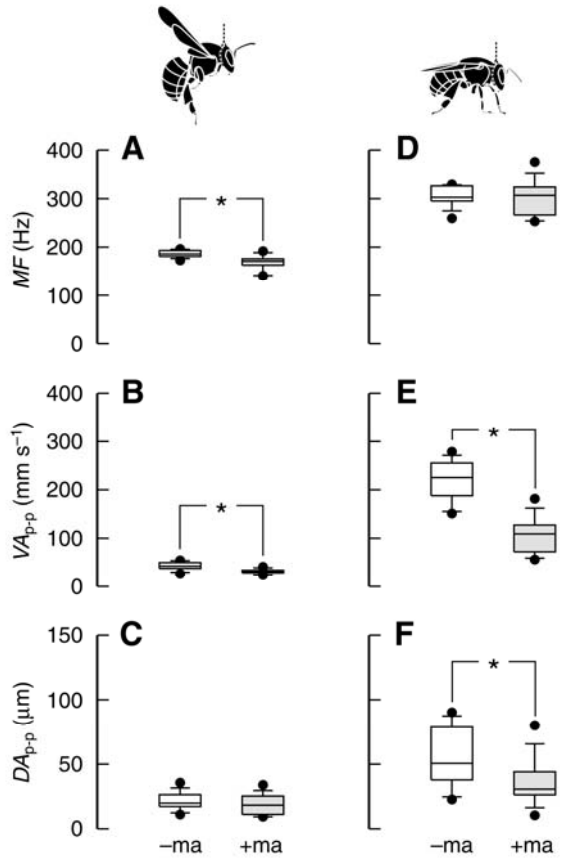

Figure 4: Effect of increasing the mass of the oscillating system on the thoracic vibrations during flight (A–C), and during annoyance buzzing (D–F) of 15 sling-tethered bees. (A,D) Main frequency; (B,E) velocity amplitude; (C,F) displacement amplitude; –ma, bees before adding mass; +ma, bees after gluing a tiny piece of lead onto the thorax. The additional mass almost doubled that of the thorax.

Asterisks indicate significant differences between the treatments (paired t-test: $P<0.05$). See text for statistics. Box plots indicate inter-quartile range (box), the median value (horizontal line), 95% range (whiskers) and outliers of all data.

CHAPTER V

E

The sound field generated by tethered stingless bees (*Melipona scutellaris*): inferences on its potential as a recruitment mechanism inside the hive

(summary of coauthor paper published in the Journal of Experimental Biology, 2008, **211**, 686-698)

Michael Hrncir, Dirk Louis P. Schorkopf, Veronika M. Schmidt, Ronaldo Zucchi and Friedrich G. Barth

Summary

In stingless bees, recruitment of hive bees to food sources involves thoracic vibrations by foragers during trophallaxis. The temporal pattern of these vibrations correlates with the sugar concentration of the collected food. One possible pathway for transferring such information to nestmates is through airborne sound. In the present study, we investigated the transformation of thoracic vibrations into air particle velocity, sound pressure, and jet airflows in the stingless bee *Melipona scutellaris* (Fig. 1). Whereas particle velocity (Fig. 2, 3) and sound pressure (Fig. 4) were found all around and above

vibrating individuals, there was no evidence for a jet airflow as with honey bees (Fig. 5). The largest particle velocities were measured 5·mm above the wings (16.0±4.8·mm·s^{-1}). Around a vibrating individual, we found maximum particle velocities of 8.6±3.0·mm·s^{-1} (horizontal particle velocity) in front of the bee's head and of 6.0±2.1·mm·s^{-1} (vertical particle velocity) behind its wings. Wing oscillations, which are mainly responsible for air particle movements in honey bees, significantly contributed to vertically oriented particle oscillations only close to the abdomen in *M. scutellaris* (distances ≤5·mm). Almost 80% of the hive bees attending trophallactic food transfers stayed within a range of 5·mm from the vibrating foragers (Fig. 6). It remains to be shown, however, whether air particle velocity alone is strong enough to be detected by Johnston's organ of the bee antenna. Taking the physiological properties of the honey bee's Johnston's organ as the reference, *M. scutellaris* hive bees are able to detect the forager vibrations through particle movements at distances of up to 2cm.

MEASUREMENT OF SOUND PARAMETERS

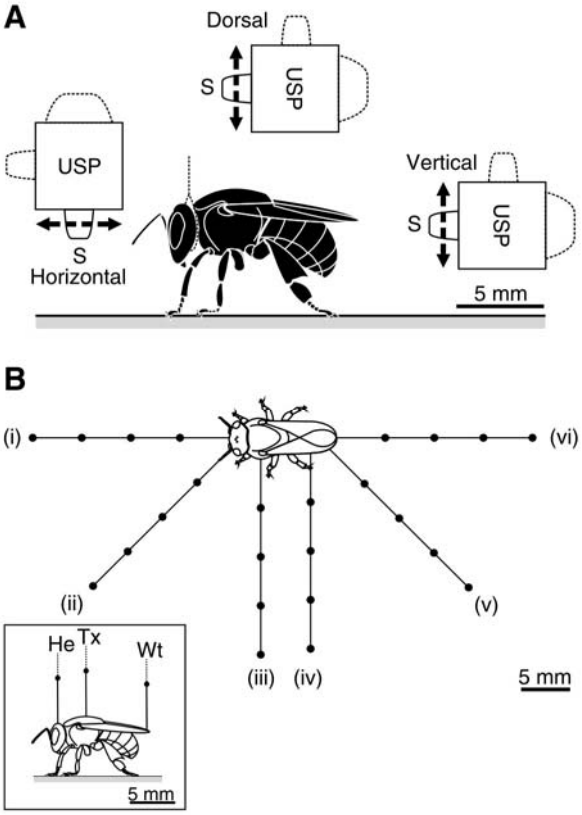

Figure 1: Airborne sound (sound pressure, mPa, and air particle velocity, mm·s^{-1}) generated by sling-tethered stingless bees was measured using a MicroflownTM USP-probe. (A) We measured particle velocity in the horizontal plane around the bee as well as above the vibrating individual. In the horizontal plane, the microphone to measure sound pressure and the airflow sensors (S) to measure air particle velocity either parallel to the substrate or perpendicular to it were kept at a constant distance of 5·mm above the plane acrylic plate used as substrate (15x15·cm2). (B) Sound pressure and air particle velocity were picked up at 24 different measurement points in the horizontal plane around the vibrating bee. The different directions of the measurement points relative to the long axis of the bee were labelled (i-vi). Inset: USP probe positions above the bee's head (He), thorax (Tx) and wingtips (Wt); only measurement points (filled circles) at 5·mm distance are shown.

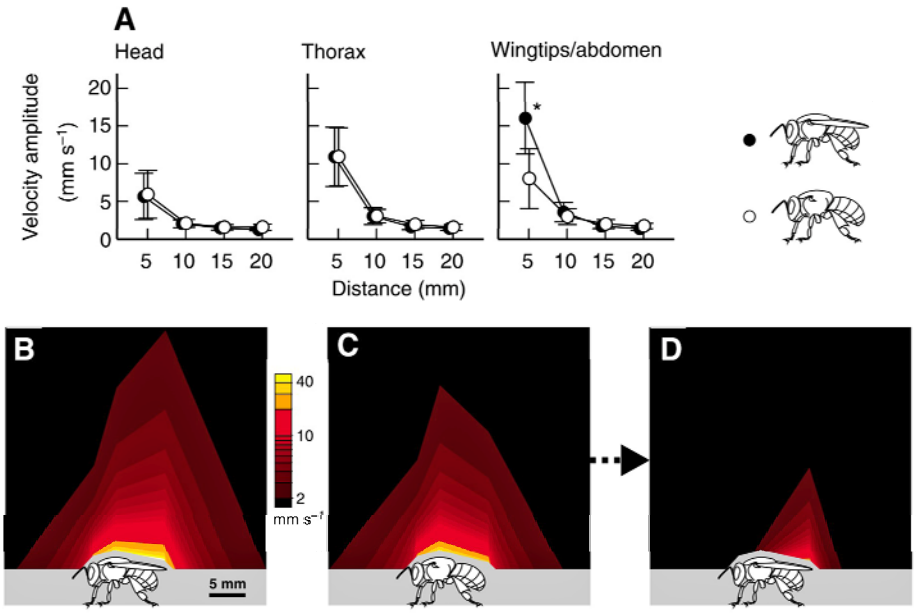

Figure 2: Vertically oriented air particle velocity amplitude VA (p-p) above a vibrating bee. (A) Mean values ±1 s.d. (N=11) above head, thorax and wingtips/abdomen, before (filled circles) and after wing removal (open circles). Circles are slightly displaced horizontally for better visibility. Asterisk indicates significant difference (paired t-test; $P_{corr.} \leq 0.025$) between intact and wingless bees. (B–D) Ranges above the vibrating bee in which air particle velocities had the same mean amplitudes. Different colours indicate mean velocity amplitudes between 2·mm·s^{-1} and 40·mm·s^{-1} as explained by the logarithmic colour scale. (B) Intact individuals, (C) wingless individuals, (D) portion of particle velocity generated solely by wings. Shaded area above bee marks the 1·mm range that cannot be accurately described by decay functions. Because the airflow sensors were positioned at least 5·mm above the substrate, no values are given for the region below 5·mm (shaded area).

HORIZONTALLY ORIENTED OSCILLATIONS

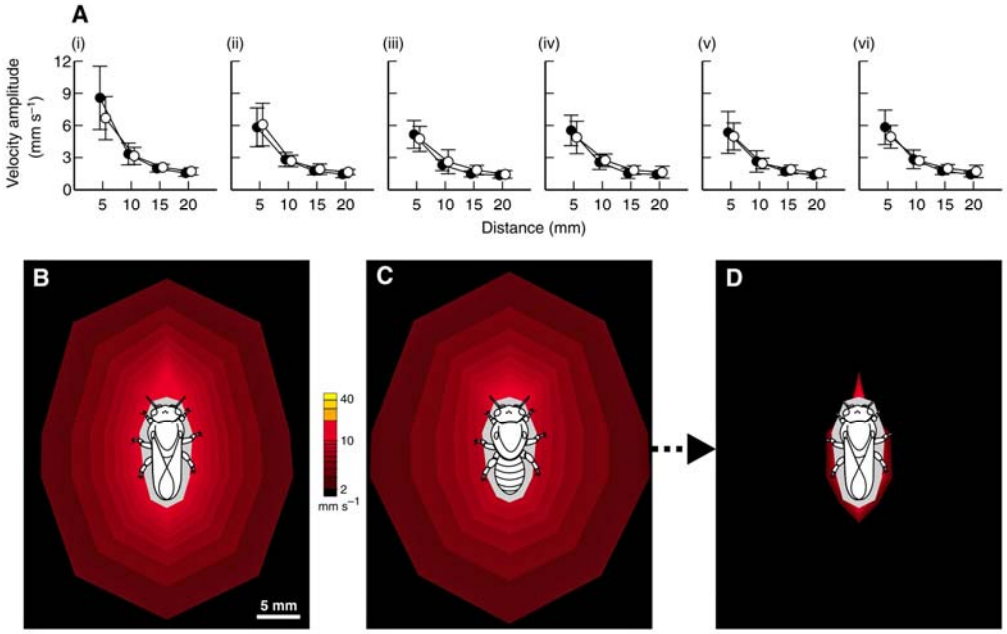

Figure 3: Horizontally oriented particle velocity amplitude VA (p-p) around a vibrating bee. (A) Mean values ±1 s.d. (N=12) at distances of 5, 10, 15 and 20·mm from the vibrating bees at measurement points in different directions relative to the long axis of the bee (i–vi; see Fig.·1); values before (filled circles) and after wing removal (open circles). Circles are slightly displaced horizontally for better visibility. There were no significant changes of values after wing ablation (paired t-test; $P>P_{corr}$, 0.025). (B–D) Ranges around the vibrating bee where particle velocities had the same mean amplitudes Colour scale as in Fig. 2. (B) Intact individuals, (C) wingless individuals, (D) portion of particle velocity generated solely by wings. Shaded area around bee marks the 1·mm range that cannot be accurately described by decay functions.

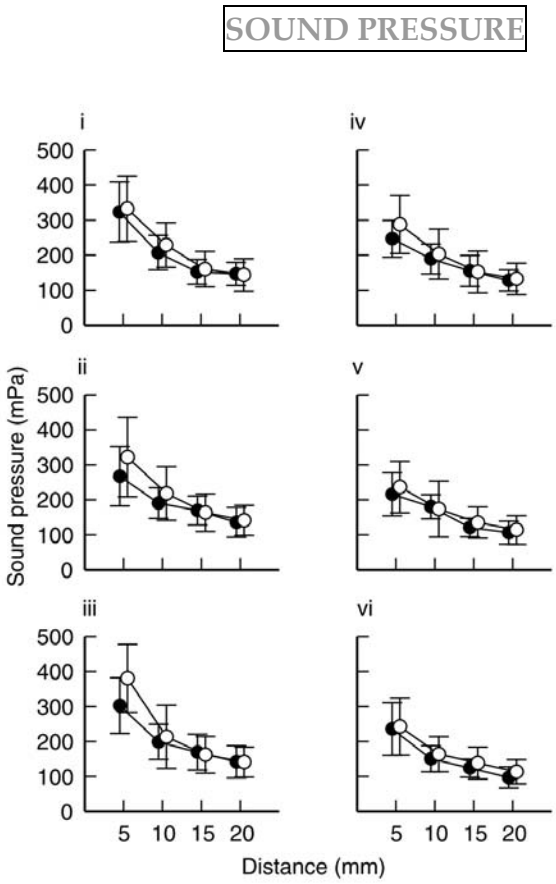

Figure 4: Sound pressure (p-p) around a vibrating bee. (A) Mean values ±1 s.d. (N=12) measured at distances of 5, 10, 15 and 20·mm from the vibrating bees and at measurement points in different directions relative to the long axis of the bee (i–vi; see Fig.·1); values before (filled circles) and after wing removal (open circles). Circles are slightly displaced horizontally for better visibility. Sound pressures generated by a bee before and after wing removal do not differ significantly between intact and wingless bees (paired t-test; $P>P_{corr}$, 0.025).

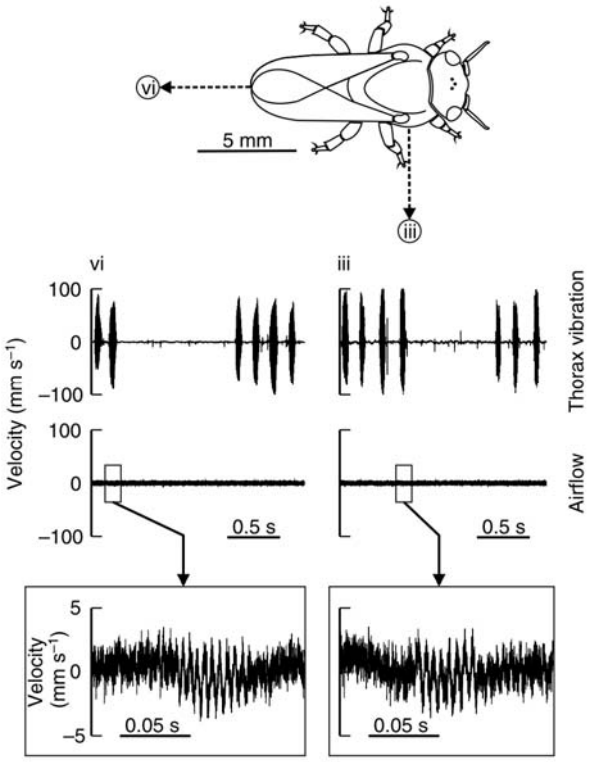

Figure 5: Simultaneously measured thorax vibrations and unidirectional air movements. A jet airflow was only to be expected behind the vibrating bees. The airflow recordings made 5·mm behind the wingtips (direction vi relative to the long axis of the bee, see Fig.·1), and those made 5·mm laterally (direction iii relative to the long axis of the bee; see Fig.·1) did not differ. Scaling for the airflow was chosen in accordance with the velocity amplitude of the air jet described in honey bees, 150·mm·s^{-1} (Michelsen, 2003).

Insets: amplified vibratory pulses showing air particle oscillations along with the thorax vibrations around the centre of the forager's thorax. Food receivers (R) were not included in the analysis. The closest position between the heads of hive bees (midpoint indicated by white dot) and the foragers served as a measure for the distance. (B, C) Distribution of 128 hive bees attending 20 trophallactic interactions (six different foragers). Different colours represent different regions around vibrating foragers; the borderlines between different regions correspond to directions (i–vi) given in Fig.·1.

DISTRIBUTION OF BEES AROUND RECRUITERS

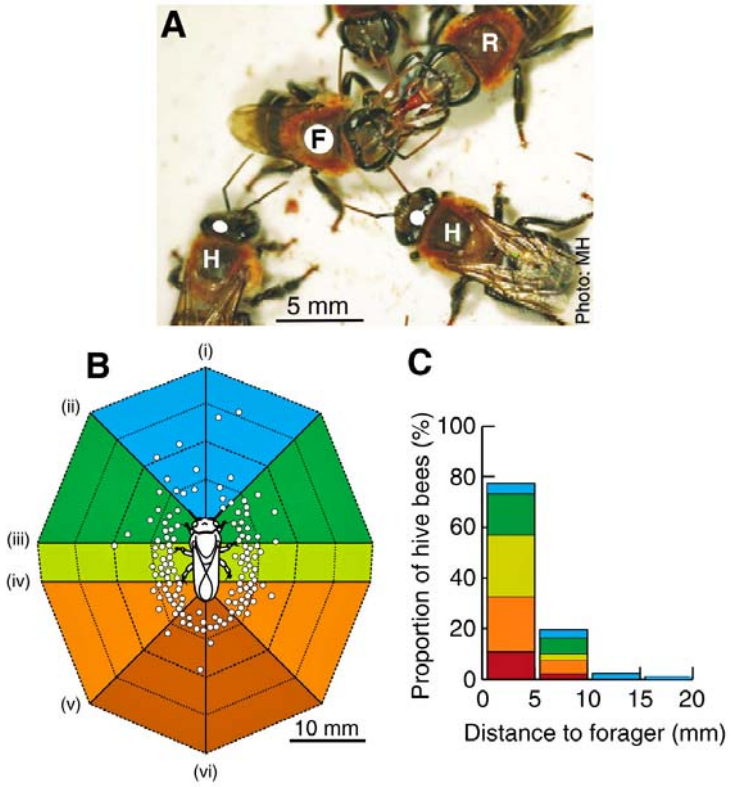

Figure 6: Distribution of hive bees (H) around a vibrating forager (F) during trophallactic food transfer measured within a circle of 2·cm radius

*"These bees also do not sting as hard[1] as they do back home. I often saw bees flying[2] at natives[3] who were retrieving honey. They stripped the bees off their naked bodies. I also grasped naked at the honey. At my first attempt I suffered great pain and had to run to the water in order to wash them off my body."**

Hans Staden (1557)

[1] Probably meaning " painful"

[2] "Flying at" probably meaning "attacking"

[3] Please note that I intentionally changed one of Staden's synonyms to describe the native inhabitants of the New World.

*Translated from: Hans Staden (2006) Brasilien: Historia von den nackten, wilden Menschenfressern [Ed.: G. Faber; Translation: U. Schlemmer]. Lenningen: Edition Erdmann. [The first Version was published in 1557 under the title "Wahrhaftige Historia und beschreibung eyner Landtschafft der wilden nacketen grimmigen Menschfresser-Leuthen, in der Newenwelt America gelegen"]

Nest entrance (diameter: ~ 4 – 11 cm) of a small to medium sized *Trigona spinipes* colony (possibly 4000-6000 bees)

CHAPTER VI

UNPUBLISHED DATA AND OBSERVATIONS

CHAPTER VI

A

How do *T. spinipes* bees react to disturbances in front of their nests other than by mandibular gland secretions?

(unpublished manuscript[1] in progress)

Little quantitative data is available in the literature about attack responses by meliponine bees defending their nest, as well as about the cues which meliponines actually use to locate their potential intruder(s). Besides the importance of such data for questions regarding the natural history of meliponines (e.g. intra- and interspecific competition) and that of their predators and

[1] The observations described in this manuscript represent an important contribution to the study of defensive and aggressive behaviour treated in chapter II.

parasites, such data is useful to design methods for the study of aggressive and defensive behaviour in these bees. For example, when studying chemical substances potentially eliciting attack responses after their application at the nest entrance, one should know to which extent defensive attack responses occur due to the mere physical manipulations at the nest entrance or to the chemical actually tested. In the following, experiments are described which analyze the effect of stimuli of different modalities on the elicitation of defensive behaviour.

METHODS

I studied the defensive attack response of *T. spinipes* bees at three different degrees of "disturbance" (no, light and heavy disturbance Figure 1) caused by human movements near the nest. In contrast to situations of "no disturbance", where no human approached the nest, "light disturbance" was caused when the experimenter cautiously approached the nest from a distance of about 8 m (walking slowly – approx. 0.2 m/s – and avoiding any sudden movements by any body part) at an angle > 30° to the nest entrance hole. The experimenter was not allowed to get closer than 0.5 m to the nest, which was the same in the "heavy disturbance" test. Here, the observer walked quickly (>1 m/s) up and down the nest entrance front (max. distance from the nest = 8m) "nervously" whilst quickly moving arms up and

down (~1-2x per s). All experiments were executed by the same experimenter (size: ~1.85 m; ~95 kg; clothed in light colours; no perfume) to avoid bias caused by potential effects originating from different body dimensions. During such disturbances the bees were obviously presented with multimodal stimuli. Visual stimuli may have predominated, but vibrational stimuli potentially contributed to a considerable extent. Even air movements, chemical and thermal stimuli emitted by the experimenter could have been perceived by the bees. To find out more about which stimuli were predominantly the cues eliciting defensive behaviour of the bees a set of experiments were carried out which are described later in this paper which first concentrates on the general disturbance experiments.

Quantification of defensive attack behaviour

To quantify the degree of defensive attack behaviour elicited by disturbances in front of the nest, I placed a clean black cotton ball [a sock stuffed with PVC foil; methodology similar to that used by Smith and Roubik (Smith and Roubik, 1983)] measuring ~10 cm in diameter as a target at a distance of 150 cm from the nest entrance during the night preceding the experiment. The ball was either suspended from a wooden broomstick or fixed onto it directly. During "non disturbance" experiments a binocular or a video camera with zoom function was used to observe the ball from a distance (~ 8 m). Disturbance experiments lasted for 2 minutes. Every disturbance

experiment showed a period when bees attacked most. Per experiment only the disturbance period with the maximum number of bees biting the cotton ball within 30 seconds was taken as a measure of aggressiveness.

RESULSTS AND DISCUSSION

As expected, in all five tested colonies no or hardly any bees attacked the black cotton ball[2] during no-disturbance periods (Figure 1). Weak disturbance provoked only a few bees to attack.

When comparing the disturbance treatments within colonies (Kruskal-Wallis analysis of variance on ranks) no statistically significant effect (Student-Newman-Keuls'pairwise multiple comparisons $P>0.05$; $N=6$ tests with each of the five colonies) could be observed between 'no disturbance' and 'light disturbance' treatments, except for the most aggressively attacking colony (D; Student-Newman-Keuls' pairwise multiple comparisons $P<0.05$; $N=6$

[2] Note that I only present the number of bees attacking the motionless cotton ball in front of the bees' nest and *not* the number of bees attacking the experimentator which actually caused the disturbances near the nest. This was done for methodological reasons and for reasons of comparability towards other experiments conducted with the same species (e.g. see experiments realized for the study of chemical substances eliciting aggressive and defensive behaviour in Chapter II).

tests with each of the five colonies). When comparing the 'no disturbance' values of all five colonies with those of the 'light disturbance' values, however, a statistically significant difference resulted (Mann-Whitney U: P <0.001; N=6 tests with each of the five colonies for each treatment). This provides evidence that weak disturbances in front of a *T. spinipes* nest can already lead to the onset of defensive behaviour.

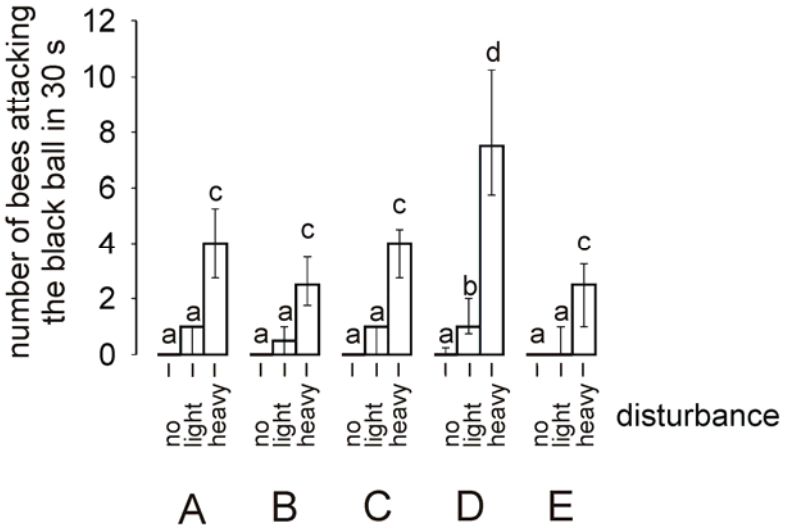

Figure 1: Number of *T. spinipes* bees attacking a dark object 150 cm away from the nest entrance after different degrees of disturbance (no, light, heavy) in front of the nest entrance. Medians (plus 1st and 3rd quartile) for five different colonies (A – E) are shown. Columns with the same letter at the top represent values that do not differ significantly from each other (alpha = 0.05).

"Heavy disturbance" in front of a *T. spinipes* nest obviously provokes some defensive attack by nest guarding bees (Figure 1). The number of attacking bees was consitently well above that for the light disturbance. Consequently statistically significant differences resulted between high disturbance and weaker disturbance treatments within each colony (Kruskal-Wallis analysis of variance on ranks: d.f.=2, H>13; P≤0.001; Student-Newman-Keuls' pairwise multiple comparisons P<0.05; ; N=6 tests with each of the five colonies for each treatment). Colony D showed significantly higher (Kruskal-Wallis analysis of variance on ranks: d.f.=4, H = 16.795; P≤0.002; Student-Newman-Keuls' pairwise multiple comparisons P<0.05;) defensive attack responses (Figure 1) compared to the other colonies. It seemed that colony D had more members at the time of experiments than any of the other colonies. Colony strength in Meliponini is a likely cause of differences in defensive attack intensity (Roubik 1989, Nogueira-Neto 1997; personal observations in *Cephalotrigona, Frieseomelitta, Geotrigona, Melipona, Nannotrigona, Scaptotrigona* and *Schwarziana*).

Which cue(s) do meliponine bees use to detect and locate a potential predator or nest intruder?

A complete answer to the above question could turn out to be highly complex. Predators, parasites or other nest intruders will mostly provide a variety of

optical, mechanical (including acoustical and vibrational) and chemical cues. However, not all cues that nest intruders provide will be of use to the attentive guard bees which must detect and locate potential threats quickly and efficiently. A few simple but reliable cues could work very well under most circumstances. Outside the nest, optical cues are probably the best choice to quickly and efficiently locate a threat during the day[3], especially among flying individuals.

If seen from the meliponine's perspective, an intruder approaching the nest entrance during the day often will appear contrasted to the sky or a comparably bright background (as seen from the inside of the nest). Therefore, a simple cue and the corresponding rule for efficient intruder localization could be accomplished by detecting dark objects (as compared to the background) and to attack them. A prerequisite for this method, of course, would be the bees' ability to distinguish "friend from foe", since nestmates will be constantly leaving and entering the nest. Again, many cues could be responsible for the

[3] Other prominent cues are less likely to play a major role for the following reasons: Current knowledge and literature (Hrncir et al. 2006, Hrncir et al. 2008) classifies meliponines as insensitive to weak and distant sounds due to the fact that their sound detecting sensory organs only detect the particle velocity of sound. Chemical cues, especially if at some distance (>1m) from the nest, will depend on air movement and in addition are slowly propagated in comparison to other cues. Finally, vibration detection could be useful and probably also plays an important role for the detection of intruders (Schorkopf, unpublished data), but will be restricted to the substrates in direct contact with the nest.

latter ability of distinction. While chemical cues or signals are probably important for nestmate recognition at close distance/contact (Breed and Page 1991; Buchwald and Breed 2005; Nunes et al. 2008, Schorkopf, personal observation), the optical appearance of the object seems to be most important for detection and localization from a distance. Here, I want to present some data restricted to objects much larger than the average bee, and which will focus on one simple aspect of test objects: their optical contrast value as compared to the background surrounding the nest. To make methods easy, I only distinguished between two different values: dark or bright as compared to the background. I achieved this by simultaneously offering two cotton balls (general method as described in the previous paragraphs on defensive attacks if not stated otherwise) at the same distance from the nest (150 cm) but with contrasting brightness values (one black and one white cotton ball, distance between both balls = 20 cm). To induce defensive behaviour, I exposed the nest entrance with one bee equivalent of *T. spinipes* "alarm" (defensive/aggressive behaviour eliciting) pheromone (see Schorkopf et al. 2009).
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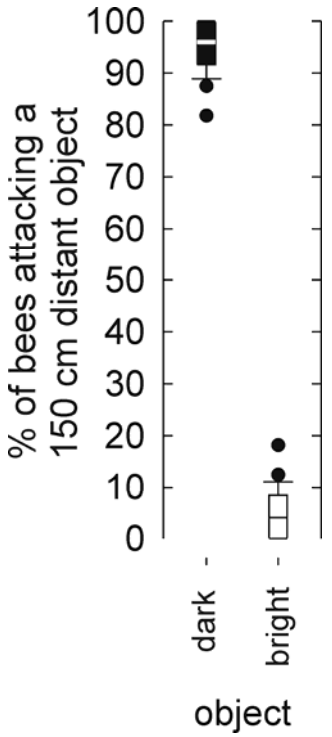

Figure 2: Preference of *T. spinipes* bees to defensively attack one of the two objects (at a distance of 150 cm from the nest) within 30s after inducing defensive attack behaviour by mandibular gland pheromones presented at the nest entrance. Box plots indicate inter-quartile range (box), the median value (horizontal line), 95% range (whiskers) and outliers of all five tested *T. spinipes* nests.

Figure 2 shows a striking preference of defensively attacking bees (>90%) for the dark object (black cotton ball) as compared to the bright object (white cotton ball). Consequently, the percentage values were significantly different (Mann-Whitney U Rank Sum Test: $P<0.001$; N=6 tests with each of the five colonies; a total of 370 bees).

Figure 3 illustrates that this preference remaine the same at different distances to the nest over a time period of 10 min. It also indicates that this preference is not dependent on the signal modality inducing the defensive attack behaviour in these bees. While mandibular pheromones represent a typical example for a signal, heavy mechanical impacts on nest structures are typical of cues. Yet, both signal and cue can induce the same type of behaviour after which the preference to attack dark instead of bright objects remains the same.

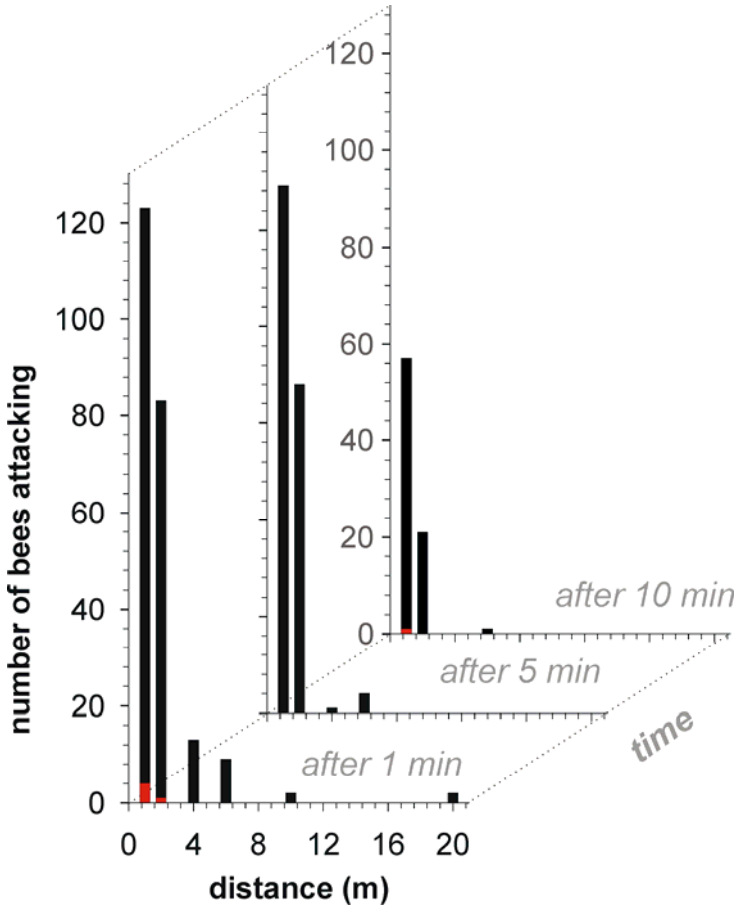

Figure 3: Illustration of the consistent preference of *T. spinipes* bees to defensively attack dark (black bars) as opposed to bright (red bars) cotton ball objects at different distances (1, 2, 4, 6, 10 and 20 m; 20 cm between equidistant dark and bright objects) and times after strong mechanical impact[4] on the nest structure.

[4] The impact by a log (force and acceleration of the impact not determined) thereby resulted in the destruction of a part (size: ~14 x 7 x 7 cm^3) of the outer nest layers. Probably no bees were located and therefore harmed in this part of the nest during the impact since no bees were observed on the respective outer layer surface shortly

Conclusions[5]

Trigona spinipes, as seen from the above given data, clearly belongs to the more agressive meliponine species. Whereas bees of "non-aggressive" species (e.g. species belonging to the genus *Melipona*, Nogueira-Neto 1997) rather tend to retreat than to attack until one actually invades their nest, *Trigona spinipes* may aggressively attack approaching objects even if these do not necessarily harm the colony. From an anthropocentric perspective the data support previous observations by researchers and bee keepers (honeybees: e.g. Crane 1990, meliponines: e.g. Nogueira-Neto 1997) suggesting that one should approach a meliponine or other bees' nest cautiously to avoid attack in species known to be "aggressive". The nearer one gets to the colony, the higher the probability of being attacked, which makes sense for the bees as fortress holders: The nest holds their brood, egg laying queen and their food reserves and therefore the regions nearest to it should be defended more fiercely than more distant

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before the impact or at any part of the resulting "crater" in the first 3 seconds after the impact.

[5] *with emphasis on the potentialconsideration of my observations for the practical handling of the bees*

regions. If attacked, the fastest and most efficient way to avoid more bites lies therefore in the simplest rule "to run away". In addition, dark cloths should be avoided when handling meliponine nests as is the case with the much better studied and described honey bees (*Apis mellifera;* e.g. Crane 1990). My data demonstrates a considerably stronger preference to attack dark objects as compared to light ones. Although quantitative data is available for only few Hymenoptera, the present study corroborates the common belief that the preference to attack dark objects is a general trait among the flying species. However, to sandbag this general view and to permit a meaningful comparison of the "aggressiveness" among different taxa, more quantitative data on other meliponines and Hymenoptera is required.

Nest entrance of
Scaptotrigona postica Latreille 1807
(diameter ~ 3 – 4.5 cm)

CHAPTER VI

B

INCIDENTAL BUT MOST LIKELY SIGNIFICANT OBSERVATIONS[6]

Additional observations in *Scaptotrigona postica* and *Trigona spinipes*

Preliminary observations in
Scaptotrigona postica Latreille 1807

After considerable difficulties, I was able to receive a colony of bees reliably identified as *Scaptotrigona postica*[7]. I could therefore examine whether there are obvious difference in the relevance of scent paths between *S.* aff. *depilis* (the "wrong *postica*" and presumably studied by Lindauer and Kerr; Lindauer and Kerr 1958) and the real *S. postica.* Together with Linde Morawetz I conducted a few experiments similar to those previously described for *S.* aff. *depilis*

[6] The observations mentioned here shall be seen as potentially significant notes to the study of pheromone paths in Meliponini, treated in chapter IV.

[7] The difficulty was due to the fact that true *S. postica* bees do not naturally occur in Piracicaba and Ribeirão Preto (see also methods in chapter IV). I therefore highly appreciate Dr. Sidnei Mateus for providing us a true *Scaptotrigona postica* colony from Mato Grosso State, Brazil (species identification was kindly confirmed by J.M.F. Camargo; specimens were deposited to his collection).

with this colony at the lake at Piracicaba (the colony was kept at the same location, for methodology see methods for *S.* aff. *depilis* in chapter IV). Due to the weakness of the colony, however, we only were able to get small numbers of newcomer bees, if any at all, recruited to the highly profitable feeders (50% w/w unscented sucrose solution) during several experiments, irrespective of the presence of pheromone paths leading towards the feeder or the presence of the lake between nest and feeder. However, it seems worth noting that some newcomer bees still were successfully recruited across the lake and without pheromone paths leading to the recruitment feeders on at least two occasions: one occasion, when 8 recruiting bees successfully recruited 6 newcomer bees within 40 min; the other, when 2 newcomer bees were recruited by 6 recruiters within 50 min[8]. *S. postica* is therefore assumed to share very similar pheromone path and recruitment mechanisms with *S.* aff. *depilis.* Although the details on the similarities or differences between both tested species need to be clarified in future studies, the few observations made on *S. postica* confirm the previously made statement of (see chapter IV): "Pheromone paths are not indispensable for successful recruitment in meliponine bees".

[8] Note that no bees (neither experienced, nor inexperienced) were ever observed to land on the control feeder (same dimensions and content but no foraging bees) positioned at the same distance, but in the opposite direction from the nest during any experiment with *S. postica* (no water barrier between nest and control feeder).

Incidental observation in *Trigona spinipes*

Considering its likely significance I report an incidental observation made in *Trigona spinipes* when I was on a boat anchored at noon at "lula beach" in the Atlantic bay at Paraty (South of Rio de Janeiro State, Brazil): At a distance of about 50 m from the beach forest (30 m water; 20 m hot sand) an individual of *T. spinipes* discovered the sweet content of a coke tin on the boat. I purposefully spilled some content around the tin can on the table (several little puddles to avoid the bee to drown either inside the tin can or in the puddles around it) on which it was originally found by the bee. I then was able to observe the individual bee to return three times (duration between visits approx. 90 - 180 s) when the bee obviously started to scent mark the tin can and the surroundings nearby. Six minutes thereafter a group of 13 recruited bees (I propose the bees to have been nest mates of the first bee) arrived. They had to fly across the hot sand on the beach in addition to the sea surrounding the boat to reach the sugary source. A noticeable wind from inland (boat flag pointed toward the sea) was no obvious challenge to the bees. They collected from the coke pads, flew back (probably) to their nest (all bees took the same direction toward the beach) and returned in similar numbers. This went on for a while. Before I unfortunately had to leave the beach for another destination, however, a larger bulk of bees returned (24 min after the first group of recruits had arrived;

estimated number of the new group: 30 – 40 bees). From this observation I tentatively conclude that *T. spinipes* and probably also other meliponines are not necessarily kept from foraging and group recruitment if they have to fly some distance without the guidance of a scent path above water, even if above the sea.

CHAPTER VII

CUES AND SIGNALS FOR ORIENTATION AND COMMUNICATION

CONCLUDING REMARKS

Main answers of this thesis

Obviously several cues and signals are used by the meliponine superorganism for efficient communication and orientation on the intra – and intersuperorganismical level. The focus on a few questions regarding foraging and defence led to the following main findings of my thesis which I briefly summarize and comment here.

Defence and aggression

One important cue for meliponines to detect and locate targets at a distance during defensive behaviour could be the "brightness" of objects as compared to other objects in their environment. Meliponine guard bees were found to attack dark or black objects much more readily and intensively than bright or white objects. Mechanical impacts on the nest lead to the attack of dark objects as did the non-invasive visual disturbance in front of the nest or chemicals exposed to the bees. This suggests that cues (e.g. the mechanical impact) alone can trigger all elements of collective defensive behaviour in meliponines as previously shown for bees having

received the corresponding chemical signals (mandibular gland pheromones). However, it seems that there are considerable differences in the number of responding bees between the above mentioned stimuli. The number of responding bees seems to be influenced by the strength and status of the colony as well. Future studies should assess this quantitatively.

Mandibular secretions

Meliponines use chemical signals originating from the mandibular glands to intraspecifically induce defensive behaviour. These pheromones, even if released in quantities well below the quantity available to single individuals, can release coordinated defensive behaviour on the level of the colony (or superorganism). The same secretions can also act between colonies (intersuperorganismic level) of the same species, which is relevant during the defence of territory and food sources. The same mandibular secretions can also act as allelochemical signal or cue between colonies of different species (interspecific communication). The effect of mandibular gland secretion regarding the induction of defensive behaviour is quickly achieved after its release and decreases within short time periods thereafter. This circumstance makes the secretions suitable for the fast arousal of defence behaviour and less susceptible to "false alarm". Mandibular secretions do not contribute to the pheromone trail or pheromone path following behaviour in

meliponine bees as previously claimed by several authors.

Scent paths and foraging

Regarding foraging, chemical signals enable the coordinated resource utilization outside the nest in cases where pheromone trails are laid down by the recruiting individuals (e.g. genera *Scaptotrigona* and *Trigona*). However, the dependence on chemical signals for recruitment to and orientation towards resources in the field could also lead to some inertia in the decision making process of a superorganism. The change of recruitment from older and less valuable targets (to which pheromone trails were established) to more profitable new resources may take longer or too long, especially in cases of short resource availability. However, this may be a negligible disadvantage in highly populous meliponine species such as those found in the genera *Scaptotrigona* or *Trigona*. The latter disadvantage also seems lessened in the light of the finding that pheromone paths are neither indispensable for recruitment nor leading to a full cessation of recruitment to other locations to which no pheromone paths lead.

Other signals

Other cues or signals in meliponines seem to be important for the recruitment to resources outside the nest. **Visual cues** or signals may contribute considerably. During our experiments with

Scaptotrigona, newcomer bees usually only appeared at the communicated food source when experienced bees accompanied them or when other bees were already present at the food source location. These facilitation mechanisms might play an even more important role in meliponines which do not construct elaborate pheromone trails towards recruitment targets. While the latter remains to be carefully treated in future research, some significant progress in our understanding of intranidal recruitment mechanisms of "non-pheromone-path" meliponines resulted from the studies of recruitment related behaviour in *Nannotrigona* and *Melipona.* **Jostling** contacts during recruitment were shown (similar to what is known for *Melipona*[1]) to be suited for coding some information about the profitability of food sources in *Nannotrigona testaceicornis* [Lepeletier 1836]. The same is true for the pulse duration of the **thorax vibrations** in this species which are produced during the recruitment behaviour inside the nest (again, as previously known for *Melipona*[2]). In *Melipona,* where the production of thorax vibrations is well documented[3], a lot of work still remains to be done to fully unveil which stimuli actually are used as signals or cues by the signal receiver bees inside the nest. One way of potential signal transmission could be added to those already described for meliponine bees[3]: **the direct**

[1] See Hrncir et al. 2000, Jarau et al. 2000 and Barth et al. 2008
[2] Hrncir 2003, Hrncir et al. 2004
[3] See Nieh 2004, Hrncir et al. 2006, Barth et al. 2008

vibration of food receivers by recruiting bees. Using *Melipona,* a method could be established which significantly increased the possibilities to study aspects related to thorax vibration and sound emission quantitatively. The **particle oscillation** field around a vibrating bee due to airborne sound was inhomogeneous, the average velocity amplitude ranging between 3 and 16 mm/s at a distance of 5 mm. The **sound pressure** field around a vibrating bee was inhomogeneous too (200-400 mPa; distance = 5mm). However, no **jet airflow** as described for the honey bee (*Apis mellifera*) was detected behind the wings of the vibrating bees.

Provided that *Melipona* possesses a Johnston organ at least as sensitive for air particle oscillations as previously found for *Apis mellifera, it* should be able to detect particle oscillations at distances up to 2 cm away from vibrating bees. About 80% of the bees interacting with recruiting foragers were found to surround the recruiting bee at distances closer than 5 mm. Most bees interacting with successfully returning foragers could therefore be able to perceive recruitment signals or cues if actually transmitted via air particle oscillations by vibrating bees.

"The meliponine superorganism puzzle"

The findings presented in this thesis show many similarities to cues and signal mechanisms already described in other, often much better studied superorganisms, such as those of honey bees and ant

or termite species (Hölldobler and Wilson 2009). Yet, the mechanisms are by no means identical, for which the non-obligatory character of the pheromone path in pheromone path laying meliponine species is a good example. Meliponines are likely to substantially contribute towards a better understanding of some principal communication mechanisms of the so called "superorganisms". The present thesis aimed to add to our understanding of the multidimensional puzzle called "the meliponine superorganism". Although I much hope that it did indeed bring us closer to the goal I also believe that the larger part of the "meliponine superorganism puzzle" still remains to be solved.

ZUSAMMENFASSUNG

HINWEISREIZE UND SIGNALE IM DIENSTE DER ORIENTIERUNG UND KOMMUNIKATION BEI DEN SUPERORGANISMISCHEN MELIPONEN

Die vorliegende Dissertation befasst sich vordergründig mit den zwei folgenden Fragen: 1) Welche Signale[4] und Hinweisreize [5] (kurz: Hinweise[6]) werden bei den superorganismisch[7] organisierten Meliponen (Staaten - bildende Bienenarten, die weltweit in den Tropen vorkommen[8]) zur und während der Verteidigung ihrer Kolonien genützt (Kapitel II)? 2) Welche Signale und Hinweise begleiten und ermöglichen die Mobilisierung, Koordination und Orientierung von

[4] Definition siehe Kapitel I

[5] Die Bezeichnung entspricht dem so genannten Kennreiz. Da die in dieser Dissertation verwendete Definition (siehe Begriff „cue“ in Kapitel I) aber nicht gänzlich dem Kennreiz gleicht, wird hier Hinweisreiz bevorzugt.

[6] Siehe Seeley (1997)

[7] Nach Wilson (2000) und Hölldobler und Wilson (2009) jedwede soziale Gemeinschaft, wie z. B. die Kolonien von eusozialen Insekten, welche Organisationseigenschaften besitzen, die analog zu den physiologischen Eigenschaften eines einzelnen Organismus bestehen (siehe auch Kapitel I). Ein Insektenstaat, zum Beispiel, ist in reproduktive Kasten (analog zu Gonaden) und Arbeiterkasten (analog zu somatischen Gewebe) aufgeteilt. Als weiteres Beispiel kann die Nahrung hierbei, funktionell ähnlich dem Körperkreislauf, z.B. durch Trophallaxis unter den Individuen unterschiedlicher Kasten aufgeteilt werden.

[8] Die Bezeichnung „Stachellose Bienen“ wird aufgrund der in Kapitel I genannten Gründe von mir vermieden.

Arbeiterinnen der Meliponenstaaten zur effizienten Nutzung von Ressourcen (Kapitel III bis V)?

Die über 400 Meliponenarten sind vor allem als Bestäuber und Blütenbesucher der weltweiten Tropen bedeutsam. Ihre im Aussehen und Verhalten recht unterschiedlichen Gattungen und Arten (siehe Kapitel I und Roubik 1989) stellen außerdem ideale Versuchsobjekte zum besseren Verständnis von tropenbiologisch relevanten Fragestellungen dar (siehe Roubik 1989). Während Nektar- und Pollensammeltätigkeit beziehungsweise daraus resultierende getrennte Honig- und Pollenvorräte auch bei den verwandten Honigbienen (*Apis sp.*) und Hummeln (z.B. *Bombus sp.*) vorkommen, zeichnen sich Meliponen ausserdem durch das zusätzliche Vorkommen fleisch-konsumierender (z. B. *Trigona hypogea*) und kleptoparasitischer Arten (z. B. Gattung *Lestrimellitta*) aus, die zur Vielfalt der faszinierenden Überlebensstrategien bei Meliponen beitragen.

Defensives und aggressives Verhalten

Im Kapitel II werden Signale und Hinweisreize identifiziert, die defensives und aggressives Verhalten bei Meliponen auf intra- und intersuperorganismischer, sowie intra- und interspezifischer Ebene ermöglichen. Zur Untersuchung wurden zwei Arten gewählt, die bereits in der Vergangenheit zu ähnlichen Fragestellungen herangezogen worden sind: *Trigona*

spinipes Fabricius 1793 und *Scaptotrigona* aff. *depilis*[9] (Artgruppe: *S. depilis* Moure 1942), welche sympatrisch in neotropischen Habitaten vorkommen. Mandibeldrüsensekrete bei Arbeiterinnen enthielten u.a. 2-Heptanol und 2-Nonanol (siehe Tabelle 1 in Kapitel II), die aggressives und defensives Verhalten auslösten. Verhaltenstests zeigten (siehe Appendix von Kapitel II), dass während auffälliger Annäherung an Meliponennester und der darauf folgenden Nestverteidigung dunkle Flächen angeflogen und auch verstärkt gegenüber hellen Flächen angegriffen werden[10]. Die oben genannten Pheromone stellten sich außerdem auch als geeignete Allelochemikalien[11] heraus, da Mandibeldrüsen-extrakte beider Arten zur Auslösung defensiver Verhaltensweisen an Futterquellen und Nesteingängen sowohl der einen wie anderen Art führten. Die Mandibeldrüsensekrete sind demnach zur intra- und intersuperorganismischer beziehungsweise intra- und interkolonialer Kommunikation auf intra- und interspezifischer

[9] Früher, wie heute fälschlicherweise oft auch als *Scaptotrigona postica* bezeichnet, da die Arten in der Gattung *Scaptotrigona* sehr ähnlich ausschauen können und z.T. auch noch nicht beschrieben worden sind (siehe Methodenteil des Kapitel II und Camargo und Pedro 2007).

[10] Meliponen benutzen v. a. ihre Mandibeln, um sich zu verteidigen. Beißen stellt ihre bevorzugte mechanische Verteidigungsform dar, da sie (im Gegensatz zu den Honigbienen und Hummeln) ihren Stachel im Laufe der Evolution reduziert haben.

[11] Im Gegensatz zu Pheromonen, die als chemische Signale innerhalb einer Spezies wirken, agieren Allelochemikalien über Artgrenzen hinweg.

Ebene geeignet. Die Mandibeldrüsensekrete der untersuchten Arten zeigten niemals anlockende Wirkung, weder an der Futterquelle, noch am Weg zwischen Nest und Futterquelle. Vielmehr lösten Mandibeldrüsensekrete in den genannten Situationen stets defensives und aggressives Verhalten aus. Deshalb kann nun, zusammen mit den davor erhobenen und wichtigen Erkenntnissen von Stefan Jarau und Kollegen (Jarau 2003, Jarau et al. 2004, Jarau et al. 2006) mit guter Sicherheit der fast fünfzig Jahre lang bestehende Irrtum (siehe Kapitel II), Meliponen nützten Mandibeldrüsen-sekrete zur Duftpfadlegung, ausgeschlos-sen werden.

Speichel als Spurpheromonträger

Dass die Absetzung attraktiver und zu Futterquellen hinführender Duftmarken bei Meliponen vor allem durch Speichel der Arbeiterinnen gewährleistet wird, zeigten Untersuchungen an *T. spinipes* (siehe Kapitel III): Extrakte (Pentan als Lösungsmittel) der speichelbildenden Labialdrüsen enthielten vor allem eine Hauptsubstanz: Octylsäure-octylester (~ 74% der unpolaren und volatilen Anteile bei gaschromatografischen Analysen; siehe auch Kapitel III). Dieser Ester ließ sich weder in den Mandibeldrüsen, noch in den Hypopharynxdrüsen nachweisen. Wohl aber wurde Octylsäure-octylester auf künstlichen Futterquellen gefunden, die davor häufig von Arbeiterinnen besucht und chemisch markiert wurden. Mittels Auftragen von Octylsäure-

octylester an zuvor unbesuchten und unbedufteten Substraten und Futterquellen konnte erfolgreich Duftspurfolgeverhalten zu diesen ausgelöst werden. Neulinge flogen gleichzeitig angebotene Futterquellen zu gleichen Anteilen an, wenn eine von ihnen mit Labialdrüsenextrakt, die andere mit gleichen Anteilen an künstlichem Octylsäure-octylester beduftet wurden. Daraus kann geschlossen werden, dass Octyl-octanoat ein Einzelkomponentenpheromon bei *T. spinipes* Arbeiterinnen darstellt. Jedenfalls handelt es sich mit großer Sicherheit um die mit Abstand wichtigste Komponente des Duftspurpheromons der letztgenannten Art. Diese Eigenschaft und die Tatsache, dass *T. spinipes* eine häufig vorkommende Meliponenart darstellt, macht sie zu einem besonders geeigneten Studienobjekt zur Untersuchung von Duftspurpheromonfolgeverhalten bei tagaktiven und flugfähigen Insekten.

Kommunikation und Orientierung mit Hilfe substratgebundener Duftpfade

Das Anlegen von Duftpfaden (substratgebundene Duftspuren[12] mit berücksichtigenswerter Länge in Richtung des für den Sender anzeigungswerten Zieles) zu Futterquellen bei flugfähigen Organismen

[12] Der von mir hier eingebrachte kleine und feine, aber für bestimmte Fragestellungen der Kommunikation, Orientierung und „Navigation" sehr bedeutsam erscheinende Unterschied zwischen Duftspuren und Duftpfaden wird im Kapitel IV näher betrachtet. Leider wird in bisher publizierten Arbeiten meines Wissens, kaum oder zu wenig auf diese Unterschiede eingegangen.

scheint in der Natur äußerst selten vorzukommen. Bei Meliponen wurde ein solches Verhalten bei manchen Arten beobachtet, allen voran bei Arten der Gattung *Scaptotrigona.* Über fünfzig Jahre alte Versuche (Lindauer und Kerr 1958) stellen bis jetzt die wichtigsten Beobachtungen dar. Da während der damaligen Versuche an einem Teich *Scaptotrigona* ausschließlich dann Neulinge erfolgreich zu Ressourcen rekrutieren konnte, wenn Duftpfadlegung zwischen Nest und Ressource möglich war, wurde bis zu den nun vorliegenden Versuchen angenommen, dass diese Duftpfade für die Rekrutierung unverzichtbar sind (Alcock 2005). Diese Tatsache ist unter anderem deshalb von Bedeutung, weil angenommen wurde (Kerr 1969), dass der in den Neotropen beobachtete Artenreichtum der Gattung *Scaptotrigona* durch die oben genannte Umstände erklärt werden könnte. Jedes Duftpfadhindernis, wie z. B. Flüsse, würde demnach zur Artbildung durch geografische Isolation beitragen. Außerdem könnten ähnlich vom Nest isolierte Blütentrachten weniger effizient von einer Kolonie bestäubt werden. Die in der vorliegenden Arbeit präsentierten Versuchsdaten zeigen jedoch, dass solche oder ähnliche Einschränkungen weder für die duftpfadlegende *Scaptotrigona* noch für *Trigona* gelten: Sowohl *S.* aff. *depilis* und *S. postica,* als auch *T. spinipes* waren in der Lage, trotz fehlender Duftpfade erfolgreich Neulinge zu rekrutieren (Kapitel IV). Duftpfade erfüllen bei Meliponen dennoch eine Funktion: Sammlerinnen

können durch Duftpfade einen Lenkungseffekt erzielen, indem sie die Wahrscheinlichkeit einer Rekrutierung zu einer durch einen Duftpfad angezeigten Futterquelle erhöhen, ohne aber die Rekrutierung zu duftpfadlosen Ressourcen zu verhindern. Die dazu angestellten Versuche (Kapitel IV) zeigten, dass bei gleichzeitiger Rekrutierung zu zwei verschiedenen Futterquellen diejenige stärker von Neulingen angeflogen wurde, zu der ein Duftpfad führte.

Eine weitere Meliponenart[13], die Duftspuren zu Futterquellen hinterlässt, ist *Trigona recursa* Smith 1863. Zu dieser Art konnten auf Versuchen basierende (Kapitel Va) Hinweise gefunden werden, dass sich ihre Duftspuren auf bisher wenig untersuchte Aspekte der Sammeltätigkeit auswirken könnten. So wurde stets zu jener von zwei gleichzeitig angebotenen Futterquellen stärker rekrutiert (Anzahl der Neulinge pro Zeit), welche bereits länger von Sammlerinnen einer Kolonie besucht wurden, auch wenn sie wesentlich weniger profitables Zuckerwasser enthielten. Bei gleichzeitigem Sammel- und Rekrutierungsbeginn wurde, wie man allgemein erwarten würde, stärker zur profitableren Futterquelle rekrutiert. *T. recursa* scheint demnach, ähnlich den duftspurlegenden Ameisen, durch ihren Rekrutierungs- und

[13] Die nun folgenden Ergebnisse und daraus gezogenen Schlüsse beziehen sich vor allem auf Arbeiten, in welche ich als Koautor mitwirkte (siehe die im Kapitel V vorgestellten Publikationszusammenfassungen).

Kommunikationsmechanismus mittels Duftspuren geprägt und in gewisser Hinsicht in der Flexibilität ihrer Entscheidungen bezüglich des bevorzugten Rekrutierungsortes auf Kolonie- beziehungsweise Superorganismusebene eingeschränkt. Die bisher bestehende Literatur stimmt mit der oben ausgeführten Hypothese überein: Eine von Biesmeijer und Ermers (1999) untersuchte Meliponenart (*Melipona fasciata*), die keine Duftpfade anlegt, sowie die als ebenfalls nicht duftpfadlegend bekannten Honigbienen (*Apis mellifera*) zeigten eine hohe Flexibilität in der Entscheidung über die Wahl verschieden profitabler Futterquellen: Anders als bei der dufptfadlegenden *T. recursa* wechselten *Melipona fasciata* und *Apis mellifera stets* zur jeweils profitableren Futterquelle, unabhängig von Sammeldauer und Sammelbeginn (Seeley 1997; Biesmeijer 1997; Biesmeijer und Ermers 1999).

Aspekte intranidaler Kommunikation

Der Schwerpunkt der nun vorgestellten Erkenntnisse liegt bei der intranidalen[14] Rekrutierungs-kommunikation. Es geht um mögliche Hinweise und Signale im Nest. Jene Meliponen, die keine Duftpfade zu Futterquellen nutzen (z. B. Arten der Gattung *Melipona, Nannotrigona*) müssen dennoch mittels Signalen auf Ressourcen außerhalb des Nestes aufmerksam machen. Wie bereits bei

[14] Intranidal = innerhalb des Nests

Melipona[15] gezeigt (Hrncir 2003, Hrncir 2004), könnte bei *Nannotrigona* die Futterqualität die Thoraxvibrationen während des Rekrutierungsverhaltens im Nest beeinflussen. Tatsächlich konnten ähnliche Änderungen im intranidalen Rekrutierungsverhaltensmuster wie bei *Melipona* aufgezeigt werden (Kapitel Vb). So stiegen mit der Profitabilität der Futterquelle die mit erfolgreicher Rekrutierung im Zusammenhang stehende Anzahl der Rempelkontakte sowie die Pulsdauer der Thoraxvibrationen während der Futterabgabe. Welche Sensorische Kanäle spielen bei der Rekrutierung von Arbeiterinnen bei Meliponen wirklich eine Rolle? Um diese Frage vollständig und stichfest beantworten zu können, fehlten und fehlen noch einige Untersuchungsschritte. So galt es herauszufinden, welche Anteile der während des Rekrutierungsverhaltens auftretenden Thoraxvibrationen tatsächlich von den Empfängern genutzt und als biologisch relevant eingestuft werden können (Kapitel Vc-e). In der Literatur wurde bis vor kurzem nicht an die Möglichkeit gedacht, dass Bienen die Thoraxvibrationen über direkten Körperkontakt wahrnehmen und als Rekrutierungssignal auswerten könnten. Wie sich herausstellte (Kapitel Vc), werden Rekrutierungskandidatinnen bei *Melipona* von den rekrutierenden und futterspendenden Bienen während der

[15] *Melipona* wurde von allen Meliponen bezüglich der oben genannten intranidalen Aspekte mit Abstand am besten untersucht (Nieh et al. 2004, Barth et al. 2008).

Futterübergabe (Trophallaxis) in Vibrationen versetzt. Die Übertragung der Vibrationen über direkten Körperkontakt war wesentlich effektiver als über das Substrat zwischen den Bienen. So betrug die Geschwindigkeits-amplitude (mm/s) der am Thorax des Empfängers gemessenen Vibrationen bei direktem Körperkontakt im Durchschnitt immerhin noch etwas mehr als 12% des am Thorax des Senders gemessenen Wertes. Am Substrat zwischen Sender und Empfänger konnten dagegen im Durchschnitt nur mehr 0,5% der Geschwindigkeitsamplitude gemessen werden (Kapitel Vc).

Neben der letztgenannten Signalübertragungs-möglichkeit durch Vibrationen könnte die Rekrutierungssignalempfängerin auch den durch die Vibrationen hervorgerufenen Luftschall nutzen. Da alle bisherigen Untersuchungen (Hrncir et al. 2006) darauf hindeuten, dass Bienen statt des Schallwechseldrucks die Schallschnelle wahr-nehmen, musste erst eine entsprechende Meßmethode entwickelt werden. Die effiziente Messung der Schallschnelle (Kapitel Ve) gelang erst durch das Festhalten einer Biene (Kapitel Vd) mit Hilfe einer „Halsschlinge“ (Befestigung der Schlinge zwischen Kopf und Thorax). Die höchsten Schallschnelleamplituden wurden über den Flügeln in der vertikal orientierten (dorsoventrale Achse) Schwingungsrichtung gemessen (Amplitudenwerte um 16 mm/s; hier wie sonst: bei Messungen 5 mm Mindestabstand zur Biene). Horizontal um eine Biene herum wurden die höchsten Werte vor dem

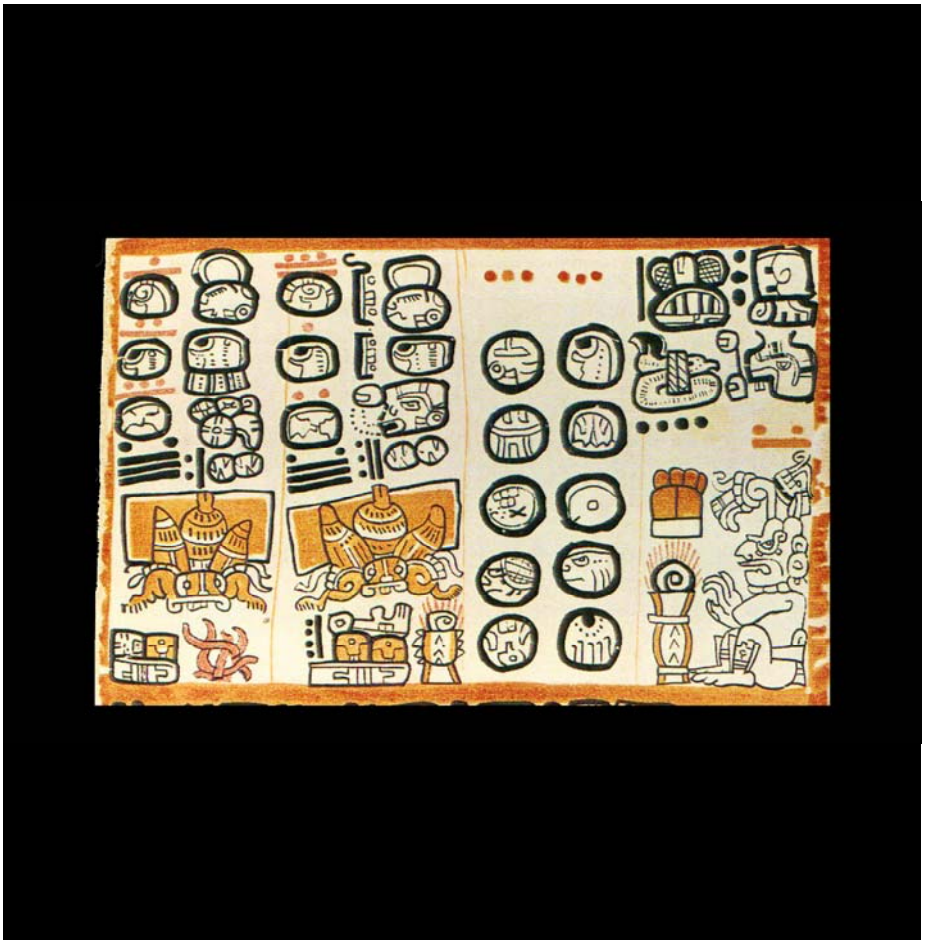
Bienenkopf gemessen (horizontale Schwingungsrichtung: 9 mm/s). Der Schallwechseldruck im Umfeld der Biene schwankte bei 5 mm Abstand zu Biene und Substrat zwischen 200 und 400 mPa (Kapitel Ve). Ein so genannter „jet airflow“[16], der bei *Apis mellifera* in caudaler Richtung angenommen wird (Michelsen 2003), wurde bei *Melipona scutellaris* nicht gefunden (Kapitel Ve). Dies könnte mit der unterschiedlichen Stellung der Flügel, die während der Thoraxvibrationen bei *Apis mellifera* (Flügel leicht gespreizt; Michelsen 2003) und *Melipona sp.* (alle bisher untersuchten Arten hielten die Flügel meistens in geschlossener Ruhestellung) auftreten, zusammenhängen.

Schließlich wurde das für die Schallperzeption relevante Verhalten von *M. scutellaris* Bienen während der Rekrutierung näher untersucht. Achtzig Prozent jener Bienen, die mit einer rekrutierenden Biene Trophallaxis durchführten, hielten einen Abstand[17] unter 5mm zur rekrutierenden Biene. Vorausgesetzt, *Melipona* besitzt ein für Schallschnelle mindestens genauso sensibles Johnston’sches Organ wie *Apis mellifera*[18], so sollten die Empfängerbienen den Schall noch bis zu einer Entfernung von etwa 2 cm wahrnehmen können.

[16] „jet airflow“ (engl.) = „Lufströmungsstrahl“ (dt.)

[17] Dieser Abstand geht oft auch, wenn auch nur kurzweilig, in direkten Körperkontakt über (Antennen berühren die Körperoberfläche der rekrutierenden Biene).

[18] Die Reizschwelle liegt nach Tsujiuchi et al. (2007) bei einer Schallpartikelelongation von etwa 60 nm (resultierende Schallschnelle bei 750 Hz: ~ 0.3 mm/s). .



Detail from the Mayan „Codex de Madrid“

From: Ferdinand, A. (1967) Codex Tro-Cortesianus (Codex Madrid), Museo de Ameríca Madrid. Graz: Akademische Druck und Verlagsanstalt

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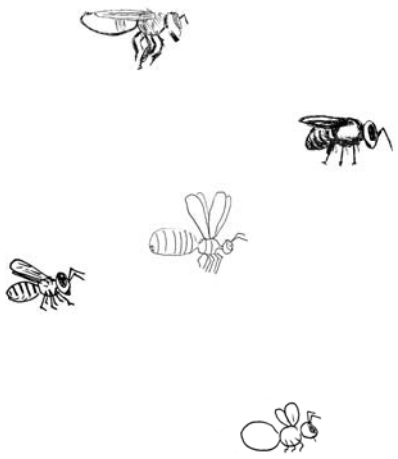

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14. 07. 1978	Born in São Paulo, Brazil
1996	Study of Biology and Ecology at the University of Vienna
2000 & 2001	Participation in the sea turtle project of the Vienna University (Austria) and Izmir University (Turkey) at the Mediterranean coast.
2001	Tropical ecology course in Uganda (Tropical Biology Association, Cambridge)
2002-2004	Master of science (University of Vienna) Thesis title: "Die Bedeutung des Honigvorrats für das Rekrutierungsverhalten einer Stachellosen Biene (*Melipona scutellaris* Latreille 1811; Hymenoptera, APIDAE, Meliponini)" [Engl.: „The importance of honey stores for the recruitment behaviour of a stingless bee (...)"] . Supervision by F.G. Barth, Department of Neurobiology at the University of Vienna. Data collection (2x 6 months) took place at the University of São Paulo/Brazil (Department of Ecology, Prof. R. Zucchi; Department of Genetics, Prof. Z. Simões).
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[1)] From: M. Lobe (1972) Das kleine ich bin ich. Vienna: Verlag Jungbrunnen.

“science

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